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Kinetics and Concentration Dependency of cAMP-Induced Desensitization of a Subpopulation of Surface cAMP Receptors in *Dictyostelium discoideum*†

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ABSTRACT: Extracellular cAMP induces the rapid activation of guanylate cyclase, which adapts within 10 s to constant cAMP concentrations. A new response can be induced either by a higher cAMP concentration or by the same cAMP concentration at some time ($t_{1/2}$ = 90 s) after removal of the previous stimulus. Stimulation of guanylate cyclase is supposed to be mediated by a subpopulation of cell surface cAMP receptors (B-sites). These sites can exist in three states, B^F, B^S, and B^{SS}, which interconvert in a cAMP and guanine nucleotide dependent manner. It has been proposed that the transition of B^S to B^{SS} represents the activation of a guanine nucleotide regulatory protein [Van Haastert, P. J. M., De Wit, R. J. W., Janssens, P. M. W., Kesbeke, F., & DeGoede, J. (1986) *J. Biol. Chem. 261*, 9604-9611]. Binding of [^{3}H]cAMP to these sites was measured after a short preincubation with an identical concentration of nonradioactive cAMP. [^{3}H]cAMP could still bind to B^F and B^S, but not to B^{SS}, indicating that the transition of B^S to B^{SS} is blocked by the preincubation with cAMP. This blockade was rapid and showed first-order kinetics with $t_{1/2}$ = 4 s. A half-maximal blockade was induced by 0.7 nM cAMP; at this concentration only 5% of the B-sites are occupied with cAMP. The blockade of the transition of B^S to B^{SS} was released by two conditions: (i) When the concentration of cAMP was increased, the blockade was released within a few seconds. (ii) When cAMP was removed, the blockade was released slowly with $t_{1/2}$ = 90 s. Finally, cAMP did not induce the blockade under conditions where guanylate cyclase did not adapt, i.e., at 0 °C and in cells starved for 2 h or less. These results suggest that the interaction of cAMP with the B-sites induces a rapid and reversible blockade of the terminal step in the generation of an active G-protein and that this blockade could be the molecular basis of adaptation of guanylate cyclase.

The eukaryotic microorganism *Dictyostelium discoideum* is a suitable organism to study desensitization of signal transduction pathways. In this organism the hormone-like substance is cAMP, which is detected by cell surface receptors. Extracellular cAMP induces the rapid activation of guanylate cyclase (Mato & Malchow, 1978) and the slower activation of adenylate cyclase (Roos & Gerisch, 1976). Intracellular cGMP reaches a peak at 10 s and declines to prestimulated levels within about 30 s (Mato et al., 1977). Intracellular cAMP reaches maximal levels after 60-120 s and is secreted, thus acting as an autocatalytic feedback loop (Gerisch & Wick, 1975).

Prolonged stimulation of *D. discoideum* cells with constant cAMP concentrations induces desensitization by at least two mechanisms: (i) One is down-regulation of cAMP-binding activity after a long incubation (5 min) with high cAMP concentrations (above 0.1 μM). After removal of cAMP, cells resensitize with a half-life of about 60 min (Klein & Juliani, 1977; Klein, 1979). (ii) Another is a rapid desensitization of the cAMP-mediated activation of adenylate and guanylate cyclases by nanomolar cAMP concentrations with the characteristics of adaptation; i.e., the activity of the cyclases fades in the presence of a constant cAMP concentration but can be reactivated by increasing the cAMP concentration (Devreotes & Steck, 1979; Van Haastert & Van der Heijden, 1983). After removal of cAMP, cells deadapt with a half-life of 1-2 min for the guanylate cyclase and 3-4 min for the adenylate cyclase. Adaptation of the cAMP-mediated cGMP accumulation is completed within 10 s, while adaptation of the cAMP accumulation is completed after about 5 min (Dinauer et al., 1980a,b; Van Haastert & Van der Heijden., 1983).

Adaptation of adenylate cyclase stimulation has been correlated with a covalent modification, presumably phosphorylation, of the receptor (Devreotes & Sherring, 1985; Klein, C., et al., 1985; Klein, P., et al., 1985, 1987). This receptor modification is probably not the molecular mechanism for adaptation of guanylate cyclase stimulation, since adaptation

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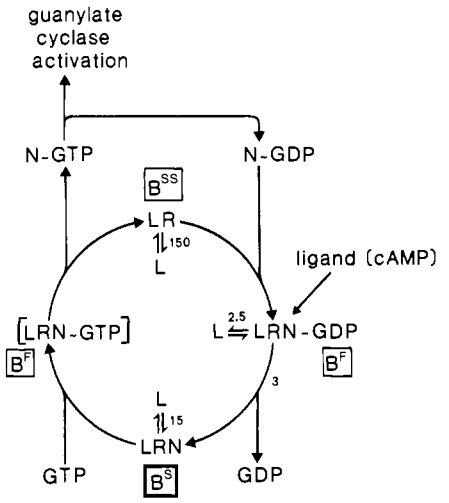

