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Molecular Basis of Transmembrane Signal Transduction in *Dictyostelium discoideum*

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INTRODUCTION

Living cells are in a constant process of information exchange with their environment. The kind of information to which cells can respond is often chemical and largely determined by the assortment of receptors they possess on their surface. Occupation of cell surface receptors by a specific ligand (or excitation by light, in the case of photoreceptors) triggers a sequence of reactions between proteins in the plasma membrane, which results in the alteration of the activity of effectors: membrane-bound enzymes or ion pores. This process is called transmembrane signal transduction or, briefly, signal transduction.

Receptors either have direct effector functions (i.e., are multifunctional molecules) (e.g., see references 43 and 93) or are coupled to effectors via transducers (14, 15, 115). The latter have been identified as guanine nucleotide binding proteins, or G-proteins for short (15, 103, 271). The heterotrimeric G-proteins, of which there are at least four types, regulate the activity of enzymes such as adenylate cyclase (15, 103), cyclic guanosine 3′,5′-phosphate (cGMP) phosphodiesterase (271), phospholipase C (170), and maybe also some ion pores (23, 233), as well as guanylate cyclase (164). The levels of the intracellular regulatory compounds (second messengers) regulated by these enzymes and ion pores have a profound influence on cellular function (41, 102, 218). Some of the protein kinases through which the second messengers exert their effects on cellular functions also phosphorylate components of the signal transduction system itself. This, together with phosphorylation by receptor-specific kinases, appears to be a crucial event in the attenuation of responsiveness, generally referred to as desensitization (10, 11, 133, 166, 237, 267, 270). Most of this knowledge has been obtained from the study of vertebrate signal transduction systems, which nowadays serve an exemplary function for research on signal transduction in other organisms, including the eucaryotic microbes.

The eucaryotic microbe *Dictyostelium discoideum* has long been used as a model system for cell differentiation and pattern formation because of its life cycle. Within 24 h after the removal of nutrients, the solitary amoebae of this slime mold aggregate and form a multicellular fruiting body which consists of two cell types (17). Obviously, a major factor in this developmental process is cell-cell communication. First, solitary cells attract one another chemotactically (53, 97, 215, 243). Second, cell differentiation and the spatial distribution of the cell types in the multicellular aggregate are regulated by cell-cell contacts and diffusable factors secreted by the cells. These cell interactions work in concert with other factors, such as cell cycle phase, to control development (176, 208, 264, 272). Since the late 1960s, several compounds have been found that transmit information between *D. discoideum* cells. The first to be identified was cyclic adenosine 3′,5′-monophosphate (cAMP) (161). cAMP is the chemoattractant that governs the aggregation of solitary living cells after food exhaustion (54, 94); furthermore, it stimulates differentiation of the starving cells (39, 208, 272). Soon after, the proteins that detect and metabolize cAMP were discovered. These include cAMP receptors (105, 110, 180), adenylate cyclase (145, 245), and cAMP-phosphodiesterase (36, 181, 229). In the 1970s, other signal molecules were discovered: compounds with chemotactic activity for solitary *D. discoideum* cells, such as folic acid

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and pterin (226, 227), and factors that affect multicellular stages, such as differentiation-inducing factor (DIF) (284), slug-turning factor (85), spore-inducing factor (329), and adenosine (216, 257). The discovery of these signal molecules together with cellular responses to each of them allowed *D. discoideum* to become a useful model system for the study of signal transduction.

PHYSIOLOGY OF SIGNAL TRANSDUCTION IN *D. DISCOIDEUM*

Vegetative amoebae of *D. discoideum* feed on bacteria. It is supposed that they find their food by means of chemotactic attraction to folic acid and other pteridines secreted by bacteria (226, 227). Mutant *D. discoideum* strains (termed axenic strains) that can grow in artificial liquid media of defined composition (89, 236) have been isolated (273; reviewed in reference 212). Use of these strains in axenic culture allows a more rigid control of growth conditions and the culture of cells on a larger scale. When food is exhausted, cells start to differentiate, resulting within 24 h in the formation of a multicellular fruiting body (17). About 5 h after the initiation of starvation, cells acquire the capacity to attract one another by means of a chemotactic machinery, acting in concert with a signal relay system. *D. discoideum* cells start to secrete the chemoattractant cAMP. Other slime molds secrete other chemoattractants such as pteridines in *D. minutum* and *D. lacteum* (67, 299) or a modified peptide in *Polysphondylium violaceum* (265). During 1 to 2 h after initiation of the chemoattractant secretion, several thousand cells aggregate and stick together by means of cell adhesion molecules located on the cell surface (8, 17, 22). This aggregate starts to behave as an organized multicellular individual, moving over the substratum seeking the best conditions for formation of a fruiting body. Within the aggregate, two major cell types become apparent: prestalk cells in the anterior and prespore cells in the posterior part. These give rise to stalk and spore cells in the fruiting body (17).

The physiology of signal transduction during the aggregation process has been described in detail in a number of reviews (39, 53, 54, 90, 94, 186, 215, 243, 287, 306). Here, only the most prominent features are dealt with.

By the time of aggregation, each *D. discoideum* cell has acquired the ability to respond chemotactically to extracellular cAMP and to transmit this signal to its neighbors (signal relay). Some cells in the population spontaneously start to emit small pulses of cAMP at a frequency of once per 6 to 9 min. Neighboring cells detect this cAMP by means of cell surface receptors and move towards the emitted signal. Concomitantly, adenylate cyclase is transiently activated at least 3- to 10-fold in these cells (53, 148, 245, 248), maximal activity being reached about 1 min after stimulus presentation (53). As a result, intracellular cAMP rises, peaking 1 to 3 min after the first contact of the cells with extracellular cAMP. This cAMP is secreted at a rate proportional to its intracellular concentration (53, 292), thereby increasing cAMP extracellularly. A neighboring cell will react chemotactically to this secreted cAMP and relay the signal by the same mechanism to the next neighbor. A wave of cAMP thus travels from cell to cell (283). When adenylate cyclase is not activated, the concentration of environmental cAMP is decreased by the action of extracellular and cell surface cAMP-phosphodiesterase (36, 181, 229). Regulation of the extracellular cAMP concentration by secreted phosphodiesterase and its inhibitor (139, 242) must be a prerequisite for proper cell aggregation, because a phosphodiesterase-deficient mutant is unable to aggregate unless exogenous phosphodiesterase is supplied (49). There is no evidence that active regulation of phosphodiesterase is necessary for the generation of the cAMP relay response.

TABLE 1. Responses known to be induced by cAMP in aggregation-competent *D. discoideum* cells

Responses	Reference(s)
Activation of adenylate cyclase and secretion of produced cAMP from the cells	53, 148, 245, 248, 292
Decrease in optical density of cell suspension	95
Fluctuations in amt of actin associated with Triton-insoluble cytoskeletons	203, 205
Increases in phosphorylation of heavy and light chains of myosin	12, 179, 237
Increase in amt of cell-associated Ca^{2+}	30, 328
Increase in intracellular concn of cGMP	201, 336
Alterations in level of methylation of phospholipids	198
Efflux of K^+ ions from the cells	2
Efflux of protons from the cells	182, 183
Alterations in no. of various intracellular vesicles	177

The responses of aggregating cells to extracellular cAMP are most conveniently investigated with cell suspensions (95) or with cells on filter disks in a perfusion apparatus (56). In both setups, large numbers of cells can be activated synchronously. Of all the responses to chemoattractants, the most thoroughly studied is the cAMP relay response. This focus of attention derives from the fact that the biological significance of this response is clear, as explained above. Other responses induced by addition of cAMP to a suspension of aggregation-competent cells are listed in Table 1. Most of the listed responses have been confirmed by various groups since their original discovery (see reviews in references 53, 90, and 306). An increase in protein carboxyl methylation has also been reported to be a response to cAMP (198, 315), but subsequent investigations suggest that this conclusion was based on artifacts (317). Except for the cAMP-induced Ca^{2+} accumulation (30), all of the responses are transient; i.e., the induced change returns partially or completely to prestimulus levels, even while the stimulus persists.

Several of the responses induced by cAMP in aggregating cells were also found to be induced by folic acid in vegetative cells, or in cells starved for a few hours (2, 53, 201, 203, 334, 335). Some of the responses have also been shown to occur in slime mold species other than *D. discoideum* following application of the chemoattractants that these species use (193, 204, 289, 310, 331). The cAMP response to folic acid of *D. discoideum* cells that have been starved for a few hours is atypical in that this response appears not to result from a direct stimulation of adenylate cyclase via folic acid receptors. Rather, it might be mediated via cAMP receptors (55). Such starved cells spontaneously secrete small amounts of cAMP; folic acid seems to make cells more sensitive to cAMP. As a result, these cells become stimulated by their own secreted cAMP when folic acid is presented (55). The other folic acid-mediated responses appear to operate differently. Folic acid or other pteridines induce responses such as chemotaxis, a transient decrease in optical density, fluctuations in the association of actin to cytoskeletons, and a

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transient increase in cGMP in vegetative *D. discoideum* cells (201, 203, 227, 334, 335) or in other slime mold species (193, 195, 204, 310), both of which are insensitive to cAMP. Furthermore, adaptation of *D. discoideum* cells to cAMP does not abolish these same responses to folic acid (see below; 55, 205, 289, 290). Finally, folic acid induces a decrease in optical density even in the presence of caffeine (55), a compound that inhibits cAMP secretion (25). Therefore, it is unlikely that these responses to pteridines are mediated via cAMP receptors.

The changes in optical density (95), in cytoskeletal actin (204), and in the phosphorylation of myosin (12, 237) following application of cAMP to aggregation-competent *Dictyostelium* cells probably reflect the chemotactic movement in response to chemoattractants. The chemotactic movement (91, 96, 98) as well as the optical density (95) and actin (203) responses are all observed within a few seconds after receptor occupation. An increase in cell-associated ^{45}Ca, accumulated from the extracellular medium, was observed within 6 s after application of chemoattractants (30). In cell-free extracts, Ca^{2+} regulates the organization of the contractile proteins actin and myosin (269) and inhibits myosin heavy-chain kinase (187). Local application of the Ca ionophore A23187 to an amoeba in the presence of extracellular Ca^{2+} causes a pseudopod to extend at that site (178). Therefore, it was proposed that the chemotactic reaction is regulated by an influx of extracellular calcium ions (e.g., see references 30 and 178). However, in a recent study Europe-Finner and Newell observed that the initial rate of ^{45}Ca uptake was independent of the presence or absence of chemoattractants (78). There was, however, a difference in the extent of ^{45}Ca uptake, which became evident only 10 to 30 s after presentation of chemoattractant to the cells (78). This result suggests that the accumulation of extracellular Ca^{2+} is a slow response, eventually involved only in the regulation of other slow responses.

