THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 9, Issue of March 3, pp. 4418–4423, 1995
Printed in U.S.A.

Occupancy of the *Dictyostelium* cAMP Receptor, cAR1, Induces a Reduction in Affinity Which Depends upon COOH-terminal Serine Residues*

(Received for publication, May 5, 1994, and in revised form, November 28, 1994)

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Many G-protein-coupled receptors display a rapid decrease in ligand binding following pretreatment with agonist. cAR1, a cAMP receptor expressed early in the developmental program of *Dictyostelium*, mediates chemotaxis, activation of adenylyl cyclase, and gene expression changes that bring about the aggregation of 10^5 amoebae to form a multicellular structure. Occupancy of cAR1 by cAMP initiates multiple desensitization processes, one of which is an apparent reduction in binding sites. In transformed cells expressing cAR1 constitutively, Scatchard analyses revealed that this apparent loss of ligand binding is largely due to a significant reduction in the affinity of cAR1 for cAMP. A parallel increase in the dose dependence of cAR1-mediated cAMP uptake was observed. Consistent with these findings, proteolysis of intact cells and immunofluorescence suggested that cAR1 remains on the cell-surface following cAMP treatment. Finally, agonist-induced loss of ligand binding is impaired in cAR1 mutants lacking a cluster of cytoplasmic serine residues, which are targets of cAMP-induced phosphorylation.

Seven-transmembrane domain, G-protein[1]-coupled receptors constitute a diverse molecular superfamily with representatives in many eukaryotic species. In mammals, these receptors regulate processes ranging from chemotaxis to synaptic signaling and vasoconstriction. Their ligands likewise range from glycoproteins to biogenic amines and lipid molecules (reviewed in Ref. 1).

Despite this heterogeneity of function and ligand structure, occupancy of these receptors elicits an apparently conserved series of activation and desensitization processes. Initially, occupancy causes the activation of receptor-associated G-proteins, which, in turn, stimulate such effectors as adenylyl cyclase, phospholipase C, and ion channels (reviewed in Ref. 2). Simultaneously, at least two different desensitization events, adaptation and "loss" of ligand binding, are observed. While most studies of these processes focused on the β-adrenergic receptor (reviewed in Ref. 3), similar observations have been made for other G-protein-coupled receptors. Adaptation, a rapid reduction in agonist-induced effector activation, has been attributed to the uncoupling of receptor from G-protein. This uncoupling is proposed to result from agonist-induced receptor phosphorylation and the subsequent association of arrestin, which appears to obstruct further receptor-G-protein interaction. The second agonist-induced desensitization process is a rapid reduction in the apparent number of surface binding sites. In some instances, this loss of ligand binding, often referred to as sequestration, has been attributed to receptor endocytosis.

Dictyostelium utilizes a family of four cell-surface G-protein-coupled cAMP receptors (cARs) to mediate a transition from a unicellular amoeboid phase to a multicellular sporogenous phase upon nutrient depletion. One of these, cAR1, is expressed shortly after the initiation of starvation and is required for aggregation (5–7, 47). cAR1 occupancy has three consequences: (i) chemotaxis of cells toward the source of cAMP, (ii) altered gene expression, and (iii) synthesis and secretion of more cAMP, which serves to propagate the signal outwardly from aggregation centers (reviewed in Ref. 4). Like other G-protein-coupled receptors, cAR1 exhibits multiple responses to cAMP binding including adenylyl cyclase activation (8), cAR1 phosphorylation (which is correlated with adenylyl cyclase adaptation) (9–11), and loss of cAMP binding (12, 13).

Constitutively expressed cAR1 undergoes phosphorylation (14, 15) and a ligand-induced reduction of ligand binding (14, 16) in the growth stage when the endogenous receptor gene is not expressed. This property has been exploited for the study of cAR1 mutants resulting in the identification of the major sites of cAMP-stimulated cAR1 phosphorylation (15). In the present study, we used this system to examine the mechanism of cAMP-induced loss of ligand binding. Our findings lead us to conclude that, following cAMP pretreatment, growth-stage cells overexpressing cAR1 exhibit reduced binding, not because of receptor internalization, but because of a reduction in the affinity of cAR1 for cAMP. We also demonstrate that this transition is defective in cAR1 mutants that lack targets of ligand-stimulated phosphorylation, suggesting a possible link between these processes.

MATERIALS AND METHODS

Cells and Cell Culture—AX-3 cells were grown in shaking culture in HL-5 (17). Transformed cell cultures were supplemented with G418 (20 μg/ml, Sigma). Cells maintained on Petri dishes were grown in suspension for at least 2 days prior to each experiment. Harvested cells were washed once in PB (5 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 6.1), and resuspended in ice-cold PB. Development in shaking culture with 50 nM cAMP pulses was initiated as described (18).

Plasmids—Met^1 and Gly^2 of cAR1 were replaced with a human c-Myc epitope (19), MAEEQKLISEEDL, by polymerase chain reaction. The

* This work was supported by National Institutes of Health Grant GM 34933 (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.