FIGURE 1: Model of interactions between cAMP (L), receptor (R), G-protein (N), GDP, and GTP leading to the activation of guanylate cyclase in *D. discoideum*. cAMP initially binds to RNGDP; the complex is designated B^F and is fast dissociating with $t_{1/2}$ = 2.5 s. GDP is released, and B^F interconverts with $t_{1/2}$ = 3 s to a state B^S, which releases bound cAMP more slowly with $t_{1/2}$ = 15 s. GTP binds to the LRN complex; although this state (LRNGTP) shows fast-dissociating properties in membranes, dissociation of the complex on cells probably does not occur, because the complex rapidly converts to the last state LR and the activated G-protein, NGTP. LR dissociates very slowly with $t_{1/2}$ = 150 s. The activated G-protein transduces the signal to guanylate cyclase. It should be noted that direct association of cAMP to B^{SS} is very slow ($t_{1/2}$ = 150 s); nevertheless, the occupied B^{SS}-state is reached within 20 s after cAMP addition, indicating that this state is almost exclusively reached via the transition of B^S to B^{SS}. Thus the analysis of occupation of B^{SS} yields information on the activation of the G-protein. Redrawn from Van Haastert et al. (1986).

of guanylate cyclase is much faster ($t_{1/2}$ = 3 s) than receptor modification ($t_{1/2}$ = 1 min). Furthermore, receptor modification does occur at 0 °C, while adaptation of guanylate cyclase does not (Devreotes & Sherring, 1984; Van Haastert, 1987). Adaptation of guanylate cyclase has been localized between the receptor (included) and the guanylate cyclase (not included) (Van Haastert, 1983a,b).

The binding of cAMP to cell surface receptors is complex. On the basis of kinetic and pharmacological studies, two subpopulations have been distinguished, A- and B-sites (Van Haastert & De Wit, 1984; Van Haastert et al., 1986). Evidence has been presented suggesting that the cAMP signal to adenylate and guanylate cyclase is mediated by respectively the A- and B-sites (Van Haastert, 1985; Kesbeke & Van Haastert, 1985). Detailed kinetic studies of the binding of cAMP to the B-sites on intact cells show interconversions of at least three states (Van Haastert et al., 1986). Initially, cAMP binds to a state (B^F) that has a half-life of dissociation of about 2.5 s. Then B^F converts with $t_{1/2}$ = 3.5 s to a state (B^S) that dissociates more slowly ($t_{1/2}$ = 15 s). Finally, B^S converts without a measurable delay to a state (B^{SS}) that dissociates very slowly with $t_{1/2}$ = 150 s. In membranes the B^S- and B^{SS}-states are converted by guanine nucleotides to the B^F-state (Van Haastert et al., 1986; Janssens et al., 1986). The nucleotide specificity suggests the involvement of a guanine nucleotide regulatory protein (G-protein). These observations have been combined in a model of B-sites G-protein interaction (Figure 1).

The transitions of the B-sites in *D. discoideum* have a characteristic not observed in other hormone receptors. On the time scale of signal transduction (seconds) the occupied B^{SS}-state does not dissociate, and it is formed only by a transition of the occupied B^S-state and not by direct association of cAMP to the empty B^{SS}-state. The model indicates that whenever an occupied B^{SS}-state is detected, it must have originated from an occupied B^S-state and thus that the G-protein has been activated. It was observed that the B^{SS}-state could not be formed in cells with an adapted guanylate cyclase. Data were interpreted as a cAMP-induced blockade in the formation of the B^{SS}-state (Van Haastert et al., 1986). In this paper experiments are described on the kinetics, reversibility, and concentration dependency of this cAMP-induced blockade, which suggests that it could be the molecular mechanism of adaptation of guanylate cyclase.

Materials and Methods

Materials. [2,8-^{3}H]cAMP (1.5 TBq/mmol) was purchased from Amersham, cAMP was from Sigma, and the silicone oils AR20 and AR200 were obtained from Wacker Chemie. *D. discoideum* NC-4(H) was grown in association with *Escherichia coli* 281 and starved for 4.5 h in 10 mM potassium/sodium phosphate buffer, pH 6.5 (Pb buffer) as described by Van Haastert and Van der Heijden (1983). Cells were collected and washed twice with Pb buffer and resuspended in this buffer at a density of 1.1×10^8 cells/mL. During the experiments cells were aerated at 20 °C at a rate of about 15 mL of air (mL of suspension)$^{-1}$ min^{-1}.