Whether or not extracellular Ca^{2+} is essential for chemotaxis and cell aggregation is a matter of debate, indeed. Some investigators have reported that chemotaxis, cell aggregation, or the light-scattering response of cells is inhibited by a Ca^{2+} chelator such as EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (178, 188). Other investigators have reported that these responses take place when the extracellular Ca^{2+} concentration is low (193, 200, 253). Part of this controversy was resolved by Europe-Finner et al. (77), who observed that the sensitivity of aggregation to EGTA was strain dependent. With some strains aggregation was inhibited by EGTA only after thorough washing of the cells in the presence of EGTA (77). Such washing might deplete intracellular Ca^{2+} pools. Thus, these results (77) might not indicate that extracellular Ca^{2+} is needed for chemotaxis and cell aggregation, but that intracellular Ca^{2+} is required. This conclusion would not be surprising because Ca^{2+} ions play a role in the cell motility apparatus (269). In fact, discussion of the possible role of Ca^{2+} in the regulation of the chemotactic and other responses is hindered by lack of measurements of cytosolic Ca^{2+} concentrations during chemotactic stimulation. This deficiency is a consequence of the problem that the widely used Ca^{2+} indicator Quin 2, and related compounds, is difficult to introduce into and is not easily hydrolyzed by *D. discoideum* cells (H. Padh and M. Brenner, Cell. Slime Mold Newsl., vol. 54, March 1984; M. van Lookeren Campagne and R. Aerts, unpublished observations). At present it cannot even be rigorously excluded that chemoattractants merely induce an increase in Ca^{2+} binding to the cell surface rather than affecting its uptake. However, this possibility seems unlikely.

Besides the cAMP relay response and the role of Ca^{2+} in signal transduction, an intensively studied response to chemoattractants is the transient rise in intracellular cGMP. The maximal cGMP concentration in wild-type *D. discoideum* cells is reached around 10 s after stimulus presentation (196, 336). This response probably results from a stimulation, at least three- to sixfold, of the activity of guanylate cyclase (197). About 20% of the accumulated cGMP is secreted; the remainder is degraded intracellularly (310). cGMP appears to have a role in regulating the chemotactic machinery, as suggested by observations on a so-called streamer F mutant (215, 252). This mutant shows dramatically prolonged periods of chemotactic movement during cell aggregation. These periods correspond with prolonged periods of elevation of intracellular cGMP, following stimulation with chemoattractant. The mutant possesses little intracellular cGMP-phosphodiesterase (252, 311). How cGMP regulates chemotaxis or other cellular processes in *D. discoideum* is unknown; maybe it acts via the intracellular cGMP receptor protein that has been demonstrated (202, 312). Whether or not this receptor protein regulates a cGMP-dependent protein kinase as do receptor proteins in other cells (102, 116) is unknown.

Less is known about the significance of the other responses to *D. discoideum* signal transduction. A study using transmethylase inhibitors suggests that there is no direct relationship between phospholipid methylation and adenylate cyclase activation or cAMP secretion (316). Phospholipid methylation (198) and also K^+ and proton efflux (2, 182, 183) might play a role in the slower responses of cells to chemoattractants, e.g., the stimulation of cell differentiation. The correlation of the signal relay process with changes in intracellular vesicles (177) points to the possibility that cAMP produced by the activated adenylate cyclase is secreted from the cells in vesicles.

As mentioned above, almost all known responses of *D. discoideum* cells to chemoattractants are transient. In fact, this is a prerequisite for the functioning of the signal transduction system, because cells can (and do) stimulate themselves via their surface cAMP receptors with the secreted cAMP, produced by the activated adenylate cyclase (53, 56). As a result of this positive-feedback loop, activation would go on indefinitely, or in practice, until the cells were exhausted, were it not that the adenylate cyclase response adapts. We define adaptation here as the characteristic of a response to terminate (not just to diminish), even though the stimulus remains present at the same level. It is meant here as a specific case of homologous desensitization, which is a type of response attenuation encountered in almost all signal transduction systems (162, 267). Activation and adaptation of the cAMP relay response in *D. discoideum* were investigated in detail by Devreotes and co-workers (58, 72–74). The response can be adequately described by assuming separate excitation and adaptation processes (53, 73, 74). Following application of a stimulus, excitation and adaptation build up to a new level that is the same for both and is determined by the magnitude of the stimulus. However, adaptation builds up more slowly than excitation. The transient excess of excitation over adaptation determines the magnitude of the response, i.e., the activation of adenylate cyclase. After excitation and adaptation have reached their equilibrium levels, further activation can only be obtained by increasing the stimulus level, up to a concentration which saturates the system (10^{-5} M cAMP; 58). After removal of the stimulus

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cells recover their ability to give a cAMP relay response to the same or lower stimulus levels with a half-life ($t_{0.5}$) of 3 to 4 min (deadaptation) (73). Other responses of *D. discoideum* for which adaptation has been studied in some detail are the chemotactic response (3, 91, 290), the optical density change (200, 333), the cGMP response (289, 308, 333), the changes in cytoskeletal actin (205), and the increase in myosin phosphorylation (12).

The availability of different chemoattractants that can be sensed by the same cell offers the possibility of investigating the interaction between various transduction pathways. A phenomenon encountered in many organisms is heterologous desensitization: prolonged application of a specific signal compound leads to a decrease in sensitivity of the cell to other signal molecules that act via receptors other than those that recognize the compound presented (267). Heterologous desensitization has not yet been demonstrated in *Dictyostelium* spp. The pathways of adaptation to folic acid and to cAMP proved to be separate for all responses investigated so far, namely, chemotactic cell movement (290), cGMP response (289), and changes in cytoskeleton-associated actin (205). Application of folic acid does not make cells insensitive to a cAMP stimulus and vice versa. This suggests that adaptation results from alterations in components which are unique to the folic acid and cAMP signal transduction pathways, for instance, the cell surface receptors for either of these compounds.

cAMP RECEPTORS IN *D. DISCOIDEUM*

Introduction

Starvation of *D. discoideum* cells triggers the appearance of cAMP receptors (105, 110, 180), adenylate cyclase (145, 224), cAMP phosphodiesterase (144, 181), and cell adhesion molecules (8, 22); all contribute to cell aggregation. The development of cAMP receptors in cell suspensions of starving *D. discoideum* cells can be accelerated by pulses of cAMP (149, 246, 338) and folate (134). The number of cAMP receptors on cells is maximal at the time of aggregation; subsequently, the number decreases (105, 110). Receptors specific for cAMP have been found on the surface of aggregating cells of four *Dictyostelium* species that use cAMP as chemoattractant; three other species that use other chemoattractants lack detectable cAMP binding activity during the aggregation period (192, 210, 256). However, all of these species acquire cAMP receptors in later multicellular phases of differentiation, during which cAMP appears to act as a morphogen (256).

A challenge after the discovery that cAMP acts as chemoattractant of *D. discoideum* during cell aggregation (161) was to measure receptors without interference by cell surface phosphodiesterase, which is abundant on aggregating cells (144, 181). Both activities were initially discriminated by exploiting the observation that cGMP is a good substrate for the enzyme, but a weak chemoattractant (110, 160, 180). Soon various other cAMP derivatives were found that discriminate between chemotaxis and receptor binding on the one hand and hydrolysis by phosphodiesterase on the other hand (97, 191, 194). The discrimination between proteins with enzymatic or binding activities (including the intracellular cAMP-dependent protein kinase) on the basis of their substrate specificity has become much easier in recent years because of the increased knowledge about the cyclic nucleotide specificities of these proteins (60, 291, 303–305). Furthermore, cAMP-phosphodiesterase activity was found to be inhibited by sulfydryl compounds such as dithiothreitol (105, 110, 229). Inclusion of this compound in binding assays enables cAMP receptors to be measured without interference by phosphodiesterase.

Ligand Specificity and Activation Mechanism of the Receptor

The structural requirements for ligand binding to the cell surface cAMP receptor were defined investigating the competition of 16 nucleotides with the binding of radioactive cAMP to cells (305). Competition was tested at two cAMP concentrations, 10^{-9} and 10^{-7} M, so that the specificity of both the low- and high-affinity receptor forms (see next subsection) was studied. The potency of inhibition for all nucleotides was the same at both cAMP concentrations, suggesting that low- and high-affinity receptor forms with different binding specificities do not exist (305). The binding affinity of various derivatives for the receptor suggests that cAMP probably binds to the receptor in the anti conformation; the adenine moiety is probably positioned in a hydrophobic cleft, while two H-bridges are formed: one with the amino group on the purine and one with the 3′-oxygen atom of the cyclic phosphate group (Fig. 1) (305).

The specificity of binding of about 10 cAMP analogs to the receptor is closely correlated with the specificity of induction of several biological responses, including chemotaxis (191, 291, 304), the cGMP response (305), the cAMP relay response (279), the stimulation of synthesis of phosphodiesterase in early development (as determined at threshold concentrations) (309), and expression of various genes later in development, determined by synthesis of specific proteins (255) or messenger ribonucleic acids (109, 221). This correlation suggests that all of these responses are triggered via a similar kind of receptor protein.

Several compounds have been found that antagonize biological responses (chemotaxis or the cGMP response) at concentrations that are approximately half-saturating for binding (291, 301, 305). Some of these compounds were classified as partial antagonists: at low concentrations they inhibited chemotaxis to cAMP, but at higher concentrations they became attractants (291, 301). Two full antagonists for chemotaxis, the cGMP response and the cAMP relay response, have been found. These compounds competitively inhibited responses to cAMP, without themselves eliciting a response at any concentration (291, 305). Both full antagonists are analogs of cAMP, modified in the phosphate moiety of the molecule, namely, the Rp isomer of cAMP-S and the Rp isomer of cAMP-dimethylamidate (291, 301). The structures of these antagonists suggest that the configuration of the cyclic phosphate group is critical for activation of the receptor. Comparison of the chemical structures of antagonists and agonists (like the Sp isomers of both cAMP analogs) led to the proposal that the receptor becomes covalently linked to the cyclic phosphate group during activation of the receptor (301).

