Differential Effects of Temperature on cAMP-induced Excitation, Adaptation, and Deadaptation of Adenylate and Guanylate Cyclase in *Dictyostelium discoideum*

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Abstract. Extracellular cAMP induces excitation of adenylate and guanylate cyclase in *Dictyostelium discoideum*. Continuous stimulation with cAMP leads to adaptation, while cells deadapt upon removal of the cAMP stimulus. Excitation of guanylate cyclase by cAMP has a lag time of ~1 s; excitation of adenylate cyclase is much slower with a lag time of 30 s. Excitation of both enzyme activities is less than twofold slower at 0°C than at 20°C. Adaptation of guanylate cyclase is very fast ($t_{½}$ = 2.4 s at 20°C), and virtually absent at 0°C. Adaptation of adenylate cyclase is much slower ($t_{½}$ = 110 s at 20°C) but not very temperature sensitive ($t_{½}$ = 290 s at 0°C). At 20°C, deadaptation of adenylate cyclase is about twofold slower than deadaptation of guanylate cyclase ($t_{½}$ = 190 and 95 s, respectively). Deadaptation of adenylate cyclase is absent at 0°C, while that of guanylate cyclase proceeds slowly ($t_{½}$ = 975 s). The results show that excitation, adaptation, and deadaptation of guanylate cyclase have different kinetics and temperature sensitivities than those of adenylate cyclase, and therefore are probably independent processes.

An intercellular signal molecule in the cellular slime mold *Dictyostelium discoideum*, cAMP, is involved in chemotaxis, differentiation, and morphogenesis (18, 24, 25). The free-living amebae of this organism feed on bacteria. Food deprivation induces a social phase in the life cycle. Some cells start to secrete cAMP in a pulsatile manner. Surrounding cells detect this cyclic nucleotide by means of cell surface receptors, which lead to two responses: chemotactic reaction towards the source of cAMP secretion, and activation of adenylate cyclase followed by secretion of the newly synthesized cAMP (reviewed in 3, 10, 39). This cAMP stimulates more distally located amebae. Finally, an aggregation center is formed, which may collect up to 100,000 amebae.

cAMP stimulates both adenylate and guanylate cyclase activity in *D. discoideum* cells (19, 21). In contrast to the synthesized cAMP, the newly formed cyclic guanosine 3′,5′-monophosphate (cGMP)[1] is not secreted but may bind to an intracellular receptor. It has been proposed that intracellular cGMP is a key component during the chemotactic reaction (22, 45). This suggests that the kinetics of the cAMP-induced activation of adenylate and guanylate cyclase are of major importance for chemotaxis-mediated cell aggregation in this organism.

The continuous stimulation of cells with cAMP leads to adaptation (i.e., adenylate and guanylate cyclase are activated transiently) and prestimulus enzyme activities are recovered even when cAMP remains present. Cells deadapt upon removal of the stimulus, and gradually regain responsiveness to cAMP. Adaptation of adenylate and guanylate cyclase have many properties in common (5–8, 32, 40, 46); (*a*) cells remain responsive to elevations of the cAMP concentration, (*b*) adaptation is complete, i.e., no residual response remains after prolonged stimulation, (*c*) adaptation is cAMP-concentration dependent, (*d*) adaptation shows additivity, (*e*) deadaptation follows first order kinetics with $t_{½}$ = 2–3 min. This may suggest that adaptation of adenylate and guanylate cyclase occurs at a common step in the signal transduction pathway. However, it has been shown that adaptation of guanylate cyclase of cells in suspension occurs much faster than adaptation of adenylate cyclase of cells on filters (8, 40).

Adaptation is probably essential for the cAMP relay mechanism (5–7), the cGMP response (40), and for chemotaxis (31). In this study, relationships of excitation, adaptation, and deadaptation of adenylate and guanylate cyclase were investigated under identical stimulus conditions at two temperatures, 20 and 0°C. The results show that these processes have widely different kinetics and temperature sensitivities. Guanylate cyclase does not adapt at 0°C, while adenylate cyclase does. In contrast, adenylate cyclase does not deadapt at 0°C, while guanylate cyclase deadapts slowly. This suggests that deadaptation of adenylate and guanylate cyclase may occur independently.

1. *Abbreviations used in this paper*: dcAMP, 2′ deoxyadenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; IP_3, inositol 1,4,5-trisphosphate; (Sp)-cAMPS, adenosine 3′,5′-monophosphorothioate, (Sp)-isomer.