Binding Assay. The exact protocol of the incubation mixtures is described in the legends of the figures. Typically, 180 μL of the cell suspension (2×10^7 cells) was preincubated at 20 °C with 20 μL of a mixture yielding Pb, 5 mM dithiothreitol, and a specific concentration of cAMP. After a specific time interval (which varies between 0 and 20 s), 100 μL of Pb, 5 mM dithiothreitol, and a specific concentration of [^{3}H]cAMP was added. At the times indicated in the figures cell-associated [^{3}H]cAMP was measured by centrifugation of the incubation mixture through 200 μL of silicone oil (AR20:AR200 = 1:2). Binding to all binding states was detected by direct centrifugation through silicone oil. Binding to B-sites was measured by adding the incubation mixture to 1 mL of a mixture placed above the silicone oil containing Pb, 5 mM dithiothreitol, and 0.1 mM cAMP. Binding to the B^S + B^{SS} states was measured by centrifugation after 10 s and binding to the B^{SS}-state by centrifugation after 2 min. Nonspecific binding was measured by including 0.1 mM cAMP during the entire incubation period. After centrifugation, the tip of the tube containing the cell pellet was cut and radioactivity was determined in 2 mL of Instagel (Packard) by liquid scintillation counting with the Rack Beta 1215 (LKB).

Results

The aim of the present study is to investigate the cAMP-induced transitions of a subpopulation of cAMP-binding sites, the B-sites, that presumably leads to the activation of a G-protein. These transitions were measured in control cells and in cells that had an adapted guanylate cyclase. *D. discoideum* cells were incubated with cAMP and [^{3}H]cAMP under two conditions. (i) Cells were incubated with cAMP at t_0; at t_1 [^{3}H]cAMP was added, and the binding of [^{3}H]cAMP to the different binding states was detected at t_2. (ii) cAMP and [^{3}H]cAMP were added simultaneously at t_0, and binding of [^{3}H]cAMP to the different binding states was also detected at t_2. Thus these experiments reveal the effect of a preincubation of cells with cAMP (which induced adaptation of guanylate cyclase) on the possibility of the different binding states to become occupied with [^{3}H]cAMP. Previously we have presented evidence that the formation of the B^{SS}-state may represent the activation of a G-protein (Van Haastert et al., 1986).

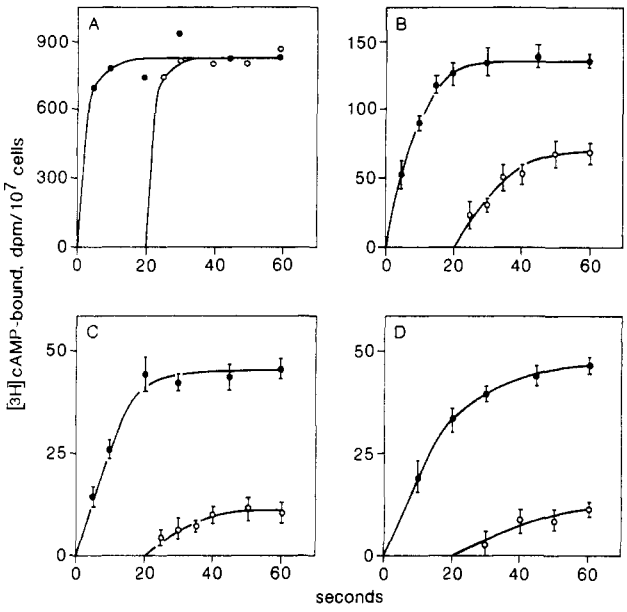

FIGURE 2: cAMP-induced blockade of the binding of [^{3}H]cAMP to B^{SS}. (A–C) *D. discoideum* cells (90 μL) were incubated at t = 0 s with 10 μL of cAMP, yielding 5 mM dithiothreitol and 10 nM cAMP. At t =0 (●) or at t = 20 s (○), 50 μL of 10 nM [^{3}H]cAMP and 5 mM dithiothreitol was added. Thus, the concentrations of cAMP and dithiothreitol did not change. In (A) 145 μL of the incubation mixture was placed above silicon oil, and cells were centrifuged through the oil at the times indicated. In (B) and (C) 145 μL of the incubation mixture was added to 1 mL of 0.1 mM cAMP and 5 mM dithiothreitol at the times indicated, and cells were centrifuged through the oil 10 s later (B) or 2 min later (C). (D) The same conditions as for (C) except that the final concentration of cAMP and [^{3}H]cAMP was 2 nM, the volume of [^{3}H]cAMP was increased to 100 μL, and 190 μL of the incubation mixture was added to the 1 mL of 0.1 mM cAMP. The results shown are the means of duplicate (A) or quadruplicate (B–D) incubations from a typical experiment repeated at least 2 times. Bars indicate the standard deviation.