Theoretically, a receptor can respond to its ligand in two ways: an occupancy receptor responds in proportion to the duration of occupation with ligand; a rate receptor responds in proportion to the number of associations with ligand (231, 288). Thus, an occupancy receptor gives the greatest response with tight-binding agonists, whereas a rate receptor gives maximal responses with agonists that rapidly exchange, i.e., that have a high dissociation rate and, consequently (most often), a low affinity. The latter type of response was found when the induction of total cellular and

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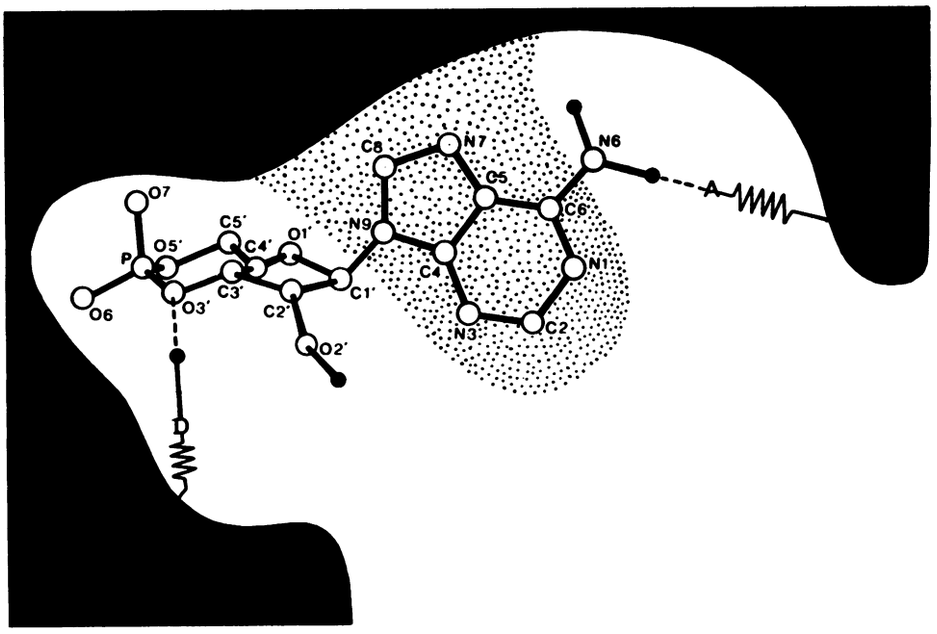

FIG. 1. Model of the binding of cAMP to the cell surface cAMP receptor in *D. discoideum* based on the study of the competition of cAMP binding by cAMP derivatives. Binding of cAMP is in the anti configuration, via two hydrogen bonds, at N_6 and $O_{3'}$, and with the adenine moiety in a hydrophobic cleft (indicated by dots). Reproduced from reference 305, with permission.

secreted phosphodiesterase by pulses of cAMP and cAMP analogs was investigated (309). At high concentrations, low-affinity analogs induced higher levels of phosphodiesterase than did high-affinity analogs. These results suggest that the *Dictyostelium* cAMP receptor functions as a rate receptor (309). It might have been useful if this hypothesis had been confirmed by investigation of the induction of markers (or cellular responses) other than phosphodiesterase. However, in the meantime we have obtained insight into the molecular interactions of receptors and G-protein(s) (see below); we presently consider such insight more fundamental than the distinction between the occupancy- and rate-receptor concepts.

Kinetic Properties of the cAMP Receptor

In the late 1970s, investigators observed that much of the cAMP-receptor complex of *D. discoideum* cells dissociates very rapidly (141, 210). Present binding assays therefore use filtration without washing (44, 105), sedimentation of cells or membranes without washing (119, 180), or sedimentation of cells or membranes through silicone oils (247). The relevance of data derived from the so-called ammonium sulfate stabilization assay (305) to the in vivo situation is not always apparent, because ammonium sulfate decreases the dissociation rate of the cAMP-receptor complex and alters the affinity and the site distribution of the kinetic receptor forms (see below). In addition, ammonium sulfate exposes latent binding sites and down-regulated receptors (120, 295, 297).

Curves relating the equilibrium binding of cAMP to intact cells or isolated membranes are nonlinear, indicating the existence of site heterogeneity or negative cooperativity or both (44, 105, 150, 210). Assuming site heterogeneity, these equilibrium binding curves are compatible with the existence of two classes of receptors with K_ds of about 10 and 150 nM (44, 105, 210). Evidence for positive cooperativity in equilibrium cAMP binding has also been found (44, 120, 207, 217).

The earlier studies on the kinetics of cAMP binding (141, 210) have recently been extended, producing direct evidence for the existence of receptor heterogeneity and cooperativity in cAMP binding. Four different kinetic receptor forms, which differ from each other in dissociation rate or apparent affinity, have been distinguished on *D. discoideum* cells (298, 300). These forms have been called H, L, S, and SS (Table 2). cAMP bound to the H and L forms dissociates relatively fast, but the apparent affinity of the L form is much lower than that of the H form (Table 2) (298). During association of cAMP with cells, the H form converts to the L form with a $t_{0.5}$ of about 10 s (298). As soon as the cAMP is removed, the affinity of cAMP binding is restored with a $t_{0.5}$ of 70 s (29); this probably reflects the formation of the original H form.

The S and SS forms have a similar affinity for cAMP, but they are easily distinguished by dissociation kinetics; both dissociate slowly, but at 10-fold different rates (Table 2). The

TABLE 2. Kinetic cAMP receptor forms observed in *D. discoideum* cells and isolated membranes[a]

Receptor form	Apparent K_D (nM)	Dissociation rate constant, k_{-1}, 20°C (s^{-1})[b]	No. of sites per cell	Effect of guanine nucleotides on abundancy
H[c]	60	4×10^{-1}	77,000	Decrease
L[c]	450	10×10^{-1}		Increase
S	6–13	4.3×10^{-2}	2,300	Decrease
SS	6–13	4.7×10^{-3}	1,100	Decrease

[a] Data were compiled from references 118, 119, 293, 298, 300.

[b] The H and L forms have recently been called A sites (A^H and A^L) and the S and SS forms have been called B sites (B^S and B^{SS}) to mark their possible coupling to adenylate cyclase and guanylate cyclase, respectively (300). At 0°C, dissociation rate constants are 1.4- to 4-fold lower (300).

[c] In preparations of isolated membranes, no discrimination was made between H and L in dissociation kinetics and forms with k_{-1} of $>10^{-1}$ s^{-1} were designed fast forms (118, 119, 300).

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apparent number of sites of the S and SS forms is much lower than that of the H and L sites (Table 2) (300).

All receptor forms have also been observed in isolated membranes (118, 119, 293, 300). The H and L forms were not always distinguished from each other and were collectively called the fast (F) form, which applies to all forms with $k_{-1} > 10^{-1}$ s^{-1} (Table 2) (118, 119, 300). In contrast to the situation in cells, the H → L transition does not appear to take place during association in isolated membranes (118, 293). In isolated membranes part of the SS form converts to faster dissociating (maybe S) forms during dissociation in the presence of high cAMP concentrations, indicating the participation of cooperative interactions (118).

Notwithstanding that the kinetics of cAMP binding to cells and isolated membranes are accurately explained by assuming the separate receptor forms H, L, S, and SS and cooperative effects, it may be noted that these are all hypothetical entities, the existence of which has been demonstrated only by kinetic means in rather complex systems. Nevertheless, it is likely that at least some of the proposed receptor forms and interconversions account for the site heterogeneity or cooperativity suggested by the equilibrium binding studies on cells and in isolated membranes.

Coupling of Receptor Forms to G-Protein and Adenylate and Guanylate Cyclase

The binding of subsaturating concentrations of cAMP to isolated membranes is decreased by guanosine di- and triphosphates (118, 119, 293, 300). The decrease results from a lowered affinity of the receptor for cAMP, not a decrease in the total number of binding sites. Furthermore, guanine nucleotides accelerate the overall dissociation of cAMP bound to isolated membranes (118, 119, 300). However, they have no effect on cAMP bound to intact *D. discoideum* cells (119), suggesting that guanine nucleotides do not compete with cAMP at the binding site of the receptor, but exert their effect via an interaction at the cytoplasmic surface of the membrane. The decrease in affinity of membranes for cAMP and the accelerated dissociation result from a reduction in the relative abundancy of the SS, S, and H receptor forms, which apparently become converted to forms with a lower affinity and higher dissociation rate (118, 119, 293, 300) (Table 2).

The effects of guanine nucleotides on cAMP receptors in *Dictyostelium* spp. are reminiscent of their effects on G-protein-coupled receptors in vertebrates (15). This similarity suggests a role for a G-protein in *Dictyostelium* signal transduction, as was first proposed by Leichtling et al. (167). The supposition that G-protein(s) functions in *Dictyostelium* signal transduction is further supported by the recent observations that guanine nucleotides modulate the activity of *Dictyostelium* adenylate cyclase (277, 307) (see below). Furthermore, both cAMP and folic acid increase the equilibrium binding of [^{3}H]guanosine 5'-triphosphate (GTP) to membranes and accelerate its dissociation (69). These results suggest that binding of agonists to receptors stimulates the exchange of free GTP with guanine nucleotides that are bound to G-protein. The same has been demonstrated to occur in vertebrates (15). Definite proof of a role for a G-protein in *Dictyostelium* signal transduction, i.e., its isolation and reconstitution into a functional system, has not yet been obtained.

In vertebrates, high- and low-affinity receptor forms are explained by the different complexes that can be formed between receptors and guanine nucleotide-occupied or empty G-proteins (15). By analogy, we proposed that the different kinetic forms of the receptor (Table 2) mirror the association states of cAMP receptors with G-protein (118, 300) (Fig. 2, inset). Binding of guanine nucleotides to the receptor–G-protein complex induces shifts in the equilibria between the various forms, which are observed experimentally as changes in abundance.

One might wonder whether in *Dictyostelium* spp. all effectors are regulated via the same G-protein and cAMP receptors. Two lines of evidence suggest that adenylate cyclase and guanylate cyclase in *Dictyostelium* spp. are activated via different pathways. First, a study of the behavior of kinetic receptor forms during down-regulation of receptors (see also below) has shown that the observed reduction of the number of cAMP binding sites results from a decrease in the number of fast-dissociating receptor forms H and L (Table 2) (137). The number of slowly dissociating S and SS sites does not decrease in down-regulated cells; rather, their affinity for cAMP decreases 10-fold (137). Measurement of the cAMP-evoked cAMP and cGMP responses showed that cells, when down-regulated, accumulate significantly decreased amounts of cAMP in response to a saturating stimulus dose. In contrast, the dose-response curve for the cGMP response was shifted to 20-fold-higher stimulus levels, but the maximally attainable cGMP response was unchanged (137). So, the capacity of both the H and L receptor forms and the cAMP response were decreased, while the affinity of both the S and SS receptor forms and the cGMP response were decreased.

Another argument against a single set of receptors and G-proteins mediating all signal transduction comes from a study of the effect of extracellular Ca^{2+}, Mg^{2+}, and Mn^{2+} ions on the cAMP-evoked cAMP and cGMP responses in *Dictyostelium* spp. It was found that the cation concentrations at which alterations in the fast-dissociating H and L receptor forms are observed correlated with the cation concentrations at which alterations in the cAMP response were induced (294). The cation concentrations at which alterations in the slowly dissociating receptors were observed correlated with the concentrations at which changes in the cGMP response were found (294).