Materials and Methods

Materials

[2,8−^{3}H]-cAMP (1.5 TBq/mmol), [8−^{3}H]-cGMP (0.8 TBq/mmol), the cGMP radioimmunoassay kit, and the cAMP-binding protein kit were purchased from Amersham International (Buckinghamshire, UK). cAMP, ATP, GTP, dithiothreitol (DTT), and 2'-deoxyadenosine 3',5'-monophosphate (dcAMP) were from Sigma Chemical Co. (St. Louis, MO). Adenosine 3',5'-monophosphorothioate, (Sp) isomer ([Sp]-cAMPS) was a generous gift of Drs. Jastorff, Baraniak, and Stec (1).

Culture Conditions

D. discoideum, NC-4(H), was grown in association with *Escherichia coli* as previously described (43). Cells were freed from bacteria by repeated washings with 10 mM KH_2PO_4/Na_2HPO_4, pH 6.5 (Pb-buffer) at 200 *g* for 2 min. Cells were starved on nonnutrient agar at a density of 1.25×10^6 cells/cm^2. After 4–5 h cells were harvested, washed twice with Pb-buffer, and resuspended in this buffer at a density of 0.5 or 1.0×10^8 cells/ml. Air was bubbled through the suspension and cells were equilibrated at the indicated temperature for at least 10 min.

The cAMP-induced cGMP response was measured essentially as previously described (40). The accumulation of cAMP levels was induced by the analog dcAMP, and cAMP levels were measured by isotope-dilution assay; cAMP-dependent protein kinase was used as a cAMP-binding protein (11). dcAMP has high affinity for the cell surface cAMP receptors, but low affinity for cAMP-dependent protein kinase (34). The absence of cross-inhibition in the cAMP assay makes purification of cAMP unnecessary. Details of the experiments are described in the legends to the figures.

Results

cGMP and cAMP Responses at 20 and 0°C

The cAMP-induced accumulation of cGMP levels is shown in Fig. 1 *a*. At 20°C, a maximum of 9 pmol cGMP/10^7 cells is obtained at 10 s after stimulation, and basal levels are recovered in ~30 s. The cGMP response is strongly retarded at 0°C; a lower maximum of 3.5 pmol/10^7 cells is obtained after 1 min, and basal levels are not reached within 5 min.

The cAMP-induced accumulation of cAMP levels is not strongly affected by the lowered temperature (Fig. 1 *b*); about the same maximal levels are obtained at both temperatures. A half-maximal cAMP accumulation is found after 75 s at 20°C and after 200 s at 0°C.

These results suggest that the cGMP response is strongly altered at 0°C, while the cAMP response is only slower at the lowered temperature. Previous work (5–8, 32, 40, 41, 46) has revealed that cGMP and cAMP levels are determined by (*a*) the stimulus concentration, (*b*) the kinetics of excitation of the cyclases, (*c*) the activity of the cyclases, (*d*) the kinetics of adaptation, which reduces the activity of the cyclases, and (*e*) the activity of phosphodiesterase. Finally, cells deadapt after removal of the stimulus; the kinetics of deadaptation determines the responsiveness of cells to newly applied stimuli.

Kinetics of Excitation

The kinetics of excitation is defined as the time period that elapses between addition of the stimulus and a steady-state activation of the cyclase. Therefore, cGMP and cAMP levels were measured at short time periods after stimulation. The accumulation of cGMP levels at 20°C is linear with time between ~1 and 8 s after stimulation (Fig. 2 *a*). The results indicate a short delay time ($\tau = 0.85$ s). This delay time is not much different at 0°C; however, the slow accumulation of