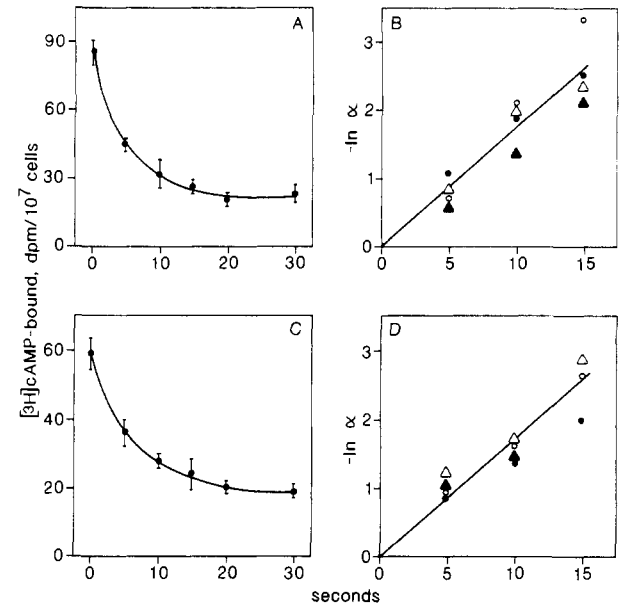

FIGURE 3: Kinetics of the blockade. (A) Cells (90 μL) were mixed with 10 μL of cAMP at t = 0, yielding 5 mM dithiothreitol and 10 nM cAMP. At the times indicated 50 μL of 10 nM [^{3}H]cAMP and 5 mM dithiothreitol was added, and binding of [^{3}H]cAMP to B^{SS} was measured at 60 s (i.e., the incubation mixture was added to 1 mL of 0.1 mM cAMP and 5 mM dithiothreitol at 60 s, and cells were centrifuged through silicon oil at 180 s). (C) Same conditions as in (A), except that the concentration of both cAMP and [^{3}H]cAMP was 2 nM and the volumes of the cell suspensions of cAMP and [^{3}H]cAMP were 2 times larger. (B, D) Logarithmic transformations of A and C, respectively α = [dpm(t) − dpm(30)]/[dpm(0) − dpm(30)]. The fact that both curves pass the origin and have identical slopes (k = 0.17 s^{-1}; $t_{1/2}$ = 4 s) indicates that the reduction of [^{3}H]cAMP bound between 0 and 30 s shows first-order kinetics. The results of (A) and (C) are the means and standard deviations of quadruplicate incubations from a typical experiment. The different symbols in (B) and (D) represent the data from four independent experiments.

Preincubation with cAMP Blocks the Binding of [^{3}H]cAMP to the B^{SS}-State but Not to the Other Binding States. *D. discoideum* cells were incubated with 10 nM cAMP plus [^{3}H]cAMP at t_0; binding to the sum of all sites rapidly increases to an equilibrium value obtained after about 10 s (Figure 2A). In the second condition, cells are preincubated with 10 nM cAMP for 20 s, during which equilibrium of binding will be reached toward the same number of sites occupied with cAMP. Then the [^{3}H]cAMP is added in such a way that the cyclic nucleotide concentration remains 10 nM. The [^{3}H]cAMP rapidly binds to the cells to the same equilibrium value as without the preincubation with cAMP. This implies that the cAMP which was bound during the preincubation did exchange with [^{3}H]cAMP during the subsequent incubation. In this experiment binding was detected as the sum of all sites.

Binding of [^{3}H]cAMP to B^S + B^{SS} is detected after a 10-s chase with excess cAMP, after which all [^{3}H]cAMP bound to the fast dissociating sites has been released. Binding to B^{SS} is detected after a chase of 2 min. An experiment with the same protocol as described above reveals that a 20-s preincubation with 10 nM cAMP results in a 50% inhibition of binding of [^{3}H]cAMP to B^S + B^{SS} (Figure 2B) and in an 80% inhibition of the binding to B^{SS} (Figure 2C). Apparently, the preincubation with cAMP prevents the binding of [^{3}H]cAMP mainly to B^{SS}. What could be the mechanism for the reduced binding of [^{3}H]cAMP to B^{SS}-sites? It has been shown that occupied B^{SS} dissociate very slowly with $t_{1/2}$ = 150 s (Van Haastert et al., 1986). Therefore, B^{SS} occupied with cAMP cannot exchange with [^{3}H]cAMP in the subsequent incubation. It could be hypothesized that [^{3}H]cAMP does not bind to B^{SS} simply because all B^{SS}-sites are occupied with cAMP. However, 10 nM cAMP is below the apparent K_d of B^{SS} (which is 15 nM), and only 40% of the B^{SS}-sites were occupied during the preincubation with cAMP. Furthermore, essentially the same result is obtained when the experiment is repeated with 2 nM cAMP, by which only 10% of the B^{SS}-sites are occupied with cAMP and 90% would still be available to bind [^{3}H]cAMP (Figure 2D). This suggests that the occupied B^{SS}-state can no longer be formed after a 20-s preincubation with cAMP. Do the other sites still bind [^{3}H]cAMP after the incubation with cAMP? Detailed calculations which correct for the partial dissociation of B^S and B^{SS} during the 10- and 120-s chases with excess cAMP have shown that the other binding states (including B^S) still could bind [^{3}H]cAMP after a preincubation with cAMP (Van Haastert et al., 1986).

These experiments reveal that a preincubation of cells with cAMP for 20 s blocks the formation of the occupied B^{SS}-state. Since these sites can only arise from a transition of the occupied B^S-state, this implies that cAMP induces a blockade in the transition of B^S to B^{SS}, which, according to our model (Figure 1), implies that a G-protein is no longer activated.