‡ Supported by a Medical Scientist Training Grant from the National Institutes of Health.

§ Supported by a fellowship from the Damon Runyon-Walter Winchell Cancer Research Fund.

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[1] The abbreviations used are: G-protein, guanine nucleotide-binding regulatory protein; cAR1, cAMP receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

final nucleotideotide sequence was as follows: 5'GATCCAAAATAAA ATG GCT GAA GAA CAA AAA TTA ATT TCA GAA GAT CTT ... 3', where the last codon originates from codon 3 in the cAR1 cDNA (5). The resulting 1.3-kilobase pair fragment was subcloned into the *BglII* site of pBS18 (5) to yield pMC8. pMC36 (16) was used to express wild-type cAR1. Both plasmids were electroporated into growth-stage *Dictyostelium* cells and transformants selected using G418 as described (20).

Loss of Ligand Binding and [^{3}H]cAMP Binding Assays—Washed cells were resuspended to 10^8/ml in PB and [^{3}H]cAMP binding measured using either a sedimentation assay (21) or a silicone oil assay (22), as indicated in the figure legends. Unless otherwise indicated, loss of ligand binding was induced with 10^{-5} M cAMP in the presence of 10 mM dithiothreitol (to inhibit endogenous phosphodiesterase; Ref. 23) and measured as described previously (16). Also unless otherwise indicated, a subsaturating cAMP concentration of 10 nM was used in the loss of binding experiments in order to allow a sensitive detection of binding affinity changes. For the concentration dependence experiment and for the experiment involving developed cells, 5 mM caffeine was included in the incubation to prevent the production of endogenous cAMP (24). For the analysis of cAMP binding parameters, [^{3}H]cAMP binding at concentrations ranging from 10^{-9} M to 2×10^{-6} M was measured using the silicone oil assay and analyzed using the program LIGAND (48).

[^{3}H]cAMP Uptake—Uptake of radiolabeled cAMP was measured as described previously (16). Washed cells (2×10^7) were shaken 15 min at 22 °C in 300 μl of PB containing 1 nM or 10 nM [^{3}H]cAMP, 10 mM dithiothreitol, and unlabeled cAMP (0–2000 nM). Nonspecific uptake was measured in the presence of 10^{-4} M unlabeled cAMP. Uptake was halted by the addition of 2 ml of ice-cold PB containing 10^{-4} M unlabeled cAMP, centrifugation (4 min, 2000 rpm, Sorvall HS-4 rotor), and three 3-ml washes with PB. Such extensive washing has been shown to remove all receptor-associated cAMP (25). Pellets were solubilized in 200 μl of 0.1 M formic acid, 4 ml of scintillation fluid was added, and radioactivity assessed. In control experiments, cells lacking cAR1 (16) showed virtually no cAMP uptake, even at the highest concentrations.

Cell Surface Trypsinization—Washed cells at 3×10^7/ml were shaken for 5 min at 0 °C. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (50 μg/ml, Worthington) was added for 0–15 min, followed by the addition of soybean trypsin inhibitor (100 μg/ml, Worthington) and two washes with 10 volumes of ice-cold PB containing 1 mg/ml bovine serum albumin (4 min, 2000 rpm, Sorvall SS34 rotor). Pellets were washed once in 5 ml of ERB containing 1.5% CHAPS (15), once in ERB, and resuspended to 10^8/ml in sample buffer (26). Control experiments indicated that all detectable tryptic activity was removed during the washes.

Generation of a Myc-specific Polyclonal Antiserum—The human c-Myc peptide (AEEQKLISEEDLLRKRREQLKHKLEQLRNSCA, Oncogene Science) was coupled to keyhole limpet hemocyanin as described (27), and injected subcutaneously into a rabbit. High-titer serum was obtained within six weeks of the initial injection.

Immunoblotting—Whole cells or the CHAPS-insoluble fraction, solubilized in sample buffer (26), were subjected to electrophoresis on 10% low-bis polyacrylamide gels (28), electrotransferred to polyvinylidene difluoride membranes (Millipore), blocked with 3% bovine serum albumin, and incubated as described (28) with cAR1 COOH terminus-specific antiserum (15) or anti-c-Myc (1:1000). Proteins were detected using alkaline phosphatase conjugated to donkey anti-rabbit-IgG antibodies (Sigma) and chemiluminescence. Quantitation of scans was performed using a digitizing scanner (Logitech) and Sigmascan Image software (Jandel).

Immunofluorescence—Washed cells (5×10^7/ml) were rapidly fixed in suspension with methanol, 1% formaldehyde (−10 °C, 15 min). Fixed cells were then pelleted (2000 rpm, 4 min, IEC rotor), washed four times with phosphate-buffered saline (PBS, 8 mM NaH_2PO_4, 2 mM K_2HPO_4, 0.8% NaCl, pH 7.4), incubated in PBS containing cAR1 antiserum (1:1000, 60 min; Ref. 29), washed four times in PBS, incubated 60 min in goat anti-rabbit fluorescein-isothiocyanate-conjugated antibodies (Cappel, Durham, NC), washed four times with PBS, and mounted on glass slides with mounting medium (PBS containing 90% glycerol and 1 mg/ml phenylenediamine, pH 8.5). Specimens were photographed using a Leitz Ortholux II fluorescence microscope. Antisera were preadsorbed with methanol-fixed vegetative AX-3 cells prior to use.