Both sets of data (137, 294) can be best explained by assuming that the H and L receptor forms are involved in the activation of adenylate cyclase while the S and SS receptor forms are involved in the activation of guanylate cyclase (Fig. 2). Such a scheme would be analogous to the scheme proposed for the coupling of folate receptors to these enzymes in *Dictyostelium* spp.: fast-dissociating receptors appear to couple to adenylate cyclase and slowly dissociating receptors appear to couple to guanylate cyclase (see next section) (65, 66). A difference between folate receptors and cAMP receptors is, however, that the fast and slowly dissociating receptors for folate can be discriminated on the basis of their analog specificities (see next section), while cAMP receptors cannot. In analogy with the folate receptors, we have termed the cAMP receptors which are probably coupled to adenylate cyclase "A sites," and those probably coupled to guanylate cyclase "B sites" (300). Thus, the fast-dissociating H and L receptor forms are the A sites and the slowly dissociating forms S and SS B sites (Table 2). Since both the H and L and S and SS receptor forms appear to be coupled to G-proteins (118, 119, 293, 300), it was proposed that both the A and B sites are coupled to adenylate and guanylate cyclases through a G-protein activation cycle (Fig. 2) (300). Such a hypothesis predicts more receptor forms than have been hitherto observed.

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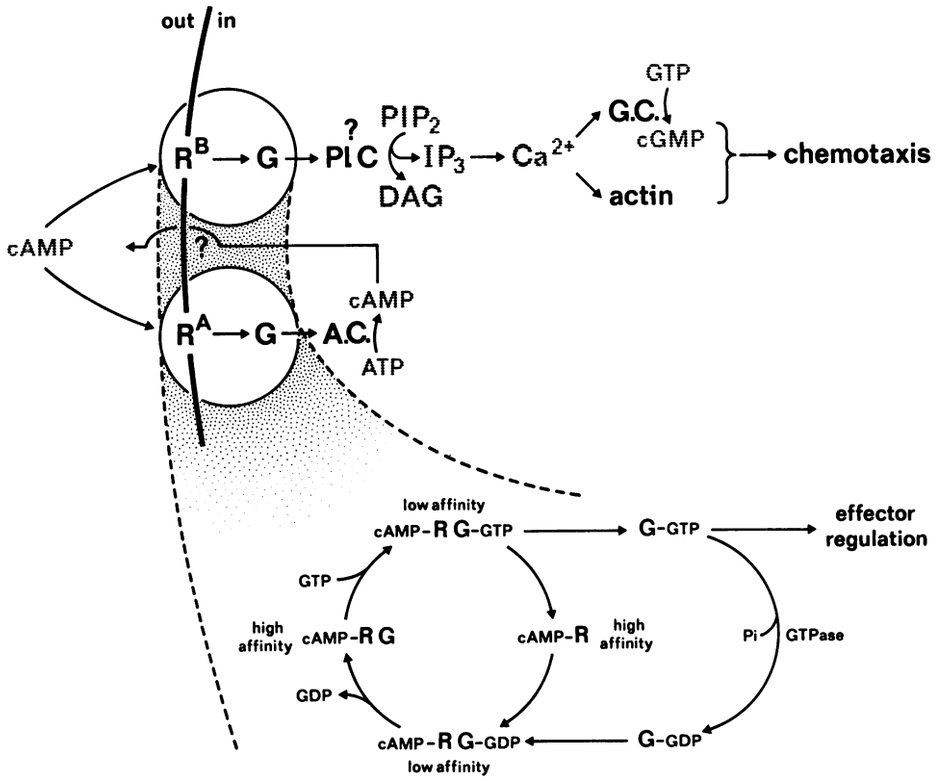

FIG. 2. Model of coupling of A and B sites to adenylate and guanylate cyclase. R, Receptor (R^A, A sites; R^B, B sites); G, G-protein; A.C., adenylate cyclase; G.C., guanylate cyclase; PlC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate. The inset gives a general G-protein activation cycle as supposed to be working at both R^A and R^B sites.

However, not all receptor forms are necessarily observable; some forms might exist only transiently for very short times after receptor occupation. Furthermore, the fast-dissociating component of the dissociation process is difficult to analyze, and it may very well be that there exist other fast-dissociating forms in addition to the H and L forms. The hypothesis that different subsets of receptors are coupled to adenylate and guanylate cyclase through different G-proteins needs to be elaborated.

Isolation of the Receptor

Addition of detergents such as Triton X-100 to *Dictyostelium* amoebae results in cell lysis and extraction of the majority of cellular proteins. The insoluble residue left is termed the cytoskeleton (28, 219). At a pH of about 6.1, a cAMP binding activity is present on such residues, which has properties of the cell surface receptor (92; unpublished observations), indicating that in vivo the receptor is attached to the cytoskeleton. Such a hypothesis agrees with the finding that cAMP receptors are found in a very high-molecular-weight aggregate in gel filtration experiments, following extraction of isolated membranes with the detergent CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate} (121; unpublished observations). Similar observations have been made with adenylate cyclase (39, 122a). Whether indeed cAMP receptors in vivo are linked to the cytoskeleton must be proven by other approaches, e.g., by receptor mobility studies with antibodies against the cAMP receptor. Such antibodies are now available (see below).

Membrane preparations enriched in cell surface cAMP receptors have been obtained by centrifugation of cell homogenates (293), sometimes followed by fractionation of the sediments in either aqueous two-phase separations (207) or sucrose gradients (118, 122a, 141). Such preparations are useful for studying the effects of regulatory compounds (e.g., guanine nucleotides) on cAMP receptors and may provide starting material for solubilization of the receptor and its purification. Alternative methods for membrane isolation, involving binding of cells to polylysine beads and subsequent cell lysis (resulting in membranes attached to the beads) (147) or membrane shedding from cells induced by guanidine-HCl (44, 111), have also been reported.

In most cases when detergents are added to receptor-enriched membrane preparations, cAMP binding activity is rapidly lost. Of 11 detergents we tested, appreciable binding activity was retained with only 2 dipolar ionic detergents (121). This could agree with data of Meyers-Hutchins and Frazier, who observed appreciable cAMP binding activity only after fractionation of Emulphogene-solubilized membrane proteins over diethylaminoethyl-Sephadex (207). These results suggest that the cAMP receptor is strongly dependent on the proper lipid environment for binding activity, a conclusion also drawn from the observation that the cAMP binding activity is very sensitive to the presence of unsaturated fatty acids (see below) (122).

When solubilization of the cAMP receptor in membranes is attempted with the dipolar ionic detergent CHAPS, cAMP becomes persistently bound to the receptor (121). This suggests that in the presence of this detergent a conforma-

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tional change of the receptor has taken place such that bound cAMP is locked in the binding site, while empty binding sites have become inaccessible (121). This behavior is reminiscent of that of the β-adrenergic receptor in the presence of deoxycholate (258).

Aiming at purification of the receptor, investigators have circumvented the problems with solubilization of the receptor by making use of photoaffinity labeling. Visualization of cAMP binding proteins in *Dictyostelium* cells and isolated membranes has been reported by three groups, using 8-azido-cAMP ($8N_3$-cAMP) as a photoaffinity label. As the receptor has about a 10-fold-lower affinity (k_d, ~300 nM) for this reagent than for cAMP (154), relatively high concentrations of labeled ligand have to be used with the risk of nonspecific labeling of proteins. Maybe for this reason the studies of Klein and co-workers (129, 151) showed labeling of many proteins in addition to the cell surface cAMP receptor. A useful method to decrease nonspecific labeling was used by Devreotes et al. (154, 278), who, after binding of $8N_3$-[^{32}P]cAMP to cells, washed the cells in the presence of high concentrations of ammonium sulfate and only then irradiated them with ultraviolet light. Ammonium sulfate retards the dissociation of the $8N_3$-cAMP–receptor complex (295, 305), but apparently not the dissociation of nonspecifically bound ligand. As ammonium sulfate decreases, but does not stop the dissociation of the ligand (295), it would be expected that predominantly slowly dissociating receptors are photolabeled. However, a comparison made between the saturation curves for binding and photolabeling suggests that all binding sites give the same product on gels (154).

Both Klein et al. and Devreotes et al., working with different techniques, have identified two closely related proteins of about 45,000 molecular weight that show properties expected for the cell surface receptor, namely (129, 154, 278); (i) competition of $8N_3$-cAMP binding and photoaffinity labeling by low concentrations of cAMP and by cAMP derivatives with a potency according to their affinity for the receptor; (ii) abundance of the products of photolabeling in *Dictyostelium* cells around the time of cell aggregation; (iii) presence in membrane preparations and not in soluble cell fractions; (iv) lack of reaction with antibodies against cAMP-dependent protein kinase; (v) difference from cell surface cAMP-phosphodiesterase as apparent from molecular weight (cf. reference 322) and from its presence in a phosphodiesterase-deficient mutant.

Evidently, both groups have identified the same two proteins as the cAMP receptor. On gels, these two proteins differ in molecular weight by about 2,000; the lower- and higher-mobility proteins were termed D or P47 and R or P45, respectively (57, 129). Both groups have presented evidence that the lower-mobility protein is a phosphorylation product of the high-mobility protein (151, 153, 173).

Devreotes and co-workers have purified the phosphoprotein to homogeneity by hydroxyapatite chromatography and two times a different one-dimensional gel electrophoresis (153). The phosphoprotein copurifies with the photoaffinity-labeled protein, which indicates that they are identical. The high- and low-mobility proteins were shown to be related by peptide mapping (153). The low-mobility protein contains about seven phosphate residues; the high-mobility protein contains about one (153). Different antibodies have been raised against the low-mobility protein that was either completely purified (155) or excised from gels on which solubilized plasma membrane proteins were electrophoresed (152). The availability of antibodies against two closely related proteins which appear to be the cell surface cAMP receptor opens the way for the molecular cloning and study of the receptor gene; in fact, a first claim for the cloning of the receptor gene has recently been made (152).

A different result was obtained by Meyers-Hutchins and Frazier (207), the third group that used photoaffinity labeling in an attempt to purify the cAMP receptor. These investigators used different cellular material for their photoaffinity labeling than Klein and Devreotes and co-workers. They started with a fraction containing cAMP binding activity, obtained from a diethylaminoethyl-Sephadex column on which Emulphogene-solubilized membranes were applied (207). A 70-kilodalton (kDa) protein from this fraction was photoaffinity labeled with $8N_3$-[^{32}P]cAMP, which labeling could partially be inhibited by excess unlabeled cAMP (207). An acidic 70-kDa glycoprotein was purified by subsequent decyl-agarose chromatography and preparative gel electrophoresis, and photoaffinity labeling of this protein was completely blocked by unlabeled cAMP. This protein was present in aggregation-competent *Dictyostelium* cells and absent in vegetative cells. The cAMP binding fraction eluted from the decyl-agarose column was partially characterized. Its affinity for cAMP and its nucleotide binding specificity matched that of the chemotactic cAMP receptor (207). However, the binding specificity was investigated with only four nucleotides, which is a rather limited survey in view of the current knowledge (see above).