Kinetics of the Blockade. The kinetics of the blockade were investigated by adding cAMP to the cells at t = 0 s and by adding [^{3}H]cAMP at different times between 0 and 30 s. Binding of [^{3}H]cAMP to the B^{SS}-state was detected at 60 s (Figure 3). The blockade is completed after 20–30 s and is equally rapid at 2 or 10 nM cAMP. Parts B and D of figure 3 reveal that the blockade follows first-order kinetics with a

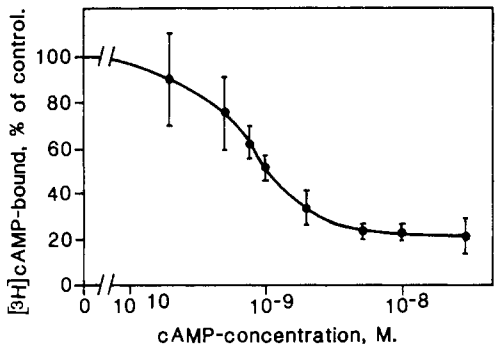

FIGURE 4: Dose–response curve of the blockade. Cells (180 μL) were incubated with 20 μL of cAMP at $t = 0$, yielding 5 mM dithiothreitol and the cAMP concentrations as indicated on the abscissa. At $t =$ 20 s 100 μL of [^{3}H]cAMP (of the same concentration as the final cAMP concentration) was added, and the binding of [^{3}H]cAMP to B^{SS} was measured at $t = 60$ s. In the control the cAMP and [^{3}H]-cAMP were added simultaneously at $t = 0$. The results shown are the means and standard deviations of three experiments, each with quadruplicate incubations.

rate constant of 0.17 s^{-1} for both cAMP concentrations ($t_{1/2}$ = 4 s).

Dose–Response Curve of the Blockade. In the previous section it was shown that 2 nM cAMP, which occupied only 10% of the sites, effectively blocked the binding of 2 nM [^{3}H]cAMP to the other B^{SS}-sites. It can be expected that the blockade cannot be induced at very low cAMP concentrations. cAMP (between 0.3 and 30 nM) was added to the cells at $t = 0$; an identical concentration of [^{3}H]cAMP was added at 20 s, and binding of [^{3}H]cAMP to B^{SS} was detected at 60 s (Figure 4). This binding is compared with the binding of [^{3}H]cAMP at 60 s when cAMP and [^{3}H]cAMP were added simultaneously at 0 s (control). This demonstrates that 0.3 nM cAMP only slightly reduces the subsequent occupation of the B^{SS}-state with [^{3}H]cAMP. A half-maximal blockade is induced by about 0.7 nM cAMP, and it saturates at about 5 nM cAMP. It should be noted that the blockade is never more than 80%.

Release of the Blockade by an Increase of the cAMP Concentration. The results, showing a blockade in a transition of the B-sites that presumably transduce the cAMP signal to guanylate cyclase, may suggest that the blockade is the basis of adaptation of the cAMP-induced activation of guanylate cyclase. The guanylate cyclase of cells adapted to cAMP can be reactivated by adding a higher cAMP concentration (Van Haastert & Van der Heijden, 1983). The question whether the blockade can be released simply by increasing the cAMP concentration was answered by the experiment shown in Figure 5. The filled circles represent the binding to B^{SS} of 2 nM [^{3}H]cAMP plus 2 nM cAMP, both added at 0 s. The filled triangles represent the binding of [^{3}H]cAMP when 2 nM [^{3}H]cAMP is added at 20 s after 2 nM cAMP; as shown previously, binding of [^{3}H]cAMP is very low due to the blockade. During this incubation (open triangles) the cAMP concentration was increased at 40 s by the addition of 25 nM cAMP (nonradioactive). Binding of [^{3}H]cAMP to B^{SS} increases immediately and reaches almost the level when cells were incubated with 2 nM [^{3}H]cAMP plus 25 nM cAMP from the beginning (open circles). Apparently, the blockade in the transition of B^S to B^{SS} that induced by 2 nM cAMP is released (temporally) by an increase of the cAMP concentration to 25 nM.

The relationship between the cAMP concentration that induces the blockade and the subsequently added [^{3}H]cAMP concentration that releases the blockade is shown in Figure

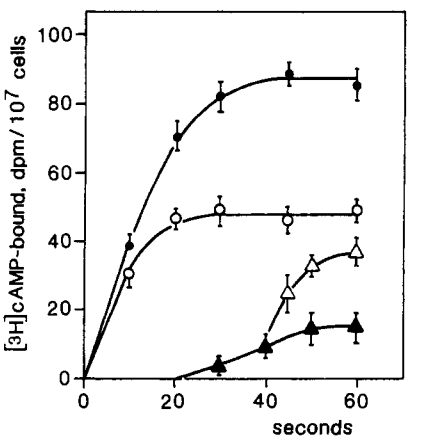

FIGURE 5: Kinetics of the release of the blockade by increasing the cAMP concentration. Cells (180 μL) were incubated with 20 μL of cAMP at $t = 0$, yielding 5 mM dithiothreitol and 2 nM cAMP. [^{3}H]cAMP (100 μL, 2 nM) was added either at $t = 0$ (●) or at t = 20 s (▲). cAMP (10 μL, yielding 25 nM) was added at $t = 40$ s to a part of the incubation that received [^{3}H]cAMP at 20 s (△). The control, in which 2 nM cAMP, 2 nM [^{3}H]cAMP, and 25 nM cAMP were added at $t = 0$ s, is also shown (○). The results are the means and standard deviations of triplicate incubations from an experiment repeated once.