RESULTS

cAMP-induced Loss of Ligand Binding Is Time- and Concentration-dependent—We first characterized this process in growth-stage AX-3 cells expressing high levels (1–5 × 10^5 sites/cell) of exogenous cAR1 from a constitutive promotor. Cells were treated with cAMP, washed extensively, and residual cAMP binding examined. As shown in Fig. 1*A*, pretreatment with 10 μM cAMP induced a rapid ($t_{1/2}$ = 1.5–2 min) 80% reduction in 10 nM [^{3}H]cAMP binding. While 1 nM cAMP elicited a detectable loss, 10 μM was required to elicit the full effect (Fig. 1*B*). The EC_{50} of this response was 25 nM. These results for overexpressed cAR1 in growth-stage cells are similar to those reported for endogenous cAR1 in developed AX-3 cells ($t_{1/2}$ = 2–3 min, EC_{50} = 50 nM; Ref. 13), suggesting that the same process is occurring at these two developmental stages. Moreover, these properties are attributable to cAR1, since vector-transformed cells lacking cAR1 (pMC34/JB4; Ref. 16) exhibit less than 1% of the cAMP binding capacity described above (data not shown).

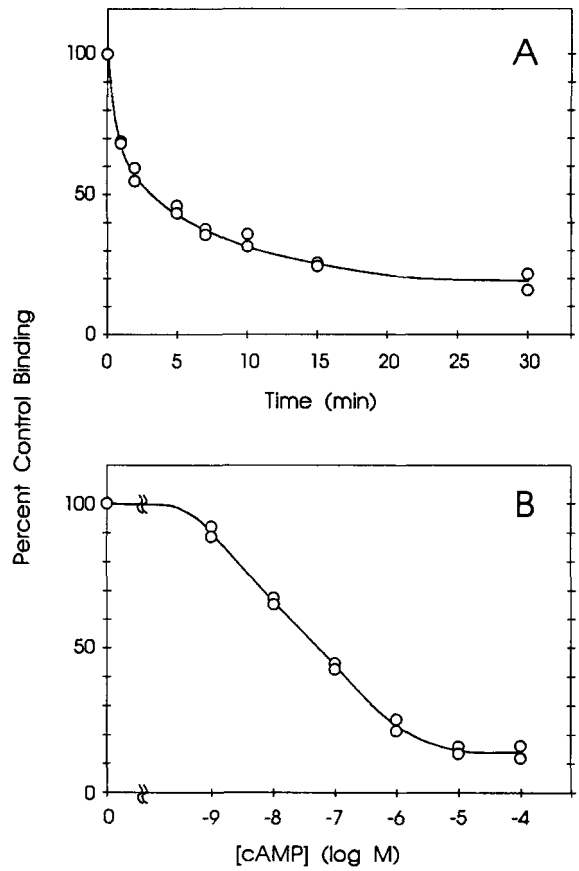

FIG. 1. **Kinetics and concentration dependence of cAMP-induced loss of cAMP binding in cells overexpressing cAR1.** *A*, washed, growth-stage cells were incubated without (0 min) or with 10^{-5} M cAMP for 1–30 min in the presence of 10 mM dithiothreitol, washed extensively, and cAMP binding measured at 10 nM by centrifugation through silicone oil as described under "Materials and Methods." *B*, washed, growth-stage cells were incubated with various concentrations of cAMP (0–10^{-5} M) for 15 min in the presence of 10 mM dithiothreitol and 5 mM caffeine, washed thoroughly, and [^{3}H]cAMP binding measured as in *panel A*. The means of triplicate determinations from each of two independent experiments are shown.

cAMP Pretreatment Results in a Reduction in cAR1 Affinity—Scatchard analyses were undertaken in order to elucidate the basis of the reduced binding observed in Fig. 1. As seen in Fig. 2*A*, untreated growth-stage cells exhibit a curvilinear cAMP binding profile. Computer-generated fits of these data (Table I, experiments 1 and 2) reveal both low and high affinity classes of binding sites with affinities consistent with those reported previously (46). Upon pretreatment with cAMP, the Scatchard plot remains curvilinear but appears more shallow than that of control cells (Fig. 2*A*). This change reflects the fact that cAMP induces a 70–80% reduction of binding when meas-