The relationship between the proteins identified by Devreotes' and Klein's groups on the one hand and by Frazier's group on the other is not clear. A 70-kDa protein was not detected by photoaffinity labeling of cells, or at most in minuscule amounts (154). The possibility that the 70-kDa protein is a precursor of the M_r-45,000 proteins is unlikely, because a 70-kDa protein was not observed after in vitro translation of messenger ribonucleic acid from preaggregation cells; rather, a product of 37 kDa was detected with the antiserum prepared against the photoaffinity-labeled product of M_r 45,000 (155). On the other hand, the 70-kDa protein might be an aggregation product of the ≃45-kDa proteins, as it has been observed that the latter have a high tendency to aggregate, even in the presence of detergent (153). The relative amounts of the two proteins of about 45 kDa vary according to the state of adaptation of the cells (see section, "Desensitization to cAMP"). This, together with the arguments given above, makes them the most interesting candidates for the cAMP receptor.

Agents That Modulate cAMP Binding

A number of compounds in addition to guanine nucleotides modulate the cAMP receptor function in *Dictyostelium* spp. (see above), but their mechanisms of action are less clear.

Millimolar concentrations of divalent cations and polyvalent anions cause a two- to threefold increase in the number of cAMP binding sites as detected on cells and in isolated membranes (120, 121, 128, 294, 295). In addition, some salts (notably ammonium sulfate) increase the affinity of receptors for cAMP (120, 295). The effect of saturating concentrations of different ions is not additive (120, 295). This, however, does not mean that all ions act by the same mechanism. For instance, Ca^{2+} ions, but not Mg^{2+} ions, counteract the effect of unsaturated fatty acids on cAMP binding, while in the absence of fatty acids both ions increase the binding of cAMP (122) (see below). Also, ammonium sulfate can induce the exposure of down-regulated cAMP receptors in cells, while Ca^{2+} ions cannot (297). The effect of Ca^{2+} on cAMP

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binding to cells is rapid (within seconds) and reversible (128); inhibitors of Ca^{2+} uptake do not counteract the effect (294). These results suggest that Ca^{2+} ions act at the extracellular surface of the cell membrane. The observation that exposure of cryptic receptors can be induced in metabolically arrested cells and in isolated membranes (120, 121, 128) suggests that these receptors are hidden within the membrane itself and are not derived from intracellular stores by some kind of exocytosis. A reasonable hypothesis seems that polyvalent cations and anions alter the structure of the membrane in such a way that hidden sites become accessible. Such a working mechanism might be similar to that proposed for the effect of cholesterol on biological membranes; stiffening of the membrane structure by cholesterol also results in exposure of hidden membrane proteins (266).

The physiological function of the ionic effects on cAMP receptors, or of the cryptic receptors themselves, is open to speculation. Extracellular ionic conditions strongly influence cellular processes related to signal transduction and cell differentiation in *D. discoideum* (171, 184, 294) (see also "Physiology of Signal Transduction in *D. discoideum*"). Some of these effects might result from altered receptor function. However, it is not clear how in vivo cells could modulate the exposure of cryptic receptors. Presently, ionic conditions are mainly used as a tool for modulating the functioning of the cAMP receptor and the processes regulated via the receptor, as was described above.

Millimolar concentrations of adenosine and related compounds inhibit the binding of cAMP to cells (217). In wild-type cells, starved for relatively short times, and in axenic cells, the inhibition is partially noncompetitive (217, 276, 291), whereas in wild-type cells starved for longer periods fully competitive inhibition is found (276). The observed noncompetitive nature of inhibition argues against the possibility that adenosine inhibits the binding of cAMP only by interacting with the cAMP binding site of the receptor. Uptake of adenosine does not appear to be required for its effect, because inhibitors of adenosine uptake did not counteract the effect on cAMP binding (313). Therefore, there might be an adenosine receptor at the outer surface of the plasma membrane which mediates the effects of adenosine. Study of the equilibrium binding of [^{3}H] adenosine to *Dictyostelium* cells reveals two binding components (216, 291), suggesting the existence of multiple adenosine receptors. The affinity of one component (K_d, 300 μM) (216, 291) is such that it could mediate the effects of adenosine on cAMP binding and on various responses. Whether this binding component indeed represents a protein needs further support, especially because its number of binding sites is rather high (7×10^6 per cell) (216, 291). If these binding sites would represent a protein with a normal molecular weight, say 50,000, they would comprise nearly 1% of total cellular protein, which seems excessive for a protein with a regulatory function.

Millimolar concentrations of adenosine inhibit the cAMP-induced cGMP and cAMP responses (25, 276, 291), chemotaxis (291), and the formation of cell aggregates (216, 217). In later developmental stages adenosine inhibits the induction of prespore cell differentiation by cAMP, which has led to the hypothesis that adenosine is a morphogen for *D. discoideum* (257, 327). All of the above-mentioned effects of adenosine might result from alterations in cAMP receptor function (either by competition with cAMP binding or via an adenosine receptor), as in each of these processes cAMP receptors play a more or less decisive role (*see* "Ligand Specificity and Activation Mechanism of the Receptor"; 53, 221, 255, 257). However, it is questionable whether adenosine ever accumulates in millimolar concentrations in the extracellular space. Indeed, this might occur in multicellular aggregates, where the extracellular space is small, but it seems unlikely in the preaggregative phase of development. In the multicellular stage a cAMP-regulated signal transduction system also appears to operate since receptors (135, 256), a cAMP response, and a cGMP response (138, 220) can be demonstrated. It might be that the effects of adenosine on signal transduction in aggregating cells merely foreshadow the role of adenosine in multicellular *D. discoideum* aggregates.

Micromolar concentrations of unsaturated fatty acids noncompetitively inhibit the binding of cAMP to isolated membranes and intact cells (122). The inhibition is counteracted by Ca^{2+} but not by Mg^{2+} ions. This and other arguments suggest that the effect of unsaturated fatty acids on the receptor results from alterations in the lipid bilayer structure of the membrane (122). Such a working mechanism has also been proposed for other systems (142, 143). Conditions to make the effect of unsaturated fatty acids on the cAMP receptor reversible have not been found (122). Reversibility would make a role in regulation of receptor functions in vivo more likely. Unsaturated fatty acids have been observed to alter various cellular functions in *D. discoideum*; e.g., they inhibit cell differentiation (326). Furthermore, inhibitors of the oxidation of polyunsaturated fatty acids inhibit the cAMP-induced light-scattering response (254). Phospholipids in *D. discoideum* membranes are unusually rich in unsaturated fatty acids (326), thus forming a large store of free unsaturated fatty acids. Whether this store is sometimes used, and whether unsaturated fatty acids play a role in the regulation of *Dictyostelium* signal transduction, is not known, as no data on the concentration of free unsaturated fatty acids in the plasma membrane are available.

The stalk cell differentiation-inducing factor (DIF) (284) is another lipophilic compound which modulates the binding of cAMP to its receptor. It decreases the affinity of the receptor for cAMP, as studied in intact aggregation-competent cells (324). Concomitantly, DIF inhibits the cAMP relay response, but not the cAMP-induced cGMP response (324). The amounts of DIF, necessary to observe effects on cAMP binding and relay with a certain amount of cells, are of the same order of magnitude as those affecting stalk cell differentiation (324), which opens the possibility that the effects of DIF on signal transduction are physiologically relevant. The interference of DIF with the signal transduction system deserves special attention, as both DIF and cAMP relay appear to be important for morphogenesis in multicellular *D. discoideum* aggregates (138, 176, 220, 256).

FOLIC ACID RECEPTORS

Vegetative *Dictyostelium* amoebae are chemotactically attracted by folic acid and pteridines (226, 227). Within several hours of starvation, amoebae lose the sensitivity to folic acid, concomitantly with their folic acid receptors, and gain receptors for cAMP (66, 214, 281, 332). Various lines of evidence suggest that folates, pteridines, and cAMP are detected by different types of cell surface receptors (53, 301, 332); however, direct binding studies on pteridine receptors have not been done yet. Initial studies on the folic acid receptors were complicated by the high activity of the folic acid-degrading enzyme folate deaminase that is present on the surface of *D. discoideum* cells (13, 330). This problem was solved by using the folate deaminase inhibitor 8-azaguanine (59) or the degradation-resistant folic acid analog methotrexate (71, 214).

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In the first binding studies a high-affinity binding site was detected that had almost the same affinity for folic acid and its deaminated product 2-deaminofolic acid (K_d, 100 to 300 nM; about 10^5 sites per cell [286, 332]). However, amoebae are chemotactically at least 10^4 times less sensitive to 2-deaminofolic acid than to folic acid (228, 301), suggesting that these binding sites are not the chemotaxis receptor for folic acid. De Wit observed another receptor which has binding affinities for folic acid and its derivatives that correlate much better with the chemotactic activities of these compounds (59, 65). This receptor was present only in small numbers (1,500 sites per cell) (71); some studies could not even detect it (281).

Detailed investigations on the pharmacology and kinetics of the folic acid binding activity of cells and isolated membranes have shown as many as five kinetic receptor forms (62, 71). Two kinetic forms, A^H and A^L, are of a "nonselective" type and bind both folic acid and deaminofolic acid. The specificity of these forms does not correlate with that of chemotaxis, but with the specificity of the folic acid-induced cAMP response, which occurs in early aggregative cells (55, 65, 66). This response probably results from an indirect activation of adenylate cyclase via cAMP receptors (55), as mentioned above ("Physiology of Signal Transduction in *D. discoideum*"). The other kinetic forms, B^F, B^S, and B^{SS}, bind folic acid more selectively and have a binding specificity correlating with that of chemotaxis, the folic acid-induced cGMP response, and the stimulation of expression of two developmental markers (64, 65, 314). The main difference between the A and B receptor types is that the A sites have much less specific requirements with respect to the structure of the pterine part of the folic acid molecule than the B sites (65). The binding specificities distinguish both A and B receptor types from each other and from the two folic acid-degrading enzymes present on cells, i.e., folate deaminase (13, 330) and folate C_9-N_{10}-cleaving enzyme (65, 67).

Many properties of folic acid receptors on vegetative *D. discoideum* cells are similar to those of cAMP receptors on aggregative cells. Similar kinetic forms exist, and different subsets of receptors appear to couple to guanylate and adenylate cyclase. Folic acid receptors probably interact with a G-protein because guanine nucleotides modulate the ligand binding to both the A and B folic acid receptor types (61, 63); vice versa, folic acid modulates the binding of GTP to isolated membranes (69). These observations have led to models explaining the different kinetic forms of the folic acid receptor by the existence of different complexes of receptors with empty or occupied G-proteins (62, 63). Similar models have been proposed for vertebrate signal transduction systems (15) and for signal transduction through cAMP receptors in *D. discoideum* (Fig. 2) (118, 300).

Relatively little progress has been made towards the molecular identification and isolation of folic acid receptors. Some folic acid receptors might be associated with the cytoskeleton (281), similar to cAMP receptors (92). Several proteins from solubilized *D. discoideum* membranes bind to folic acid-derivatized Sepharose and are specifically eluted (286). Among these might be the folic acid receptors.