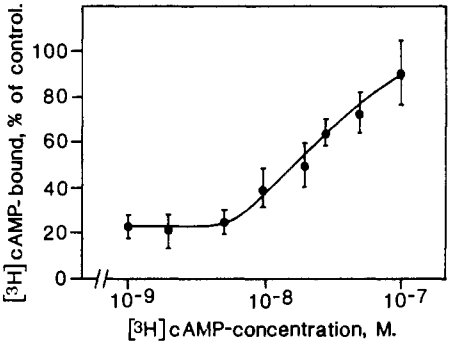

FIGURE 6: Release of the blockade by increasing the cAMP concentration; dose–response curve. Cells (180 μL) were incubated with 20 μL of cAMP at $t = 0$, yielding 5 mM dithiothreitol and 5 nM cAMP. At $t = 20$ s 100 μL of [^{3}H]cAMP was added (with the concentrations as indicted on the abscissa). Binding of [^{3}H]cAMP to B^{SS} was detected at 60 s. In the control incubation cAMP and [^{3}H]cAMP were added simultaneously at $t = 0$ s. The results shown are the means and standard deviations of three experiments, each with triplicate incubations.

6. Cells were incubated with 5 nM cAMP at $t = 0$. At $t =$ 20 s different concentrations of [^{3}H]cAMP (1–100 nM) were added, and the binding of [^{3}H]cAMP to B^{SS} was detected at 60 s. This binding is compared with the binding of [^{3}H]cAMP when cAMP and [^{3}H]cAMP were added simultaneously at 0 s (control). The preincubation with 5 nM cAMP inhibits the subsequent binding of 5 nM [^{3}H]cAMP by about 80%. This inhibition remains identical when the concentration of [^{3}H]cAMP is lower than 5 nM (by which the total cAMP concentration is reduced). The inhibition is reduced when the concentration of [^{3}H]cAMP is higher than 5 nM. The blockade is removed almost completely by an increase from 5 to 100 nM. A half-maximal release of the blockade is obtained at about 30 nM.

Release of the Blockade by Removing All cAMP. It can be expected that the blockade will be released when all cAMP is removed (e.g., by degradation by cell surface phosphodiesterase). If adaptation of the cAMP-mediated activation of guanylate cyclase is caused by the blockade of the transition of B^S to B^{SS}, then the rate of deadaptation ($t_{1/2}$ = 90 s) should be identical with the rate at which the blockade is released.

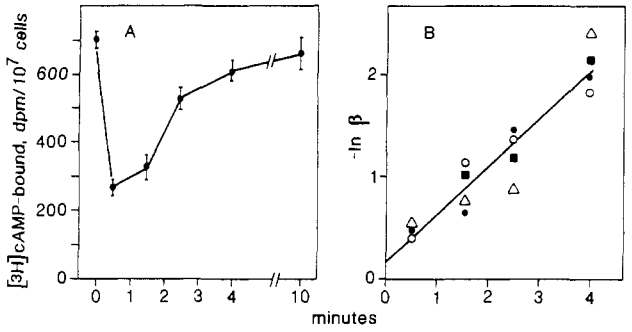

FIGURE 7: Release of blockade by reducing the cAMP concentration. (A) Cells (1 mL) were incubated with 10 μL of cAMP without dithiothreitol (the cAMP concentration after addition was 10 nM). Due to the absence of the phosphodiesterase inhibitor, cAMP is degraded with a half-life of about 10 s. At 0.5, 1.5, 2.5, 4, and 10 min portions of the cell suspension (180 μL) were incubated with 20 μL of [^{3}H]cAMP (yielding 5 mM dithiothreitol and 10 mM [^{3}H]-cAMP), and binding to B^{SS} was measured 30 s later. The [^{3}H]cAMP bound at 0 min represents the binding of 10 nM [^{3}H]cAMP to B^{SS} when cells were not prestimulated with cAMP. (B) Logarithmic transformation of the data of (A). $\beta = 1 - \mathrm{dpm}(t)/\mathrm{dpm}(0)$. The straight line indicates that the recovery of bound [^{3}H]cAMP has first-order kinetics. The slope is 7.6×10^{-3} s^{-1}, which yields a half-life of about 90 s. The results in (A) are from a typical experiment with triplicate incubations. The results in (B) are from four independent experiments.