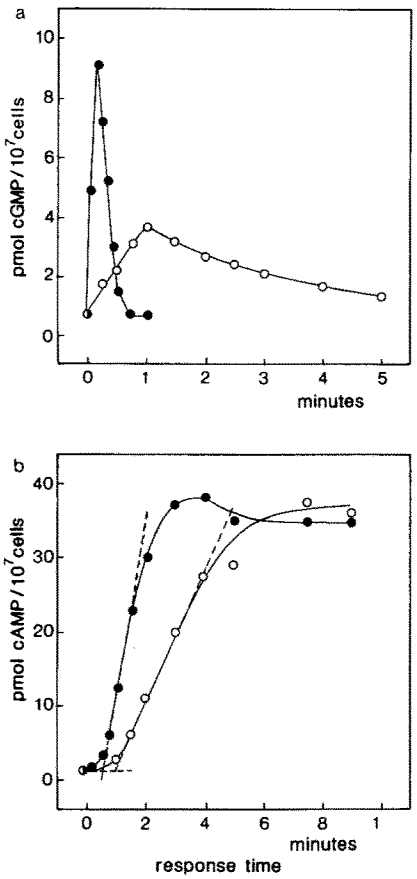

Figure 1. cGMP response (*a*) and cAMP response (*b*) at 20 (●) and 0°C (○). (*a*) Aggregative *D. discoideum* cells (5×10^7 cells/ml) were stimulated with 0.1 μM cAMP. At the times indicated 20 μl of the cell suspension was added to 20 μl perchloric acid (3.5%, vol/vol). The lysates were neutralized, and the cGMP content was measured by radioimmunoassay. (*b*) Cells were stimulated with 10 μM dcAMP and 5 mM DTT, and lysed with perchloric acid at the times indicated. The cAMP content was measured with a binding protein assay. The results shown are the means of duplicate determinations in an experiment reproduced four times.

cGMP levels at 0°C makes it difficult to obtain an accurate estimate of τ at 0°C (Fig. 2 *b*).

Adenylate cyclase is activated more slowly than guanylate cyclase; the delay time between stimulus addition and full expression of adenylate cyclase activity is ~30 s at 20°C (Fig. 1 *b*). At 0°C the delay time is ~55 s.

These results suggest that signal transduction up to activation of adenylate or guanylate cyclase does not contain a step that is very temperature sensitive. These data agree well with altered kinetics of cAMP binding to cell surface receptors, which is slowed down two- to threefold upon a lowering of temperature from 20 to 0°C (38; data not shown).

Kinetics of Adaptation

The kinetics of adaptation of guanylate and adenylate cyclase were investigated as follows. Cells were stimulated at 20 or at 0°C with (Sp)-cAMPS for different time periods. Then (Sp)-cAMPS was removed by washing the cells at 0°C, and cells were restimulated at 20°C. cGMP and cAMP levels were measured at 10 s and 5 min, respectively, after restimulation. The rationale of the experiment is as follows. First,

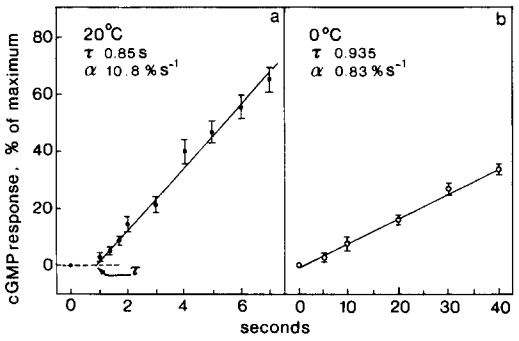

Figure 2. The kinetics of excitation of the cGMP response at 20 (*a*) and 0°C (*b*). Cells were stimulated at $t = 0$ s with 0.1 μM cAMP and lysed at the times indicated. Data were subjected to linear regression analysis; the data at $t = 0$ s were excluded from this analysis. τ is the intercept with the abscissa, and α is the slope. The data of three independent experiments with triplicate determinations were combined; the means and SEM are shown. The cGMP content at 0 and 10 s after stimulation at 20°C was set at 0 and 100%, respectively. See Fig. 1 *b* for the kinetics of excitation of adenylate cyclase.

(Sp)-cAMPS is a full agonist of cAMP; about 100-fold higher concentrations of (Sp)-cAMPS induce the same effects as cAMP (30, 38, 40; unpublished observations). Furthermore, (Sp)-cAMPS is degraded very slowly by cell surface phosphodiesterase (23, 43); the half-life of 10 μM cAMPS is ~15 h (operationally this is called nonhydrolyzable). Second, deadaptation does not occur or is very slow at 0°C (see below). Thus, cells remain adapted during the washing step at 0°C. Third, cells that have been at 0°C for a longer period and then transferred to 20°C show the typical response of cells at 20°C (Fig. 3).