ADENYLATE CYCLASE

Basal Activity

All adenylate cyclase activity in *D. discoideum* is particulate (107, 224). Histochemical data suggest that the enzyme is localized on the inner side of the plasma membrane (48, 84). However, adenylate cyclase does not copurify on sucrose gradients with plasma membrane marker enzymes such as alkaline phosphatase and 5'-nucleotidase (112, 122a, 212, 230). This could imply that *Dictyostelium* adenylate cyclase is localized in specialized domains of the plasma membrane that contain little of these plasma membrane markers (cf. references 39 and 122a). Such uneven distribution of different plasma membrane-bound proteins has also been found in other cells (104, 126).

The adenylate cyclase activity in homogenates of aggregating cells is very unstable (107, 206, 224). The basal enzyme activity is about 10 pmol min^{-1} mg of $protein^{-1}$ with Mg-adenosine 5'-triphosphate (ATP) as substrate (45, 53, 248). This activity is stimulated by Mn^{2+} ions (45, 47, 171) and inhibited by Ca^{2+} ions (45, 145, 171, 248); the latter inhibition is antagonized by Mn^{2+} ions (45, 171). Basal adenylate cyclase has a K_m of 0.4 mM for Mg^{2+}-ATP (171). In the presence of Mn^{2+} ions the kinetics are non-Michaelian, being best described by two K_m values of 0.02 and 0.4 mM ATP (107, 239). The basal enzyme activity is inhibited by preincubation with a heat-stable inhibitor, present in soluble as well as particulate fractions of vegetative cells (47). The roles of Ca^{2+}, Mg^{2+}, or Mn^{2+} ions and the heat-stable inhibitor in the regulation of adenylate cyclase in vivo are unknown. Compounds such as NaF (or AlF_3) and Forskolin that modulate the activity of, respectively, G-protein-coupled and uncoupled adenylate cyclase in vertebrates (15) do not modulate *Dictyostelium* cyclase (24, 119, 145, 206, 245). Similar observations were made in other eucaryotic microbes (32, 35, 50, 86, 123, 124, 156, 225, 249, 323; exceptions, in which NaF inhibits, are reported in references 40, 42, 106, 185).

Adenylate cyclase activity remains associated with an insoluble protein residue following extraction of membranes with the detergent CHAPS (39, 122a). Up to 60% of basal adenylate cyclase is solubilized by this detergent when membranes are preincubated with 1 M NaCl before the addition of CHAPS (108). Lubrol PX is probably a better solubilizing detergent than CHAPS, as assessed from the size of adenylate cyclase determined by gel filtration chromatography (122a).

Stimulated Activity

The periodic activation of adenylate cyclase in aggregating *D. discoideum* cells is the central event in the cAMP relay response and is responsible for the propagation of the cAMP signal from cell to cell. Activation of adenylate cyclase in vivo may require the movement of proteins in the plasma membrane, because treatment of cells with cross-linking agents such as lectins, antibodies, or chemical compounds inhibits the cAMP relay response and prevents activation of adenylate cyclase (88). Some important components of the signal transduction system are probably associated with the cytoskeleton, for instance, cAMP receptors (92) (see above) or adenylate cyclase (39, 122a). The submembrane cytoskeleton might have to move for adenylate cyclase activation, and this movement would be inhibited by the cross-linkers. Interestingly, the cAMP-mediated cGMP response is not inhibited but is potentiated by the lectin concanavalin A (200).

Until very recently, the only way to demonstrate the activated state of adenylate cyclase was by stimulating the enzyme with cAMP in vivo, rapidly lysing the cells, and instantaneously measuring enzyme activity in vitro (53, 148,

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171, 245, 248). The 3- to 10-fold increase in enzyme activity thus observed rapidly decays to basal activity, a process which can be retarded at 0°C (39, 246). More detailed investigations of the "in vivo stimulation-in vitro measurement" approach were recently reported by Padh and Brenner (223). They observed that inclusion of [α-^{32}P]ATP in the mixture during cell lysis resulted in an eightfold increase in basal adenylate cyclase activity as compared to the situation normally used, in which [α-^{32}P]ATP is added after cell lysis. As a result, the stimulus-induced increase in the adenylate cyclase activity in homogenates was relatively much lower when the substrate [α-^{32}P]ATP was added prior to cell lysis instead of thereafter. The authors presented evidence suggesting that a significant fraction of the enzyme becomes latent shortly after cell lysis, more so in homogenates from unstimulated cells than from stimulated cells. This might indicate that activation of adenylate cyclase in vivo is correlated with an increased accessibility of ATP to the enzyme (223).

Several observations challenge the interpretation of the "substrate accessibility" studies of Padh and Brenner (223) and their relevance to the regulation of adenylate cyclase in vivo. First, there is almost no difference in cAMP production between lysis in the presence and absence of [α-^{32}P]ATP, when enzyme activity is measured with Mn^{2+} ions instead of Mg^{2+} ions; in parallel experiments with Mg^{2+} ions (as used by Padh and Brenner [223]), the differences are 7- to 15-fold (A. Theibert and P. Devreotes, personal communication). As in many other systems, *Dictyostelium* adenylate cyclase is stimulated by Mn^{2+} ions and uncoupled from guanine nucleotide regulatory proteins (277; see below). It would be expected that latency of adenylate cyclase is not affected by its intrinsic activity. Second, even when cells are lysed in the presence of Mg^{2+} [α-^{32}P]ATP, an increase is observed in the formation of [^{32}P]cAMP in homogenates from stimulated cells compared to unstimulated cells, although this increase is greater when cells are lysed in the absence of [α-^{32}P]ATP (223). Third, higher adenylate cyclase activity is also observed in homogenates from stimulated cells, as compared to unstimulated cells, when cells are lysed with the detergent CHAPS (88). It is unlikely that after lysis by detergents differences in enzyme sequestration are preserved. This suggests that altered substrate accessibility, when occurring, is not the sole regulatory mechanism of adenylate cyclase activity in vivo.

The activation of adenylate cyclase in vivo probably results, at least partly, from coupling of the enzyme to a G-protein (277, 307). While many investigators tried in vain to activate the enzyme with guanine nucleotides (cf. reference 53), this now appears to be possible under specific conditions. About 10- to 20-fold stimulation of adenylate cyclase occurs when guanine nucleotides are present during cell lysis or within 5 min thereafter; a preincubation of about 5 min at 0°C of cell homogenate with guanine nucleotides is required to obtain maximal stimulation (277). Alternatively, a twofold activation of adenylate cyclase in crude membrane preparations is observed when enzyme incubations are performed at temperatures between 0 and 10°C (307). In addition, inhibition of adenylate cyclase by guanosine triphosphates becomes detectable when membranes are preincubated with ATPγS, suggesting that an inhibitory G-protein may also be present (307). It is not clear why the specific conditions are necessary to observe effects of guanine nucleotides on adenylate cyclase. They might relate to necessary coupling/uncoupling events of G-protein to adenylate cyclase or to a transient latency of adenylate cyclase or G-protein. Also, it should be noted that the *Dictyostelium* adenylate cyclase system is rather unusual, compared to other systems, in that the product of the enzyme, cAMP, is the agonist of the receptor which regulates the enzyme. The possibility of a positive-feedback loop in vivo, with cells stimulating themselves with their secreted cAMP (53, 56), may require more control elements than in other systems.

The observations of Theibert and Devreotes and ourselves regarding the stimulation of adenylate cyclase are qualitatively similar in many respects (277, 307). Adenylate cyclase, as measured with Mg-ATP, was stimulated by micromolar concentrations of guanosine triphosphates, nonhydrolyzable analogs being better than GTP. The nonhydrolyzable guanosine diphosphate GDPβS did not stimulate adenylate cyclase and antagonized the stimulation by guanosine triphosphates. In the presence of Mn^{2+} ions, micromolar concentrations of guanine nucleotides did not stimulate enzyme activity, but were slightly inhibitory, especially at higher concentrations (50 to 1,000 μM). Such observations have also been reported by Khachatrian et al. (140). Because millimolar concentrations of Mn^{2+} ions are unphysiological (222), and guanosine diphosphates tended to inhibit more strongly than guanosine triphosphates in the presence of Mn^{2+} ions (140), it is unlikely that these inhibitory effects are related to a physiologically significant regulation via G-proteins. Guanine nucleotide stimulation of adenylate cyclase in thoroughly washed membranes depended on the addition of a cytosolic fraction (307). This was especially apparent from studies with a mutant which has lost the cAMP relay response in vivo. Adenylate cyclase activity in this mutant could not be stimulated by guanine nucleotides, unless cytosol from wild-type cells was added (277, 307). Various lines of evidence suggest that the cytosolic factor is not identical to a soluble G-protein (307), but more definite conclusions have to await its further characterization.

In both types of preparations (277, 307), cAMP stimulated the activity of adenylate cyclase 1.3- to 4-fold in both the presence and the absence of guanine nucleotides. Theibert and Devreotes could demonstrate this stimulation of activity only when cAMP was added to cells shortly prior to lysis (277). This in fact is the in vivo stimulation-in vitro measurement approach, mentioned above. In contrast, we could observe stimulation of cyclase by adding cAMP to our crude membrane preparations (307).

In conclusion, the guanine nucleotide stimulation of adenylate cyclase and its potentiation by cAMP support the concept of a receptor-G_s protein-adenylate cyclase coupling. However, the observation that cAMP must be added to intact cells and guanine nucleotides directly after cell lysis (277), or alternatively that all measurements must be done below 10°C to observe regulation of the enzyme (307), suggests that other control mechanisms operate in vivo which are incompletely controlled in vitro. Such mechanisms might also involve a special architecture of the adenylate cyclase system, i.e., linkage to the cytoskeleton or compartmentalization of the enzyme.

GUANYLATE CYCLASE AND THE INOSITOL PHOSPHATE PATHWAY

Guanylate cyclase activity is present in both vegetative and aggregating *D. discoideum* cells and is stimulated via folic acid and cAMP receptors. Basal enzyme activity in aggregating cells is two- to sixfold higher than in vegetative

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cells (199, 222, 325). Guanylate cyclase is found in soluble and particulate cell fractions (122a, 197, 222, 325), as in many higher organisms (211). It is not known whether these activities represent different molecular entities. Soluble guanylate cyclase has an M_r of ≅250 kDa (222). Attempts to further purify this enzyme are hampered by the instability of the enzyme (222).

As in vertebrates (211), *Dictyostelium* guanylate cyclase in vitro can as yet only be measured in the presence of Mn^{2+} ions (K_M, 0.7 mM Mn^{2+}) (222). This seems unphysiological, because the intracellular Mn^{2+} concentration in *D. discoideum* is 5 to 10 μM (222). Apparently, the in vitro enzyme measurements are not representative of the in vivo conditions, which is a major obstacle for the study of the regulation of this enzyme. The activity as measured with Mn^{2+}-GTP is stimulated by micromolar concentrations of ATP and AppNHp (190, 222). ATP, but not AppNHp, added to intact aggregation-competent cells increases the cAMP-induced cGMP response (189). The relevance of these observations to the regulation of the enzyme in vivo is unknown. Only Mato and Malchow (197) have measured the activated state of guanylate cyclase in vitro, following the same approach as used for adenylate cyclase. After stimulation of cells with cAMP and rapid cell lysis by sonication, three- to sixfold activated guanylate cyclase activity persisted in homogenates for about 1 min, as measured with Mn^{2+}-GTP (197).