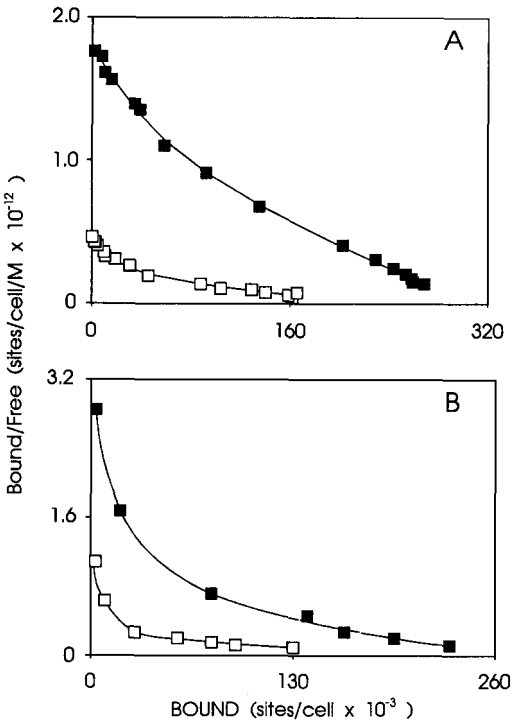

FIG. 2. **Scatchard analysis of [^{3}H]cAMP binding to cAR1 with and without cAMP pretreatment.** Growth-stage (*A*) or developed (*B*) cells overexpressing cAR1 were treated without (*filled squares*) or with (*open squares*) cAMP (10^{-5} M, 15 min, with 10 mM dithiothreitol), washed, and [^{3}H]cAMP binding measured at cAMP concentrations from 10^{-9} M to 2×10^{-6} M by centrifugation through silicone oil as described under "Materials and Methods." Data shown are the means from one of four (*A*) or two (*B*) independent experiments performed in triplicate. The lines represent the computer-generated fit for each set of data (Table I, experiments 1 and 3).

ured at 10 nM cAMP (as in Fig. 1) but only a 40% reduction of binding when measured at 2 μM cAMP (*rightmost points* in Fig. 2*A*). Fitting of these data also reveals two classes of binding sites (Table I, experiments 1 and 2), both of which appear to be affected by cAMP pretreatment. First and more dramatically, there is a 3–5-fold reduction in the cAMP affinity of the major, lower affinity class (from 293 ± 33 to 1117 ± 276 nM and from 454 ± 106 to 2442 ± 950 nM in experiments 1 and 2, respectively) without an appreciable loss of binding sites. Second, there is a small but reproducible reduction in the number of sites of the high affinity class (31,000 ± 10,000 to 13,000 ± 5000 sites/cell and 10,000 ± 6000 to 1000 ± 1000 sites/cell in experiments 1 and 2, respectively) with no consistent change in their affinity. There is relatively little reduction in the total number of cAMP binding sites (12 ± 7% and 5 ± 19% in experiments 1 and 2, respectively), suggesting that most cAR1 molecules remain on the cell surface following exposure to cAMP. Thus, while some of the loss of binding observed at very low cAMP concentrations (<35 nM) results from changes in both affinity components, the loss seen at higher subsaturating concentrations is largely attributable to the affinity reduction exhibited by the low affinity sites.

To assess whether this pattern of binding changes is a property unique to growth-stage cells, an identical analysis was performed using developed cells (Fig. 2*B*). As with growth-stage cells, the Scatchard plot of the untreated developed cells is curvilinear and cAMP pretreatment causes a shallowing of the plot. Again, much of the observed change results from a reduction in the affinity of the low affinity receptor form (Table I, experiments 3 and 4). The total loss of cAMP binding sites may be slightly greater than in growth stage cells (32 ± 6% and 15 ± 11% in experiments 3 and 4, respectively) but does not fully explain the loss of binding. It is unclear whether, in developed cells, there is a reduction in the number of high affinity sites analogous to that observed in growth-stage cells. An overall difference between growth-stage and developed cells is an apparent trend toward increased affinities of all receptor classes in the latter. This increase may reflect the expression of the G-protein subunit, Gα2, which mediates cAR1 signaling during aggregation (49).

Dose Dependence of cAMP Uptake Is Altered by cAMP Pretreatment—Based on the Scatchard results, we predicted that cAMP pretreatment should increase the dose dependence of cAR1-mediated responses without altering maximum responsiveness. To test this prediction, we examined the effect of cAMP pretreatment on the subsequent "uptake" of [^{3}H]cAMP. This cAR1-dependent process occurs more slowly than cAMP binding, is very slowly reversible, and does not represent persistent binding to the receptor (16, 25, 30). As shown in Fig. 3, we examined this process in growth-stage cAR1-expressing cells and found that, following cAMP pretreatment, little change in the uptake of high cAMP concentrations occurred while uptake at lower cAMP concentrations is significantly decreased (57% decrease at 10^{-9} M cAMP). This response profile is consistent with a reduction in the affinity of cAR1.

Localization of cAR1 Is Unchanged by cAMP Treatment—The Scatchard analysis indicates that cAMP pretreatment causes a marked alteration in the affinity of most of the receptor molecules on the cell-surface with little or no reduction in the total number of surface binding sites. To extend this observation, we assessed the subcellular localization of cAR1 in cAMP-treated cells by proteolytic treatment of intact cells and by immunofluorescence. For these experiments, we expressed cAR1 containing a c-Myc epitope at its amino terminus in cells. Immunoblots of extracts prepared from these cells revealed a 47-kDa protein with immunoreactivity toward both anti-cAR1 serum and a polyclonal anti-Myc serum (Fig. 4*A*). Its migration is slightly slower than that of wild-type cAR1, reflecting the addition of 12 amino acids. The Myc-tagged cAR1 was comparable to wild-type cAR1 in binding affinities (data not shown) and extent of loss of ligand binding (80 ± 6% *versus* 77 ± 1%, measured at 16 nM [^{3}H]cAMP).