The kinetics of adaptation of guanylate cyclase is shown in Fig. 4 *a*. At 20°C adaptation is very fast; a preincubation of cells with 10 μM (Sp)-cAMPS for 10 s results in the attenuation of the response to cAMP. Adaptation shows first order kinetics with $t_{½} = 2.4$ s (Fig. 4 *a*, *inset*). Apparently, adaptation of the cGMP response does not occur at 0°C. Preincubation of cells at 0°C with (Sp)-cAMPS for 3–7.5 min does not result in a diminished response to cAMP.

Adaptation of adenylate cyclase activation is a relatively slow process (Fig. 4 *b*). At 20°C (Sp)-cAMPS induces a 96% attenuation of the activation of this enzyme. Adaptation shows first order kinetics with $t_{½} = 2$ min. At 0°C the attenuation of adenylate cyclase by (Sp)-cAMPS is not complete. In three independent experiments the response induced by a new stimulus after a 30-min preincubation with (Sp)-cAMPS at 0°C was 14, 20, and 16%. Adaptation of adenylate cyclase at 0°C shows first order kinetics with $t_{½} = 5$ min, thereby being ~2.75-fold slower at 0°C if compared with 20°C.

Kinetics of Deadaptation

Adaptation of guanylate and adenylate cyclase was induced at 20°C by (Sp)-cAMPS during a preincubation of 30 s and 5 min, respectively. Then, (Sp)-cAMPS was removed by washing the cells at 0°C, and cells were resuspended in buffer at 0°C. One portion of the cells was kept at 0°C, and another portion was transferred to 20°C. At various time periods after resuspension, cells were restimulated at 20°C. cGMP and cAMP levels were measured at 10 s and 5 min, respectively, after restimulation.

The results (Fig. 5) show that cells that were preincubated with (Sp)-cAMPS for 30 s at 20°C and subsequently washed at 0°C show a strongly reduced cGMP response upon restimulation with cAMP. This response gradually recovered when cells were transferred to 20°C. Deadaptation shows first order kinetics with $t_{½} = 95$ s at 20°C (Fig. 5 *a*, *inset*). The cGMP response recovers more slowly when cells are kept at 0°C. When it is assumed that the response will recover to the same level at 20 and 0°C, it has been calculated that deadaptation at 0°C has first order kinetics with $t_{½} = 975$ s; thus deadaptation of the cGMP response at 0°C is ~10-fold slower than at 20°C (Fig. 5 *a*, *inset*).

Deadaptation of the cAMP response (Fig. 5 *b*) shows approximately the same kinetics as deadaptation of the cGMP response both at 20°C. The $t_{½}$ is ~190 s (Fig. 5 *b*, *inset*). In contrast, deadaptation of the cAMP response does not occur at 0°C within the time period of the experiment, indicating that it is at least 30-fold slower than at 20°C.

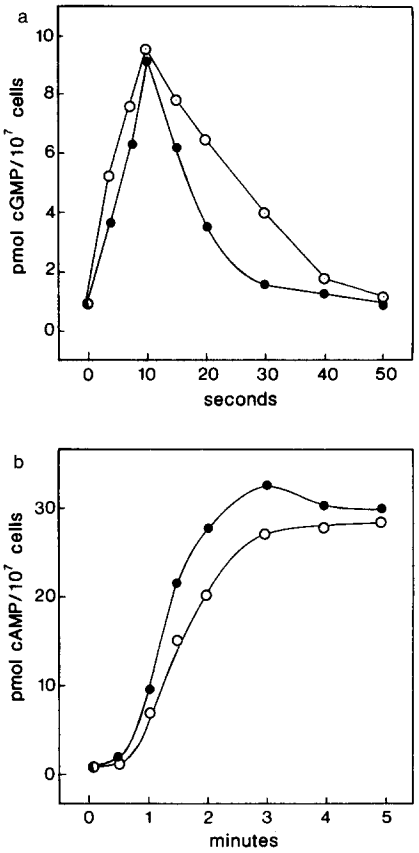

Figure 3. cGMP response (*a*) and cAMP response (*b*) at 20°C in cells that were preincubated at 20 (●) or 0°C (○) for 10 min. Equal volumes of a cell suspension and a stimulus solution were mixed in a tube equilibrated at 20°C. The temperature of the cells and stimulus solutions was 20°C (●), and 0 and 40°C, respectively, (○). The stimulus was 0.1 μM cAMP (*a*), and 10 μM dcAMP with 5 mM DTT (*b*). At the times indicated, samples of the cell suspensions were added to perchloric acid and the cGMP or cAMP content was measured in the neutralized lysates. The results are the means of duplicate determinations from an experiment reproduced two times.

