Evidence that the Rate of Association of Adenosine 3',5'-Monophosphate to Its Chemotactic Receptor Induces Phosphodiesterase Activity in *Dictyostelium discoideum*

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Adenosine 3',5'-monophosphate (cyclic AMP) mediates cell aggregation in *Dictyostelium discoideum*. Cell aggregation is enhanced by pulses of cyclic AMP. Application of pulses of cyclic AMP to cells that were starved only for 1 h (postvegetative cells) induces enzyme activity. One of the enzymes induced by cyclic AMP pulses is phosphodiesterase. We pulsed postvegetative cells with a set of cyclic AMP derivatives that were selected according to certain conformational and physical-chemical properties, and we measured their effect on the induction of phosphodiesterase activity. The cyclic nucleotide specificity for chemotaxis in the aggregative phase was similar to the specificity for phosphodiesterase induction in the postvegetative phase. The shape of the dose-response curves shows a paradox: the activity of a derivative, when applied at receptor-saturating concentrations, is inversely related to its affinity. These results can be explained by the assumption that the response of the chemoreceptor to different cyclic AMP derivatives is proportional to the frequency of associations (rate receptor) and not to the proportion of occupied receptors (occupation receptor). The characteristics of rate receptors and occupation receptors during chemosensory transduction will be discussed.

In the presence of nutrients the cellular slime mold *Dictyostelium discoideum* grows as single cells. When the food supply is exhausted, the cells pass a transient phase, after which aggregation starts, followed by the formation of a fruiting body consisting of stalk cells and spores. Aggregation-competent cells react chemotactically to cyclic AMP (21), which is excreted in pulses by neighboring cells (8). Extracellular cyclic AMP either is hydrolyzed by a cyclic nucleotide phosphodiesterase (PDE; EC 3.1.4.17) (25, 33) or binds to nonhydrolyzing receptors (9, 12, 24, 27), which results in a fast, transient increase of cyclic GMP levels inside the cell (29, 31, 40) and, ultimately, in a directed pseudopod formation.

During the transition period from the vegetative phase to the aggregative phase, amoebae undergo drastic changes. The activity of adenylate cyclase (15), membrane-bound PDE (25), and extracellular PDE inhibitor (35) increases; also the numbers of cyclic AMP receptors (9, 12, 24, 27) and contact sites A (1) increase. Addition of pulses of cyclic AMP to postvegetative cells decreases the length of the interphase (5, 6, 16, 17) and induces an earlier increase of PDE activity, cyclic AMP receptors, and contact sites A

(6, 16, 17). Pulses, rather than a continuous flow, of cyclic AMP induce a reduction of the interphase and membrane differentiation (40).

To investigate the mechanism by which cyclic AMP pulses enhance differentiation, it is necessary to identify the cyclic AMP receptor which is transducing the signal.

PDE induction by pulses of a set of selected cyclic AMP derivatives (13, 14) was measured. Their specificity for PDE induction in the postvegetative phase was similar to the specificity of the derivatives for chemotaxis of aggregative cells (26). Experimental dose-response curves were compared with theoretical dose-response curves of rate receptors and occupation receptors (38). The results suggest that PDE activity is induced by activation of a rate receptor. The consequences of activation of chemotactic receptors by the rate of association with the chemoattractant will be discussed. An abstract of this work was published previously (P. J. M. Van Haastert, R. C. Van Der Meer, and T. M. Konijn, Adv. Cyclic Nucleotide Res., in press).

MATERIALS AND METHODS

Chemicals. Cyclic [8-³H]AMP was purchased from Amersham, and snake venom (*Ophiophagus hannah*)

was obtained from Sigma. The set of cyclic AMP derivatives (14) was a gift of B. Jastorff.

PDE induction. *D. discoideum* NC-4(H) cells were grown on nutrient agar and harvested as described (20). Cells were starved by shaking in 10 mM sodium-potassium phosphate buffer (pH 6.0) at a density of 10^7 cells per ml. After 1 h, cells were centrifuged, washed twice with 10 mM phosphate buffer (pH 7.0), and resuspended in the same buffer at a density of 1.5 $\times$ 10^7 cells per ml. Cell suspensions (100 μl) were continuously shaken in small conical tubes and stimulated by 12 pulses of each derivative added at 5-min intervals. The chemoattractants were dissolved in 10 mM phosphate buffer (pH 7.0). Corrections were carried out for the volume of the liquid added. The volume of a pulse was always equal to 1/9 times the volume of the cell suspension before addition of that pulse. Fifteen minutes after the addition of the last pulse, cells were homogenized by freezing and thawing. Homogenates were incubated at 22°C for 2 h to hydrolyze still-intact cyclic AMP or cyclic AMP derivatives. The volume of the homogenates was adjusted to 1 ml. The experiments were repeated at least two times.