Unlike the wild-type cAR1, the Myc-tagged cAR1 is sensitive to surface trypsinization. Within 15 min, more than 87% of the molecules are cleaved to yield slightly smaller products which have lost the Myc epitope (Fig. 4*B*, *left*). The time dependence of the cleavage strongly suggests that trypsinization is occurring on intact cells, and not during subsequent sample preparation. Co-incubation of samples from trypsin-treated and untreated cells results in no further loss of Myc-tagged cAR1 (data not shown), further supporting this conclusion. When the cells were pretreated with cAMP, the mobility of the Myc-tagged cAR1 on SDS-PAGE was observed to further decrease (Fig. 4, *right*). This mobility alteration, previously reported for wild-type cAR1, is due to serine phosphorylation on the cytoplasmic COOH-terminal domain (15). When the pretreated cells were subjected to proteolysis, removal of the Myc epitope occurred as extensively as it did in untreated cells (Fig. 4*B*, *right*). The slight decrease in the amount of Myc-cAR1 detectable with anti-cAR1 serum (approximately 30% at 15 min, Fig. 4*A*) might reflect cleavage at basic residues within the cAR1 sequence or an inherent instability in the NH_2-terminally cleaved Myc-cAR1. The failure of cAMP pretreatment to protect Myc-cAR1 from proteolysis is consistent with the notion that the reduction in cAMP binding observed upon pretreatment can occur without significant receptor internalization.

This interpretation was corroborated by immunofluores-

TABLE I
Scatchard analysis of cAMP binding data

The binding data illustrated in Fig. 2 were subjected to computer fitting using the program LIGAND. The K_d (nM) and B_{max} (sites/cell $\times 10^{-3}$) values for each site (± standard error) are given. *Results* from four independent experiments are shown. Also shown is the total B_{max} for untreated (control) and cAMP-treated (cAMP) cells in units of sites/cell $\times 10^{-3}$ and as a percentage of control cells.

	Growth stage				Developed			
	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Control	cAMP	Control	cAMP	Control	cAMP	Control	cAMP
K_d 1	35 ± 10	47 ± 20	30 ± 30	25 ± 90	7 ± 3	9 ± 3	8 ± 11	8 ± 3
B_{max} 1	31 ± 10	13 ± 5	10 ± 6	1 ± 1	17 ± 5	9 ± 2	16 ± 9	10 ± 2
K_d 2	293 ± 33	1177 ± 276	454 ± 106	2442 ± 950	313 ± 60	866 ± 177	183 ± 71	874 ± 276
B_{max} 2	265 ± 8	247 ± 17	135 ± 5	136 ± 26	237 ± 12	164 ± 13	152 ± 11	134 ± 15
B_{max} total	296 ± 13	260 ± 18	145 ± 8	137 ± 26	255 ± 13	173 ± 13	168 ± 14	144 ± 15
% control		88 ± 7		95 ± 19		68 ± 6		85 ± 11

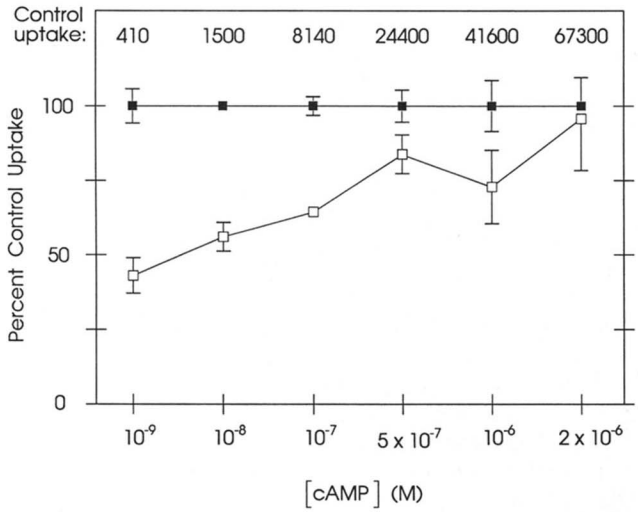

FIG. 3. **Effect of cAMP pretreatment on the dose dependence of [^{3}H]cAMP uptake.** Cells were incubated without or with cAMP (10^{-5} M, 15 min, with 10 mM dithiothreitol), washed, and [^{3}H]cAMP uptake determined at the various concentrations shown on the *abscissa* (10^{-9} to 10^{-6} M) as described under "Materials and Methods." Plotted for each concentration is the percentage of control uptake exhibited by control cells (*filled squares*, 100 by definition) and cAMP-pretreated cells (*open squares*). Values shown are the means ± standard deviations of nine (10^{-9} M and 2×10^{-6} M points) or two (remaining points) determinations pooled from three independent experiments. The mean absolute uptake exhibited by control cells (in units of molecules per cell) is indicated above each data point. Because the absolute uptake varied by as much as 2-fold from day to day, all uptake values were normalized to the average level of uptake exhibited by control cells for each experiment prior to the calculation of standard deviation.