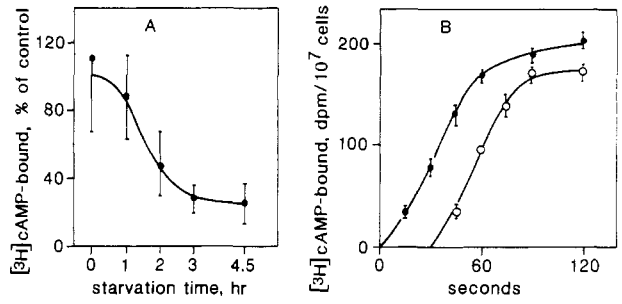

FIGURE 8: Reduced blockade in undifferentiated cells (A) and at 0 °C (B). (A) Cells were starved in suspension for different times. For each starvation period cells (180 μL) were incubated at 20 °C with 20 μL of cAMP at $t = 0$ s, yielding 5 mM dithiothreitol and 4 nM cAMP. At $t = 20$ s 200 μL of 4 nM [^{3}H]cAMP was added, and binding of [^{3}H]cAMP to B^{SS} was measured at 60 s. The control contained cAMP and [^{3}H]cAMP at $t = 0$. (B) Aggregative cells (90 μL) were incubated at 0 °C with 10 μL of cAMP at $t = 0$ s, yielding 5 mM dithiothreitol and 4 nM cAMP. At $t = 0$ s (●) or at $t = 30$ s (○) 100 μL of 4 nM [^{3}H]cAMP was added, and the binding of [^{3}H]cAMP to B^{SS} was measured at the indicated times. The results shown are the means and standard deviations of triplicate (A) or quadruplicate (B) incubations from an experiment repeated once with similar results.

The results of Figure 7 indeed show that this is the case. Cells were incubated at $t = 0$ with 10 nM cAMP without the phosphodiesterase inhibition dithiothreitol, by which cAMP is degraded with a half-life of about 10 s. At different times thereafter, the cells were incubated with 10 nM [^{3}H]cAMP for 30 s, and binding to B^{SS} was measured (Figure 7A). At 30 s cells show a strongly reduced ability to bind [^{3}H]cAMP. This ability is recovered slowly, and restored only after 4–10 min. Apparently, the blockade induced by cAMP is released only slowly after removal of the cAMP. This release shows first-order kinetics (Figure 7B) with a rate constant of 7.6×10^{-3} s^{-1} ($t_{1/2} = 90$ s).

There Is No Blockade When Cells Do Not Adapt. Experiments on the cAMP-mediated cGMP response have revealed that *D. discoideum* cells show a strongly reduced adaptation at two conditions. First, aggregative cells at 0 °C adapt at least 30-fold slower than at 20 °C (Van Haastert, 1987). Second, undifferentiated cells do not show adaptation, cells starved for 1–2 h adapt partially, and adaptation is complete after a starvation period of about 2 h (Wuster & Butz, 1983, and unpublished results). The results of Figure 8A show that this developmental regulation of adaptation correlates well with the blockade of [^{3}H]cAMP binding after a preincubation with cAMP.

At 0 °C the kinetics of association and dissociation of the cAMP-binding sites is only 2–3-fold slower than at 20 °C (Van Haastert et al., 1986). Nevertheless, adaptation of the cAMP-mediated cGMP response is strongly reduced (Van Haastert, 1987). A preincubation of cells with 4 nM cAMP at 0 °C inhibits the subsequent binding of [^{3}H]cAMP to B^{SS} by only 15% (Figure 8B). At 20 °C this inhibition is about 80%.

Discussion

The cAMP-mediated activation of adenylate and guanylate cyclase in *D. discoideum* is regulated by an adaptation process (Devreotes & Steck, 1979; Van Haastert & Van der Heijden, 1983). Cells adapt to constant cAMP stimuli but remain responsive to increments of the cAMP concentration. Adaptation of adenylate and guanylate cyclase stimulation are probably independent. (i) Adaptation of guanylate cyclase at 20 °C is 20 times faster than adaptation of adenylate cyclase (half-times are 3 s and 1 min, respectively). (ii) Adaptation of guanylate cyclase is absent at 0 °C, while adaptation of adenylate cyclase is only 2-fold slower than at 20 °C. (iii) Guanylate cyclase does deadapt at 0 °C, while adenylate cyclase does not (Van Haastert, 1987). (iv) Cells transformed with a mutated *ras* gene show an increased adaptation of guanylate cyclase, while adaptation of adenylate cyclase is not affected (Van Haastert et al., 1987). It has been suggested that covalent modification of the surface cAMP receptor (presumably phosphorylation) is the molecular mechanism of adaptation of adenylate cyclase. This hypothesis is based on the observation that receptor modification and adaptation of adenylate cyclase show similar kinetics, dose dependency, reversibility, and temperature dependency (Devreotes & Sherring, 1985). It should be noted that the phosphorylation state of the receptor does not influence the cAMP-mediated activation of guanylate cyclase. First, the stimulation of guanylate cyclase has adapted after a few seconds when the receptor has not yet been phosphorylated. Second, the receptor becomes phosphorylated at 0 °C, but guanylate cyclase stimulation does not adapt at this lowered temperature.