Europe-Finner and Newell recently obtained important evidence which suggests that guanylate cyclase is regulated via the inositol phosphate pathway. They reported that the addition of inositol 1,4,5-trisphosphate to saponin-permealized cells results in a rapid transient elevation of cGMP (79) and a permanent increase in cytoskeleton-associated actin (80). Similar responses can be evoked by addition of Ca^{2+} ions to permeabilized cells (80, 268). In higher organisms, as well as in *Dictyostelium* spp., inositol trisphosphate stimulates the liberation of Ca^{2+} ions from cellular, nonmitochondrial stores (14, 81, 115). Taken together, these data might suggest that Ca^{2+} is the direct activator of *Dictyostelium* guanylate cyclase.

In ciliates, Ca^{2+} ions activate guanylate cyclase via the universal Ca effector protein calmodulin (131, 157, 259). Padh and Brenner, however, observed that neither Ca^{2+} ions nor calmodulin could stimulate *D. discoideum* guanylate cyclase activity in vitro with either Mn^{2+}-GTP or Mg^{2+}-GTP as substrate (222). These and some other aspects of the regulation of *Dictyostelium* guanylate cyclase remain to be clarified. For instance, inositol 1,4,5-trisphosphate also evoked a (small) cGMP and an actin response in nonpermeabilized cells (80). Furthermore, the inositol 1,4,5-trisphosphate-induced cGMP response in permeabilized cells did not require the addition of GTP or ATP (79), although it might be expected that cellular GTP, ATP, and other small molecules should have leaked out of the cells.

Dictyostelium cells contain polyphosphoinositides (M. M. van Lookeren Campagne, unpublished observations) and phosphatidylinositol kinase (318). Direct evidence for the existence of a cAMP receptor and G-protein-regulated phosphatidylinositol cycle was recently presented by Europe-Finner and Newell (82, 83). They reported that in [^{3}H]inositol-labeled intact or permeabilized aggregation-competent *Dictyostelium* cells cAMP and guanosine triphosphates induce the accumulation of radio-activity that coelutes with inositol 1,4,5-trisphosphate and other inositol phosphates on anion-exchange columns (82, 83). Basal or activated phospholipase C activity, i.e., the turnover of (poly)phosphatidyl inositol, has not yet been demonstrated directly (cf. references 117 and 302).

In summary, although some important regulatory mechanisms are still unknown, recent progress is bringing new impetus into guanylate cyclase research in *D. discoideum*. *Saccharomyces cerevisiae* (130) and *Dictyostelium* spp. are the only eucaryotic microorganisms for which evidence for a phosphatidylinositol cycle has been obtained so far. *Dictyostelium* is probably the first organism in which the inositol phosphate pathway is linked to guanylate cyclase, actin polymerization, and cell locomotion (Fig. 2).

DESENSITIZATION TO cAMP

Homologous (or agonist-specific) desensitization in vertebrate signal transduction systems results primarily from alterations in the receptors (267). The same may be the case in *D. discoideum*. This is suggested by studies of the binding characteristics and the physicochemical properties of the cAMP receptor during signal transduction and desensitization.

Alterations in Binding Properties

Ligand-induced alterations in ligand binding in *D. discoideum* were first observed by Klein and Juliani (146, 150). cAMP induces a decrease in the number of cAMP binding sites on cells (146, 150), a phenomenon termed down-regulation (267). Originally, high cAMP concentrations (10^{-4} to 10^{-3} M) were reported to be necessary (146, 150), but much lower ligand concentrations are sufficient when the phosphodiesterase activity is reduced by using a phosphodiesterase-deficient mutant, a phosphodiesterase inhibitor (dithiothreitol), or a nonhydrolyzable analog of cAMP (146, 297). The minimal concentration of cAMP necessary to induce down-regulation was found by maintaining the extracellular cAMP concentration constant, i.e., by inhibition of phosphodiesterase with dithiothreitol and inhibition of adenylate cyclase with caffeine (25, 275). Under these conditions a half-maximal loss of cAMP binding sites was induced by exposing cells for 15 min at 20°C to 50 nM cAMP (297). The molecular mechanism of down-regulation in *D. discoideum* and the fate of down-regulated receptors are not known. It has been suggested that accessible receptor sites are lost as a result of the formation of an extremely slowly dissociating cAMP-receptor complex (146). Following removal of cAMP, lost sites reappear slowly with a $t_{0.5}$ of about 1 h at 20°C (137, 150, 297). Protein synthesis is not required for the reappearance of the receptors, suggesting that down-regulated receptors are not degraded. Binding studies of the residual receptors imply that the H and L kinetic forms of the receptor are reduced in number, whereas the S and SS forms are reduced in affinity (Table 2), as mentioned above (137). Down-regulation affects the cAMP relay response by decreasing the amount of cAMP accumulated in response to a saturating stimulus dose, whereas the dose-response curve for the cGMP response is shifted to higher stimulus concentrations (137) (see subsection, "Coupling of Receptor Forms to G-Protein and Adenylate and Guanylate Cyclases").

Recently, a more rapid alteration of cAMP binding was observed that may be related to desensitization. A short preincubation of cells with cAMP prevented the subsequent binding of ligand to the SS form of the receptor (300). From analysis of the association kinetics of cAMP binding to cells it was inferred that occupied SS sites originate from the

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conversion of other sites, which have higher "on" rates (300). Apparently, cAMP-occupied SS sites can only be formed when the cAMP concentration is raised. Thus, formation of occupied SS sites depends on the prevailing cAMP concentration, which is akin to adaptation at the receptor binding level. Based on the assumption that the SS receptor form is involved in guanylate cyclase activation (see above), it was proposed that cessation of new formation of occupied SS sites is directly responsible for the cessation of activation of guanylate cyclase, i.e., adaptation of the enzyme (300).

A third ligand-induced change in ligand binding was already mentioned and is the conversion of the H receptor form to the L form shortly after initiation of cAMP binding (29, 137, 298). Assuming that the H and L forms are involved in adenylate cyclase regulation (see above), this change is too fast ($t_{0.5}$ of 10 s [298]) to be related to adenylate cyclase adaptation ($t_{0.5}$ of 3 min [53, 74]).

Covalent Modification of Receptors

Devreotes et al. and Klein et al. have identified by photoaffinity labeling two closely related proteins of M_r ≅45,000 which are different forms of the same cAMP receptor protein, as described above (subsection, "Isolation of the Receptor") (129, 151, 154, 278). The low-mobility protein in gels is a phosphorylation product of the high-mobility protein (151, 153, 173). Photoaffinity labeling and in vivo phosphorylation experiments revealed that the low-mobility strongly phosphorylated protein predominates in cells after stimulation by cAMP and the high-mobility weakly phosphorylated protein is abundant in cells that are not stimulated (57, 151, 154). As a result, in autonomously oscillating cell suspensions the abundancy of the low- and high-mobility receptor proteins alternates with the frequency of the oscillatory activation of the cells (154). Apparently, occupation of the receptor by cAMP triggers a signal that gives rise to increased receptor phosphorylation.

The appearance and disappearance of the low- and high-mobility receptor proteins during cAMP stimulation of cells correlate well with adaptation of adenylate cyclase (57). First, the cAMP-induced transition of high- to low-mobility receptor protein occurred with the same rate as adaptation of adenylate cyclase, both having a $t_{0.5}$of 2.5 to 3 min (57, 74). This rate is compatible with the rate of appearance of the strongly phosphorylated protein, as observed by Klein et al. (151, 173). Second, the amount of low-mobility receptor protein in stimulated cells was proportional to the concentration of extracellular cAMP presented and matched the dose-response curve for adaptation. Finally, the low-mobility protein persisted as long as the cAMP concentration was unchanged, while as soon as cAMP was removed, the high-mobility receptor protein returned with a $t_{0.5}$ of 5 to 6 min at 20°C (57). About the same rate is observed for de-adaptation of adenylate cyclase ($t_{0.5}$ of 3 to 4 min [73]). Mathematical models have been designed that could explain both adenylate cyclase adaptation and autonomous oscillatory signalling in cell suspensions through receptor modification and positive-feedback stimulation (104, 158, 263).

The transition of the receptor protein from the high- to the low-mobility form is probably not related to activation of adenylate cyclase. Receptor modification takes place in the presence of caffeine (154), whereas this compound blocks activation of adenylate cyclase, but not adaptation (25, 275). Furthermore, it is also unlikely that the high- to low-mobility form transition is related to either guanylate cyclase activation or adaptation, because both of these processes are >10-fold faster (308) than the receptor transition.

The relationship between the physical and binding characteristics of the receptor have been investigated by Devreotes and co-workers (57, 154). They reported that phosphorylated receptors have a lower affinity for cAMP. However, interpretation of this result is complicated because cAMP binding, like the photoaffinity labeling, was done in the presence of ammonium sulfate, which profoundly alters the receptor binding properties and exposes down-regulated receptors (120, 295, 297). The relationship between receptor phosphorylation and the various kinetic forms of the receptor (Table 2) is not obvious. We might suppose that the photolabeled products on gels are representative of the majority of cAMP binding sites on cells and that, as a result, most of the receptors undergo modifications (154; see subsection, "Isolation of the Receptor"). This would suggest that the H and L kinetic forms of the receptor, which together comprise 96% of the total number of binding sites on *Dictyostelium* cells, are the subjects of receptor modification and phosphorylation. However, it is unlikely that the H and L forms are identical to the high- and low-mobility proteins, respectively, because the transition of H to L following application of cAMP to cells is much faster ($t_{0.5}$ of 10 s [298]) than the transition of the high- to low-mobility protein ($t_{0.5}$ of 2.5 min [57]). In addition, it is not known whether the S and SS forms of the receptor have been detected in photoaffinity labeling and phosphorylation experiments. Thus, a comparison between photoaffinity labeling data and binding characteristics of the receptor presently cannot clarify the relationship between modification and the kinetic forms of the receptor.

Incubation of membranes under conditions designed to stimulate protein phosphorylation alters the binding properties of both the H and L and S and SS kinetic forms or their putative coupling to G-proteins or both (174, 296, 302). In addition it abolishes GTP stimulation of adenylate cyclase (307). These observations, however, give no insight into the relationship between binding and receptor modification, because possible physical alterations in the receptors resulting from phosphorylation were either not observed (296) or not investigated (174, 302). The altered receptor binding properties were assumed to be caused by phosphorylations catalyzed by different kinases, such as protein kinase C (302), kinase A (174), or an endogenous membrane-bound kinase (296). It is likely that phosphorylation of receptors and possibly other transducing elements by various kinases can occur in *Dictyostelium* spp. and plays a role in the regulation of desensitization, as also occurs in vertebrate systems (10, 11, 133, 136, 166, 270). At present we are only at the beginning of unraveling the complex phenomena related to phosphorylation and desensitization in *Dictyostelium* species.