PDE assay. PDE was assayed according to the procedure of Thompson et al. (37). The incubation mixture of 350 μl contained: 0.1 μM cyclic AMP, 150,000 dpm of cyclic [8-^{3}H]AMP, 1 mM MgCl$_2$, and 10 mM phosphate buffer (pH 7.0). Incubations at 22°C were started by the addition of 50 μl of homogenate and terminated by boiling for 2 min. An incubation time of between 5 and 20 min was chosen to assure hydrolysis of cyclic AMP between 10 and 50%. The boiled samples were incubated at 22°C for 30 min with 100 μl of snake venom (1 mg/ml). Nonhydrolyzed cyclic AMP was removed by the addition of 1 ml of ion-exchange slurry (one part of AG 1X2 in water plus two parts of ethanol). After centrifugation the radioactivity of 500 μl of the supernatant was measured. PDE was assayed in duplicate.

Assay of cyclic GMP. Cyclic GMP accumulation was measured by a modification of the assays of Mato et al. (29) and Wurster et al. (39). Cells were shaken for 1 h, washed twice with 10 mM phosphate buffer (pH 6.0), and resuspended at a density of 10^8 cells per ml. After air was bubbled through the suspensions for 10 min, 100-μl samples were pipetted into conical tubes. While shaking was continued, 20 μl of chemoattractant was added. At the time indicated, 100 μl of cold perchloric acid (3.5%, vol/vol) was added. Cell lysates were neutralized with 50 μl of potassium bicarbonate (50% saturated solution at 20°C) and shaken for 5 min to allow CO$_2$ to escape. Samples were centrifuged for 2 min at 8,000 $\times$ *g* to remove cell debris and potassium perchlorate. The cyclic GMP content was measured in duplicate in 100 μl of the supernatant with a radioimmunoassay kit provided by Amersham.

Preparation of 5'-AMP derivatives and nucleoside derivatives. Cyclic AMP derivatives (300 μl, 10^{-4} M, in 10 mM phosphate buffer, pH 7.5) were incubated with 5 μl of PDE (Boehringer, 1 mg/ml, 10 mU/mg). Ten hours later, proteins were precipitated by the addition of 1 ml of ice-cold ethanol. After centrifugation, the supernatant was concentrated at low pressure and dissolved in 300 μl of water.

Degradation products were analyzed with high-performance liquid chromatography on Partisil SAX (Whatman Inc., Clifton, N.J.) in 15% methanol and 50 mM KH$_2$PO$_4$. This system separates nucleosides, cyclic nucleotides, and 5'-mononucleotides. Due to phosphate impurities in the PDE preparation, a mixture of 5'-AMP derivatives and nucleoside derivatives was found. Cyclic AMP derivatives were not detected. Cells were pulsed at 5-min intervals with these mixtures for 1 h at a final concentration of 10^{-5} M.