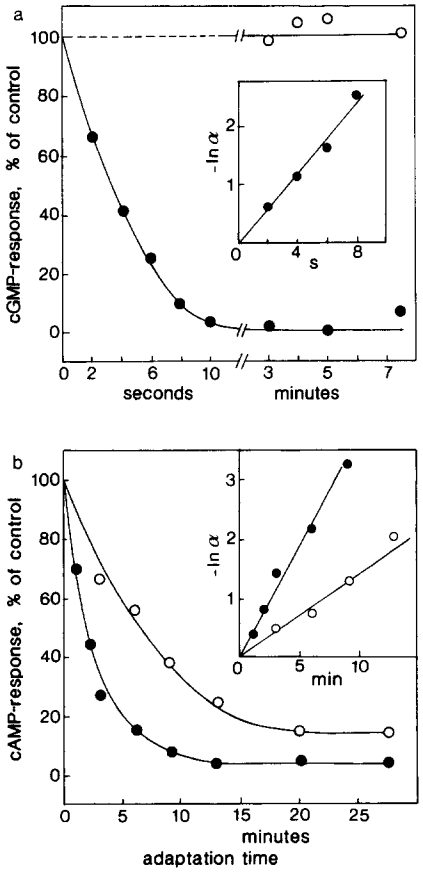

Figure 4. The kinetics of adaptation of guanylate cyclase (*a*) and adenylate cyclase (*b*) at 20 (●) and 0°C (○). Cells were stimulated at $t = 0$ s with 10 μM (Sp)-cAMPS in a total volume of 120 μl; the temperature was 20 (●) or 0°C (○). At the times indicated, cells were added to 6 ml ice-cold buffer and centrifuged at 0°C for 1 min at 300 *g*. The pellet was resuspended at 0°C into 120 μl buffer. Duplicate samples (20 μl) were added to perchloric acid for the determination of basal cGMP or cAMP levels (0% response). Triplicate samples (20 μl) were mixed in a tube at 20°C with 20 μl stimulus solution, which had a temperature of 40°C. The stimulus was 0.1 μM cAMP (*a*) or 10 μM dcAMP with 5 mM DTT (*b*) (final concentrations). Cells were lysed at 10 s after stimulation (*a*) or at 5 min after stimulation (*b*) and the cGMP and cAMP content was measured in the neutralized lysates. In the control experiment, cells (without [Sp]-cAMPS) were added to 6 ml ice-cold buffer that contained 0.2 μM (Sp)-cAMPS, and further treated as indicated above. (*Insets*) Semi-logarithmic plots of the data from the main figures. $\alpha = (R^t - R^\infty)(R^\circ - R^\infty)^{-1}$, where R indicates the response, and the superscript indicates the time period that cells were preincubated with (Sp)-cAMPS (∞ = 3–7 min [*a*] and 20–25 min [*b*]). The slopes in these figures yield the rate constants of adaptation (*a* [●], $k = 0.29\ s^{-1}$; *a* [○], $k \leqslant 5 \times 10^{-3}\ s^{-1}$; *b* [●], $k = 6.2 \times 10^{-3}\ s^{-1}$; *b* [○], $k = 2.3 \times 10^{-3}\ s^{-1}$). The results from two independent experiments were combined.