The differential adaptation of adenylate and guanylate cyclase agrees with, but does not prove, the hypothesis that signal transduction of extracellular cAMP to adenylate and guanylate cyclase proceeds via two subpopulations of surface receptors, which we designated A- and B-sites, respectively (Van Haastert, 1985; Kesbeke & Van Haastert, 1985). The B-sites show a series of transition upon binding of cAMP (Van Haastert et al., 1986). Initially, cAMP binds to a fast-exchanging state (B^F), which has a half-life of dissociation of about 2.5 s. B^F is converted rapidly ($t_{1/2} = 4$ s) to a more slowly dissociating state (B^S) ($t_{1/2} = 15$ s), which is subsequently converted to a very slowly dissociating state (B^{SS}) ($t_{1/2} = 150$ s). The B^S- and B^{SS}-states are sensitive to guanyl nucleotides and are transformed to the B^F-state. A cycle of interactions between receptor, G-protein, and guanyl nucleotides has been proposed to account for these observations (Figure 1). In this cycle, and probably in any cycle of receptor conversion, the response induced by cAMP is proportional to the rate at which the cycle turns around. Therefore, it is probably more important to measure the transitions of occupied binding states than binding to these sites per se. The

transition leading to an activated G-protein is especially important. According to the model this transition equals the conversion of B^S to B^{SS}. In *D. discoideum* this transition is easily measured, because the occupied B^{SS}-state arises only by the transition from the occupied B^S-state and not by association of cAMP to empty B^{SS} (Van Haastert et al., 1986).

In this paper we have further investigated the hypothesis that the activation of guanylate cyclase is directly or indirectly mediated by the B-sites. The interconversions of the different states of the B-sites were examined under conditions in which the stimulation of guanylate cyclase was adapted. The major findings are the following: (i) A short incubation of *D. discoideum* cells with cAMP prevents the subsequent binding of [^{3}H]cAMP to B^{SS} if the total cAMP concentration is not increased by the addition of [^{3}H]cAMP. (ii) The cAMP-induced blockade of [^{3}H]cAMP binding is rapid, showing first-order kinetics with a half-time of 4 s. (iii) The blockade is induced by low cAMP concentrations. A half-maximal effect is induced by about 0.7 nM cAMP; at this concentration only 5% of the B^S- and B^{SS}-sites are occupied with cAMP. (iv) The cAMP-induced blockade of the binding of equimolar concentrations of [^{3}H]cAMP is released upon addition of a higher concentration of cAMP. The release is fast because [^{3}H]cAMP immediately starts to bind to B^{SS} after the increment of the cAMP concentration. (v) The cAMP-induced blockade is released slowly after removal of cAMP. Binding of equimolar concentrations of [^{3}H]cAMP to B^{SS} reappears with a half-time of about 90 s. (vi) Conditions that prevent full adaptation of the cGMP response result in only a partial blockade of the transition fo B^S to B^{SS}.

The characteristics of the cAMP-induced blockade of cAMP binding to the B^{SS}-state of the B-receptor population are essentially identical with the cAMP-induced adaptation of guanylate cyclase. In contrast, these characteristics are quite different from those of the cAMP-induced adaptation of adenylate cyclase or the cAMP-induced modification (presumably phosphorylation) of the major cell surface receptor, which occurs with a half-time of a few minutes and takes place at 0 °C.

Although cAMP-induced adaptations of adenylate and guanylate cyclase probably occur independent of each other, they may proceed via a similar mechanism, i.e., an alteration of receptor G-protein interaction. Recently it has become possible to detect GTP-stimulated adenylate cyclase activity in *D. discoideum* membranes (Theibert & Devreotes, 1986; Van Haastert et al., 1987). In both studies it was observed that GTP stimulation of adenylate cyclase was lost in membranes that were derived from cells with an adapted adenylate cyclase.

The mechanism by which cAMP stimulates guanylate cyclase in *D. discoideum* is emerging. Recently, Europe-Finner and Newell (1985) and Small et al. (1986) have shown that IP_3 and Ca^{2+} activate guanylate cyclase in permeabilized cells. Furthermore, these investigators have shown that GTPγS induces the accumulation of IP_3 in these cells (Europe-Finner & Newell, 1987), suggesting a direct or indirect role for the G-protein-mediated activation of phospholipase C in the stimulation of guanylate cyclase in *D. discoideum*. It is tempting to suggest that adaptation of guanylate cyclase is associated with the inability to activate this G-protein, which is detected as the cAMP-induced blockade in the formation of the SS-state of the B-sites. Further work on the molecular mechanism of adaptation of guanylate cyclase will focus on the physical identification of G-proteins and receptor subclasses and on their modification by cAMP.

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