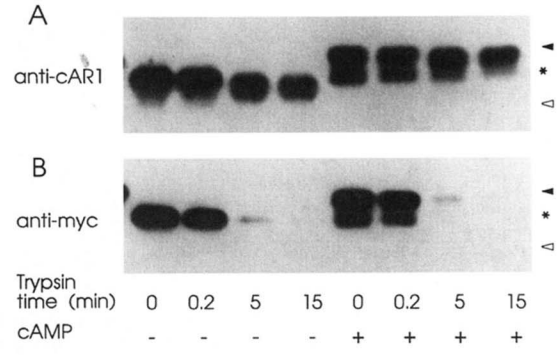

FIG. 4. **Effect of cAMP on protease accessibility of epitope-tagged cAR1.** Washed, growth-stage cells expressing Myc-cAR1 were shaken without (−) or with (+) cAMP (10^{-5} M, 15 min, 22 °C, with 10 mM dithiothreitol) and washed four times in ice-cold PB. They were then incubated in the absence (0 min) or presence of trypsin (50 μg/ml, 0 °C) for the indicated times. After addition of trypsin inhibitor (100 μg/ml) and extensive washing, a CHAPS-insoluble fraction was prepared, subjected to SDS-PAGE and immunoblotted with cAR1 COOH terminus-specific antiserum (*A*) or Myc-specific antiserum as described under "Materials and Methods" (*B*). The electrophoretic positions of wild-type cAR1 (*open arrowhead*, not shown), Myc-cAR1 (*asterisk*), and phosphorylated Myc-cAR1 (*filled arrowhead*) are indicated.

cence studies. When untreated cells expressing Myc-cAR1 were rapidly fixed in suspension, they exhibited a predominantly peripheral pattern of immunofluorescent staining with anti-cAR1 antibodies (Fig. 5*A*). This pattern is similar to that described previously for endogenous cAR1 in developed cells and suggests that most receptor molecules are within the plasma membrane. Despite a 74% reduction of binding at 16 nM [^{3}H]cAMP, cAMP-pretreated cells revealed the same pattern of cAR1 distribution as untreated cells (Fig. 5*B*), suggesting that little if any relocalization had occurred. Vector control cells expressing no cAR1 displayed no peripheral staining under these conditions (data not shown).

Defective Loss of Ligand Binding in Serine Substitution Mutants—cAMP stimulates the addition of 3–4 phosphates (29) to serines within the cytoplasmic carboxyl terminus of cAR1 (15). This domain contains 18 serine residues organized into four clusters (15). To determine whether these serines and perhaps their phosphorylation play a role in the agonist-induced loss of ligand binding, we examined this process in cAR1 mutants in which combinations of these four serine clusters had been deleted or substituted with alanine and glycine residues (15). As shown in Fig. 6, substitution of all of the serine clusters (mutant cm1234) completely abolishes cAMP-induced affinity reduction. Furthermore, while the elimination of serines in clusters 2, 3, and 4 (mutant cm234) has little effect, substitution of the serines of cluster 1 alone (mutant cm1) results in a drastically reduced response. Thus, serines in cluster 1 appear to play a major and specific role in the modulation of cAR1 affinity classes.

DISCUSSION

The nature of loss of ligand binding by G-protein-coupled receptors has been difficult to ascertain. Often, this process has been equated with receptor internalization. Studies using immunofluorescence (31, 32) and cell fractionation (33, 34) have revealed a strong correlation between the loss of ligand binding and the "sequestration" or movement of receptors to a new, apparently intracellular compartment. Consistent with this interpretation, cells expressing β-adrenergic and muscarinic receptors, upon pretreatment, lose their ability to bind hydrophilic but not hydrophobic ligands (35). While the reduction in binding and internalization may be tightly correlated, however, they might be separate processes. Several studies, in fact, have suggested that loss of binding can occur without receptor internalization (36, 37). Previous studies of cAR1 (38, 39) and our present results support this hypothesis.