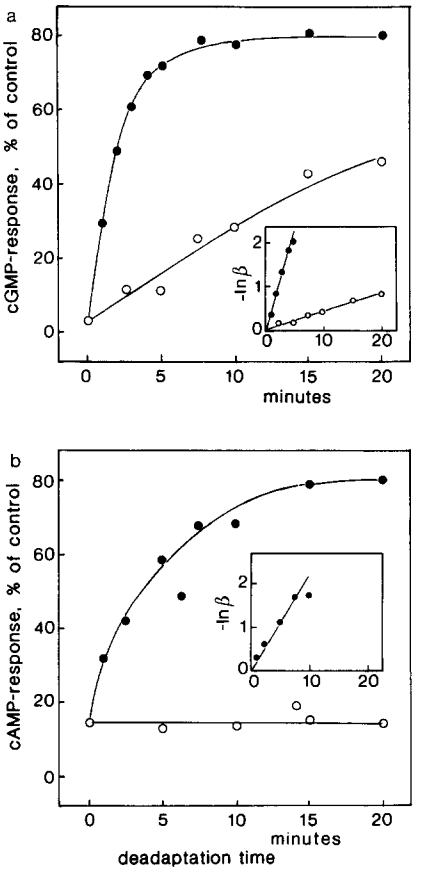

Figure 5. The kinetics of deadaptation of guanylate cyclase (*a*) and adenylate cyclase (*b*) at 20 (●) and 0°C (○). Cells were preincubated with 10 μM (Sp)-cAMPS at 20°C in a total volume of 2 ml. The preincubation period was 30 s (*a*) and 5 min (*b*). Then, cells were diluted 50-fold with ice-cold buffer, centrifuged at 0°C for 1 min at 300 *g*, and the pellet was resuspended in 2 ml ice-cold buffer (at $t = 0$ s in the figure). Part of the suspension (1 ml) was incubated at 20°C, the remaining part was kept at 0°C. At the times indicated in the figure, duplicate samples were added to perchloric acid, and triplicate samples were stimulated at 20°C. The stimulus was 0.1 μM cAMP (*a*), or 10 μM dcAMP with 5 mM DTT (*b*). Cells were lysed at 10 s (*a*) or 5 min (*b*) after stimulation. The control was taken as was indicated in the legend of Fig. 4. (*Insets*) Semi-logarithmic plots of the data from the main figures. $\beta = (R^t - R^\circ)(R^\infty - R^\circ)^{-1}$, where R indicates the response, and the superscript indicates the time period of deadaptation (∞ = 15–20 min [●]). The slopes in these figures yield the rate constants of deadaptation (*a* [●], $k = 7.3 \times 10^{-3}\ s^{-1}$; *a* [○], $k = 7.1 \times 10^{-4}\ s^{-1}$; *b* [●], $k = 3.7 \times 10^{-3}\ s^{-1}$; *b* [○], $k < 1 \times 10^{-4}\ s^{-1}$). The results from two independent experiments were combined.

Discussion

Chemosensory transduction in *D. discoideum* is a complex process and includes the stimulation of adenylate and guanylate cyclase by extracellular cAMP. cAMP binds to cell surface receptors, which transduce the signal to the cyclases, probably via a guanine nucleotide regulatory protein (12, 13, 29, 33, 42, 44). This transduction step is called excitation. Adenylate and guanylate cyclase are only transiently activated even when the stimulus is present continuously. Enzyme activities decay by a process called adaptation. When the stimulus is removed cells gradually regain responsiveness to newly applied stimuli; this process is called deadaptation (5–8, 32, 40, 46).

The major findings of the present report are as follows (Table I). (*a*) Excitation of guanylate cyclase is very fast (delay time ~1 s); excitation of adenylate cyclase is much slower (delay time ~30 s). Excitation of both cyclases is not very temperature sensitive. (*b*) Adaptation of guanylate cyclase is very fast ($t_{1/2}$ = 2.4 s), and is virtually absent at 0°C. In contrast, adaptation of adenylate cyclase is much slower ($t_{1/2}$

Table I. Kinetics and Temperature Dependency of Signal Transduction in D. discoideum

Process	Units	Values at 20°C		Fold reduction at 0°C	
		GuCy	AdCy	GuCy	AdCy
Excitation	τ, s	0.85	30	~1.1	1.8
Activity	α, pmol/s/10^7 cells	1.0	0.36	13	2.4
Adaptation	k, s^{-1}	0.29	6.3×10^{-3}	≥200	2.7
Deadaptation	k, s^{-1}	7.3×10^{-3}	3.6×10^{-3}	10	≥30

GuCy, guanylate cyclase; AdCy, adenylate cyclase; k, rate constant of the reaction; $t_{1/2} = \ln 2 \cdot k^{-1}$.

= 110 s), and not very sensitive to a lowered temperature. (*c*) Deadaptation of guanylate and adenylate cyclase shows slightly different kinetics at 20°C ($t_{1/2}$ values are 95 and 190 s, respectively). However, deadaptation of guanylate cyclase proceeds slowly at 0°C, while deadaptation of adenylate cyclase is virtually absent at this reduced temperature.