COMPARISON WITH OTHER EUCARYOTIC MICROBES

The eucaryotic microbes form a vastly divergent group, including organisms as different as fungi, protozoa, and algae. A large number of behavioral studies are at hand (159, 165, 169), and in many organisms alterations of cyclic nucleotide levels have been correlated with a specific growth phase or behavior (225). The enzyme activities involved in cyclic nucleotide metabolism have often been demonstrated

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and partly characterized, but the biochemistry underlying the regulation of the enzymes is still largely unknown (1, 42, 113, 123, 175, 225). Among the eucaryotic microbes, molecules possibly involved in signal transduction have been studied in some detail in only a few organisms besides *Dictyostelium* spp., notably, *Paramecium*, *Tetrahymena*, *Saccharomyces*, *Neurospora*, *Mucor*, and *Trypanosoma* species.

The study of extracellular messenger molecules and cell surface receptors in lower eucaryotes has been approached from two directions. First, evidence for chemoattractants or pheromones has been found in various species, and some compounds have been identified (16, 132, 159, 165, 169, 209, 280). In a few organisms, including *Dictyostelium* spp., these studies have been followed by binding studies, giving direct evidence for cell surface receptors (31, 51, 125, 262). To our knowledge, besides the *Dictyostelium* cAMP receptor, no membrane receptor has been isolated from eucaryotic microorganisms. However, in *S. cerevisiae* the genes of the receptors for the pheromones α-factor and **a**-factor have been cloned and sequenced (30a, 107a, 213a).

The second approach is based on the observation that microorganisms can either produce or respond to substances that are very similar to vertebrate signal molecules such as hormones (168). This suggests an early evolutionary appearance of vertebrate signal compounds and their receptors (cf. references 168 and 172). However, this approach has not been very fruitful for the understanding of signal transduction mechanisms, because either the vertebratelike substances produced were almost never found to affect the microorganisms themselves (106, 168) or specific vertebrate compounds that affected microorganisms were not produced in sufficiently high concentrations by these microorganisms to be important (4, 42, 127).

Until a few years ago, the regulation of adenylate cyclase in microorganisms was largely unknown; guanine nucleotides had no influence on the enzyme activity, and the unphysiological substrate Mn-ATP was often strongly preferred over Mg-ATP (32, 123, 175, 225, 260). More recently, however, data have accumulated that suggest the regulation of adenylate cyclase by GTP-binding regulatory proteins in *Phycomyces* (42), *Saccharomyces* (20, 35), *Neurospora* (86, 249, 250), *Trypanosoma* (75, 76), and *Dictyostelium* (see subsection, "Stimulated Activity") species. The characterization and isolation of the presumed GTP binding regulatory proteins are still in a preliminary phase. At best, reconstitution experiments with crude fractions have been performed (20, 250). In *Neurospora* and *Trypanosoma* species, reconstitution of guanine nucleotide and hormone-sensitive adenylate cyclase could also be accomplished with G-proteins and hormone receptors from detergent extracts of vertebrate membranes (75, 86). These unique observations suggest a close homology between fungal and vertebrate signal transduction systems. This seems to contrast with a study using antibodies against subunits of purified G-protein: even antibodies against the most homologous subunit of different G-proteins, β (101, 244), do not recognize a similar protein in invertebrate organisms (5). Although information about the homology of adenylate cyclase systems in higher and lower eucaryotes may be inconclusive and scarce, it is likely that the overall regulation of adenylate cyclase in some eucaryotic microorganisms is the same as in vertebrates.

Not all adenylate cyclase activity in microorganisms is regulated by guanine nucleotides. For instance, adenylate cyclase in trypanosomes is stimulated by Ca^{2+} ions (319, 320) and may resemble the Ca/calmodulin-regulated enzyme prominent in vertebrate brain tissue (27, 37). However, the calmodulin antagonist trifluoperazine had no effect on activation of *Trypanosoma* adenylate cyclase by Ca^{2+} ions (320). It is not clear whether the Ca^{2+}-stimulated adenylate cyclase in *Trypanosoma* spp. is the same enzyme as the one that can be activated by (vertebrate) G-proteins (75). In other eucaryotic microbes adenylate cyclases are inhibited by Ca^{2+} ions (1, 42, 213). Adenylate cyclase of *Paramecium* spp. is peculiar, because its activity seems to be regulated by K^+ ions (156). In *Neurospora* spp. a soluble adenylate cyclase has been purified to homogeneity (238). This enzyme appears to be different from the G-protein-regulated adenylate cyclase in this organism (250).

A most exciting development has come with the discovery of the *ras* protooncogenes in divergent eucaryotes including *Dictyostelium* spp. and yeasts (52, 232, 234, 235, 241). The products of *ras* genes are proteins of M_r ~21,000 having guanosine triphosphatase activity, as do the α subunits of G-proteins (100, 274). In *S. cereviseae*, strains deficient in *ras* function lack GTP-stimulated adenylate cyclase and have a phenotype similar to that of adenylate cyclase-deficient strains (282). The guanine nucleotide sensitivity of cyclase in *ras*-deficient strains can be restored by reconstitution with yeast or human *ras* proteins (26, 282). The identification of the gene encoding the catalytic subunit of adenylate cyclase in *S. cerevisiae* (21, 34) has enabled the successful reconstitution of adenylate cyclase and the *S. cereviseae RAS* gene products, using extracts of transformed bacteria in which each of the genes was expressed (285). These observations have raised the question whether in *S. cerevisiae* the *RAS* protein is identical to the endogenous GTP binding regulatory protein (18). However, mammalian adenylate cyclase is not affected by reconstitution with *ras* proteins (9), suggesting that *ras* is not a regulatory component of the mammalian enzyme. In addition, overexpression of endogenous or mutated *ras* genes in *Dictyostelium* spp. does not alter the regulation of adenylate cyclase by cAMP in vivo or by guanine nucleotides in vitro (204a, 240). *Dictyostelium* cells transformed with mutated *ras* genes show altered adaptation of the cAMP-induced cGMP response (204a). This may indicate that in *Dictyostelium* cells *ras* proteins are involved in the regulation of guanylate cyclase or phosphatidylinositol turnover, which is linked to guanylate cyclase (see section, "Guanylate Cyclase and the Inositol Phosphate Pathway"). Recently, data have been presented which suggest that also in vertebrates *ras* proteins are involved in the regulation of phosphatidylinositol hydrolysis (38, 87, 321), and this may be more general than the involvement of *ras* in the regulation of adenylate cyclase occurring in yeasts.

Guanylate cyclase has been thoroughly investigated in the ciliates *Paramecium* and *Tetrahymena*. This enzyme is regulated by Ca^{2+} ions via the Ca^{2+} regulatory protein calmodulin (131, 157, 259). *Paramecium* calmodulin can activate a vertebrate enzyme (phosphodiesterase) and calmodulin from soya bean, pig brain, or *Tetrahymena* will activate guanylate cyclase in *Paramecium* membranes (157, 261). These data suggest a strong conservation of the Ca^{2+}/calmodulin regulatory mechanisms during evolution. Intracellular Ca^{2+} concentrations in ciliates fluctuate in response to various stimuli, giving rise to a Ca^{2+}/K^+ action potential across the plasma membrane (163). Investigations with several Ca^{2+} channel mutants indicate that the activity of guanylate cyclase in the cilia (cf. reference 260) is directly determined by the Ca^{2+} fluxes across the ciliary membrane (261).

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In many of the eucaryotic microbes studied so far, it is unclear whether adenylate and guanylate cyclase form a part of a signal transduction system, e.g., in *Neurospora* (249), *Mucor* (33), *Acanthamoeba* (1, 40), and *Alternaria* (123) species (cf. reference 225). The currently most advanced model systems for transmembrane signal transduction in lower eucaryotes are the ciliates *Paramecium* and *Tetrahymena* and the slime mold *Dictyostelium*. Rapid progress is also being made with yeasts (cf. reference 19). Prominent in the ciliates are electrical phenomena, such as the Ca^{2+} action potential, that result from chemical or mechanical stimuli (163). *Paramecium* therefore aphoristically has been called "a swimming neuron" (163). Chemical phenomena are prominent in *Dictyostelium* spp. following stimulation by chemoattractants. Therefore, *Dictyostelium* sp. may be considered the model hormone-responsive cell among the eucaryotic microbes.

PROSPECTS

In recent years much of the biochemical basis of transmembrane signal transduction in *D. discoideum* has been defined. The pharmacology and the kinetics of ligand binding to cAMP and folic acid receptors have been described and the cAMP receptor has been purified. Strong evidence for a G-protein(s) has been obtained from the study of cAMP and folic acid receptors as well as adenylate cyclase. Evidence for the regulation of guanylate cyclase via a phosphatidylinositol phosphate cycle has been obtained, and some light has been shed on the mechanism of adaptation in *Dictyostelium* spp. This has led to a model of *Dictyostelium* signal transduction which has close analogies with vertebrate systems (Fig. 2). Apparently, the signal transduction systems in vertebrates are of very ancient evolutionary origin.

Now that the homology with vertebrates seems clear, the peculiarities of the *Dictyostelium* signal transduction system may also be discovered. For instance, why does *Dictyostelium* adenylate cyclase resist activation in vitro by receptor ligands and guanine nucleotides, under conditions which operate in vertebrates? This may be related to the architecture of the system, e.g., coupling of its elements to the cytoskeleton. The answer may be valuable for the study of other eucaryotic microbes.

The isolation of components presently suggested to operate in signal transduction is a major task to perform in the coming years: receptors, G-proteins, adenylate cyclase, phospholipase C, Ca^{2+}-regulated guanylate cyclase, and protein kinases regulating the system, to mention some of them. Significant progress has only been made with the cell surface cAMP receptor. The experience in vertebrates shows that isolation is not always an easy task, and it may take several years before reconstitution of various components will be accomplished. However, only this approach will give definite proof of the existence of the components now supposed to be involved and their interactions.The discovery of new factors involved in the regulation of signal transduction may be expected. The role of some of other factors needs to be more clearly defined, e.g., protein kinases and *ras* proteins. The regulation of guanylate cyclase via receptors coupled to G-proteins remains to be substantiated. The mechanism of cAMP secretion is still largely unknown. Insight into this mechanism could provide essential information on the compartmentation of receptors, adenylate cyclase, and produced cAMP. The study of mutants may be of great help in defining the function of the components and regulatory mechanisms involved in *Dictyostelium* signal transduction. In general, mutants are more easily obtained in this lower eucaryote than in vertebrates, and many *Dictyostelium* mutants are already available (cf. references 6, 7, and 46). With these tools at hand, a detailed description of *Dictyostelium* signal transduction in molecular terms may not be so far away.

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