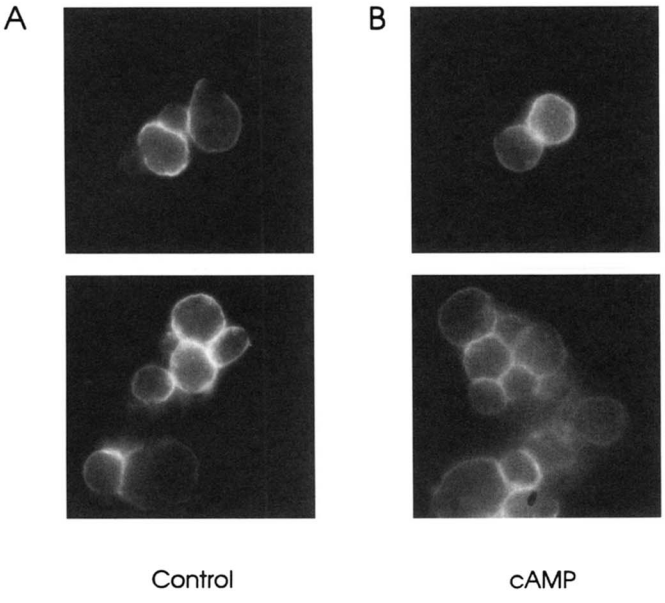

FIG. 5. **Effect of cAMP pretreatment on cAR1 immunofluorescence.** Washed, growth-stage cells expressing Myc-cAR1 were shaken without (*A*) or with (*B*) cAMP (10^{-5} M, 12 min, 22 °C) and washed four times with ice-cold PB. They were then fixed in suspension with methanol, 1% formaldehyde, stained with cAR1 antiserum (29) and fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies as described under "Materials and Methods." Pretreated cells in this representative experiment displayed 74% less binding to 16 nM [^{3}H]cAMP than control cells, when assessed at the time of fixation. The cells shown are approximately 12 μm in diameter.

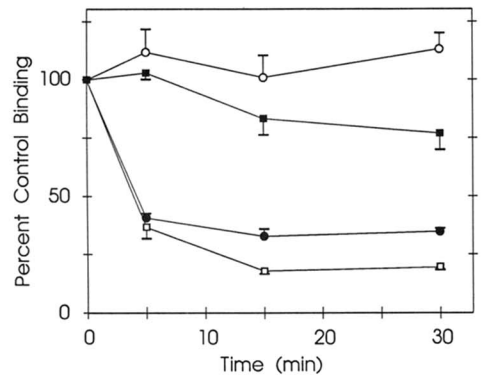

FIG. 6. **Loss of ligand binding in cAR1 mutants lacking cytoplasmic serine residues.** Washed growth-stage cells expressing wild-type cAR1 (*open squares*), cm234 (*filled circles*), cm1 (*filled squares*), or cm1234 (*open circles*) were shaken without (0 min) or with cAMP (10^{-5} M, 22 °C) for 5, 15, or 30 min and washed extensively with PB. [^{3}H]cAMP binding was then measured at 16 nM by the sedimentation assay, as described under "Materials and Methods." Data shown represent the means ± S.E. of three experiments performed in triplicate.

We demonstrate here that in response to cAMP pretreatment, growth-phase *Dictyostelium* cells overexpressing cAR1 exhibit a reduction of cAMP binding similar in rate, extent, and concentration dependence to that displayed in developed cells expressing endogenous cAR1. Scatchard analysis and computer fitting of cAMP binding data suggest that this binding alteration is due predominantly to a reduction in the affinity of the lower affinity receptor form, which represents the bulk of cell surface cAR1, with a concomitant small change in the number (but not the affinity) of high affinity binding sites. It is unclear whether this latter change represents an interconversion of high to low affinity sites or a selective removal of high affinity sites from the cell surface. Nevertheless, there is a minimal reduction in the total number of binding sites under these conditions, supporting the hypothesis that even at saturating cAMP concentrations, at least 80% of cAR1 molecules are neither internalized (38, 39), "irreversibly" occupied with ligand (25) or incapable of binding for other reasons. Developed cells overexpressing cAR1 exhibit a similar pattern of affinity changes, demonstrating that this phenomenon is not a function of the developmental stage. There is some suggestion from the present data, however, that in these cells, cAMP might induce a slightly higher fractional receptor internalization than in growth-stage cells.

The binding affinity changes described above are mirrored functionally by changes in cell sensitivity to cAMP. The concentration dependence of cAR1-mediated cAMP uptake, a process distinct from binding, is altered by pretreatment. As with binding, pretreatment causes an apparent transition from high to low sensitivity of uptake, with little or no change in maximal uptake.

A different conclusion was drawn in previous studies involving endogenous cAR1 in developed cells. There, Scatchard analysis suggested that loss of ligand binding was due to a reduction in total cAMP binding sites, with little change in affinity (12, 13, 25). We attribute these differences to our use of cells overexpressing cAR1, which enhances the detection of low affinity binding sites. In cAMP binding experiments involving endogenous cAR1, the expression levels are lower than those presented here, resulting in a lower signal-to-noise ratio at higher cAMP concentrations, and thereby precluding the detection of low affinity binding sites. In addition, our initial analysis of growth-stage cells, which exhibit less curvilinear Scatchard plots and less reduction in total binding, also facilitated the detection of changes among the low affinity sites.

The protease accessibility and immunofluorescence experiments described here provide physical evidence that nearly all cAR1 molecules remain on the cell surface following cAMP pretreatment. The immunolocalization of cAR1 in cAMP-treated and untreated cells is virtually indistinguishable when these cells are rapidly fixed in suspension, suggesting there is not significant internalization. Furthermore, even after maximal induction of the response, nearly all epitope-tagged cAR1 molecules remain susceptible to surface trypsinization. Consistent with these results, sustained chymotrypsin sensitivity of endogenous cAR1 following cAMP pretreatment has been previously reported (39). Our results with epitope-tagged cAR1 strengthen the proposal put forth by these authors that loss of ligand binding can occur without cAR1 internalization.