These data allow the selection of conditions in which adenylate and guanylate cyclase are regulated differently. At 2 s after stimulus addition guanylate cyclase is activated while adenylate cyclase is not. At 20 s after stimulation at 20°C guanylate cyclase has adapted while adenylate cyclase has not (adenylate cyclase has not yet been completely activated). The reversed situation is present at 5–10 min after stimulation at 0°C; adenylate cyclase is adapted while guanylate cyclase is not. Finally, deadaptation of guanylate cyclase does occur at 0°C while that of adenylate cyclase does not. These data strongly suggest that excitation, adaptation, and deadaptation of adenylate and guanylate cyclase proceed by largely independent mechanisms.

Adaptation of adenylate cyclase has been related to the covalent modification, presumably phosphorylation, of the cAMP surface receptor (15–17). This hypothesis is based on similar kinetics and concentration dependencies of these reactions (4). The present observations that adaptation of adenylate cyclase does and deadaptation does not occur at 0°C agree well with the temperature dependency of the receptor modification. Recently it has become possible to detect GTP-stimulated adenylate cyclase in *D. discoideum* membranes, suggesting the involvement of the stimulatory G protein (29, 44). Both studies revealed that GTP could not stimulate adenylate cyclase in membranes that were derived from cells in which adenylate cyclase was adapted. Desensitization of hormone-stimulated adenylate cyclase by receptor phosphorylation and receptor G protein adenylate cyclase uncoupling appears to have become a general mechanism (27).

It should be noted that the phosphorylation state of the receptor apparently does not influence the receptor-mediated activation of guanylate cyclase. This enzyme is adapted after a few seconds when the receptor is not yet phosphorylated, whereas the receptor becomes phosphorylated at 0°C while guanylate cyclase does not adapt. The phosphorylation of the receptor is accompanied by a shift of its apparent molecular mass from 40 to 43 kD in SDS-PAGE. It is surprising that such a drastic modification of the receptor conformation would not affect a signal transduction to guanylate cyclase. Indeed, we have proposed recently that the cAMP-binding activity of *D. discoideum* cells is composed of two subclasses of binding sites, A- and B-sites, which represent ~95 and 5%, respectively, of the total cAMP-binding activity on *D. discoideum* cells (37, 38). It is possible that only the major cAMP receptor population was detected in the receptor-modification experiments. Some evidence has been presented that A- and B-sites transduce the signal to adenylate and guanylate cyclase, respectively (14, 35). Detailed kinetics of the binding of cAMP to the B-sites indicate different forms of this receptor which interconvert in a cAMP- and guanine nucleotide–dependent manner (42). It was observed that one of these interconversions, which was supposed to represent the activation of a G protein, did not occur under conditions that specifically induced the adaptation of guanylate cyclase (36).

Our current working model for the initial steps of signal transduction in *D. discoideum* is composed of two subpopulations of cAMP surface receptor, both of which interact with G proteins, leading directly or indirectly to the activation of adenylate and guanylate cyclase, respectively. Adaptation of both signal transduction pathways is localized at the interaction between receptor and G protein, but they are essentially independent of each other. Support for this working model should come from the physical identification of G proteins and receptor subpopulations.

Recently the interesting observation was made that inositol 1,4,5-trisphosphate (IP_3) or Ca^{2+} stimulate guanylate cyclase in permeabilized *D. discoideum* cells (9, 28). In other organisms it has been shown that IP_3 can be formed by receptor and G protein–mediated stimulation of phospholipase C, which hydrolyses phosphatidylinositol 4,5-bisphosphate into diacylglycerol and IP_3 (reviewed in 2). IP_3 induces the release of Ca^{2+} from internal stores, and diacylglycerol stimulates the Ca^{2+}/phospholipid–dependent protein kinase C (20). It is tempting to suggest that the regulation of guanylate cyclase activity in *D. discoideum* is a consequence of the regulation of the phospholipase C/protein kinase C pathway.

The major functions of cAMP in *D. discoideum* are the induction of chemotaxis and prestalk- and prespore-specific gene expression. Mutant studies indicate the involvement of the cGMP response in chemotaxis, whereas the activation of adenylate cyclase appears to be nonessential for either chemotaxis or gene expression (26). Therefore, further research will focus on the transduction pathway(s) leading to the formation of IP_3 and cGMP. The present results, which show that the cGMP pathway can be manipulated independent of the activation and adaptation of adenylate cyclase, could be helpful to elucidate the molecular mechanisms of cAMP-induced chemotaxis and differentiation.

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