Our immunofluorescence results appear to contradict the previously reported observation that cAR1 localization changes upon cAMP pretreatment from a peripheral pattern to a more punctate, possibly vesicular, one (32, 38). We believe that this difference arises from the distinct treatment of the cells used in each case. In the previous studies, cells were allowed to adhere to glass slides, flattened with a sheet of agar, and then methanol-fixed. In the present study, in contrast, shaking cells were rapidly fixed in suspension. We too have found that adherent cells exhibit an apparent cAMP-induced redistribution of cAR1 immunofluorescence (data not shown), unlike cells fixed in suspension. Perhaps cAMP treatment normally induces cAR1 relocalization in the physiological context of a cell attached to a solid surface, while in suspension, the internalization process is uncoupled. Thus, the transition in cAR1 affinity appears not to depend upon internalization, though it might reflect an early step in this process, such as the binding of a component of the endocytic machinery to the receptor.

Several domains within G-protein-coupled receptors have been described as important for agonist-induced reductions in ligand binding. Mutant adrenergic, muscarinic, or cAMP receptors bearing substitutions or deletions within the putative sec-

ond and third intracellular loops have shown drastic impairment in this process (16, 40–42). Replacement of a human β_2-adrenergic receptor tyrosine (Tyr^{326}) with an alanine has no influence on G-protein activation or desensitization with respect to adenylyl cyclase activation but blocks loss of ligand binding, receptor relocalization, and resensitization of the cyclase response (43). This tyrosine occurs within a sequence motif near the COOH-terminal end of the putative seventh transmembrane domain of many G-protein-coupled receptors ($NPX_{2-3}Y$, where X represents aliphatic residues). A similar phenotype is seen with β-adrenergic receptor mutants in which putative targets of protein kinase A phosphorylation have been changed to alanines (44, 45). Nevertheless, removal of all serines from the COOH terminus of this receptor does not prevent the agonist-induced reduction of ligand binding (44), suggesting that β-adrenergic receptor phosphorylation is unnecessary for loss of binding.

Our present studies with cAR1 suggest that a subset of COOH-terminal serines is required for loss of cAMP binding. A cAR1 mutant lacking all serines in the cytoplasmic COOH terminus cannot undergo this process. Substitution of the 5 serines that comprise cluster 1 (serines 299, 302, 303, 304, and 308) with alanine and glycine residues (mutant cm1) results in a severe reduction in both the rate and extent of the agonist-induced binding changes, while substitution of all other serines in the COOH-terminal tail has virtually no effect. We previously demonstrated (15) that approximately two-thirds of the cAMP-induced phosphorylation of cAR1 occurs within serine cluster 1 and the remainder occurs within cluster 2 (serines 324, 325, and 331). Which of these residues are modified has not yet been determined. The remaining 10 serines of the COOH-terminal domain, which comprise clusters 3 and 4 (as defined in Ref. 15) are not phosphorylated in response to cAMP binding. Phosphorylation occurring within cluster 1 is strongly correlated with the adaptation of cAMP-stimulated adenylyl cyclase (11, 29). Thus, the affinity transition and phosphorylation undergone by cAR1 in response to cAMP appear to share either common or overlapping structural requirements within this domain.

cAR1 contains two NPX_nY motifs similar to that implicated in β_2-adrenergic receptor loss of ligand binding (5, 43). The first of these ($NPLMWRY^{277}$) like those of other receptors is located at the COOH-terminal portion of the seventh transmembrane domain. The second ($NPSPY^{301}$) includes Ser^{299} of serine cluster 1 in the COOH-terminal cytoplasmic domain. It is tempting to speculate that the latter is important for the loss of ligand binding in light of its proximity to cluster 1. Note, however, that in mutant cm1, which is impaired in loss of ligand binding, this sequence ($NPAPY^{301}$) resembles the proposed consensus sequence even more closely than does the wild-type sequence. The mechanisms by which serine replacement affects cAR1 loss of ligand binding therefore remain to be clarified.

In summary, agonist-induced loss of cAMP binding sites appears to involve a 3–5-fold reduction in the affinity of the predominant low affinity receptor form and is not dependent upon receptor internalization. The reduction in affinity could, however, reflect an intermediate step in the internalization process. Furthermore, this affinity transition depends upon a domain in the cAR1 cytoplasmic carboxyl terminus, which is also a target of cAMP-induced phosphorylation. Continued analysis of these processes should allow a detailed molecular description of the various adaptive changes undergone by cAR1 upon agonist binding.

Acknowledgments—We thank Dr. Stephen Gill and Dr. Geoffrey Pitt for helpful discussions regarding epitope tagging and immunofluorescence, Ji Yun Kim for help with the preparation of the anti-Myc antiserum, and Dr. Peter Munson for providing the LIGAND software.

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