RESULTS AND DISCUSSION

The PDE activity of cyclic AMP-pulsed cells remained equal to the activity of phosphate-pulsed cells during the first 30 min of pulsation. Thereafter cyclic AMP pulses induced PDE activity. The induction of PDE was approximately linear with time for at least the next 60 min of pulsation (data not shown). Induction of PDE by cyclic AMP pulses was concentration dependent (Fig. 1). The concentration for half-maximal PDE induction was 1.4 $\times$ 10^{-8} M. This concentration has an order of magnitude similar to the dissociation constant of the cyclic AMP receptor of aggregative cells (9, 12, 24, 27). To obtain more information on the identity of this receptor, postvegetative cells were pulsed with a set of cyclic AMP derivatives (14). The cyclic AMP derivatives (Fig. 2) were selected on the following grounds: (i) only one derivative for each changed atom of the cyclic nucleotide was chosen; (ii) if an atom or atom group of the cyclic AMP molecule can form a hydrogen bond with the receptor, this hydrogen bond formation cannot take place with the cyclic AMP derivative; (iii) the syn-anti equilibrium is changed to the synconformation in 8-bromo-cyclic AMP; (iv) derivatives and cyclic AMP have similar size and polarity; (v) all derivatives, except 3'-deoxy-3'-amino-cyclic AMP and cyclic adenosine 3',5'-
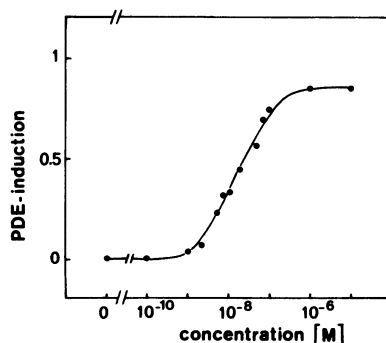

FIG. 1. *Semilogarithmic dose-response curve of PDE induction by cyclic AMP pulses. PDE induction is defined as (X − Y)/Y, where X is PDE activity of cells pulsed with chemoattractants and Y is PDE activity of cells pulsed with 10 mM phosphate buffer.*

monophosphorothioate are easily hydrolyzed by living cells (unpublished data). Application of this small set of selected cyclic AMP derivatives is sufficient to reveal the atoms and atom groups of the cyclic AMP molecule which are essential for an interaction with the receptor (13, 14, 26).

Pulsation with different concentrations of these derivatives led to quite different dose-response curves (Fig. 3). Pulsation with a mixture of a 5'-AMP derivative and a nucleoside derivative of each cyclic AMP derivative (see Materials and Methods) did not result in PDE induc-

tion, indicating that the cyclic nucleotide structures and not a degradation product is responsible for PDE induction (data not shown). The cyclic nucleotide specificities of PDE induction in postvegetative cells and of chemotaxis in aggregative cells were nearly identical (Fig. 4). Only 3'-deoxy-3'-amino-cyclic AMP is much more active in PDE induction than in chemotaxis. This compound, however, is hydrolyzed by living cells at a very low rate (unpublished data), so that the concentration is higher at each subsequent pulse of this derivative. According to the criteria proposed (13, 14, 26), the cyclic AMP receptors for PDE induction and chemotaxis both bind cyclic AMP in the anti conformation by hydrogen bonds at positions N^6H_2, N^7, and 3'-O. These data (Fig. 4) suggest that the cyclic AMP receptor for PDE induction in the vegetative phase is identical to the cyclic AMP receptor for chemotaxis in the aggregative phase.

Hayashi and Yamasaki (10) have proposed that the cyclic AMP binding sites for the induction of PDE may be different from the cyclic AMP binding sites for chemotaxis. This assumption was based on the observation that cyclic GMP and dibutyryl-cyclic AMP are as active as cyclic AMP for the induction of PDE, but are chemotactically far less active than cyclic AMP. These authors added these cyclic nucleotides not as pulses, but once at only one high (1 mM) concentration, which could explain their different conclusions.

The dose-response curves of the PDE induction by cyclic AMP derivatives (Fig. 3) are paradoxical: addition of a high concentration of a cyclic AMP derivative with a high threshold
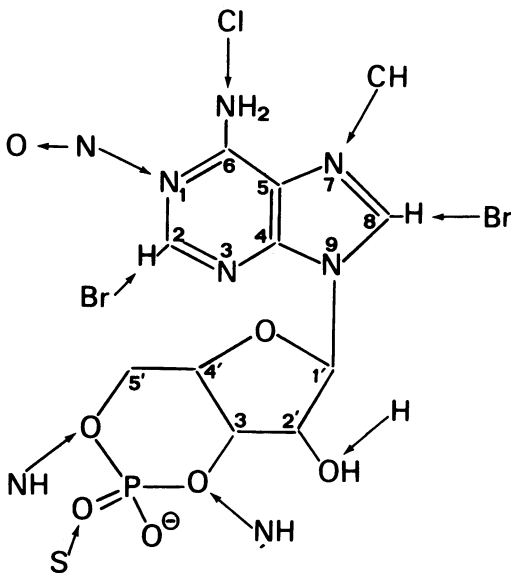

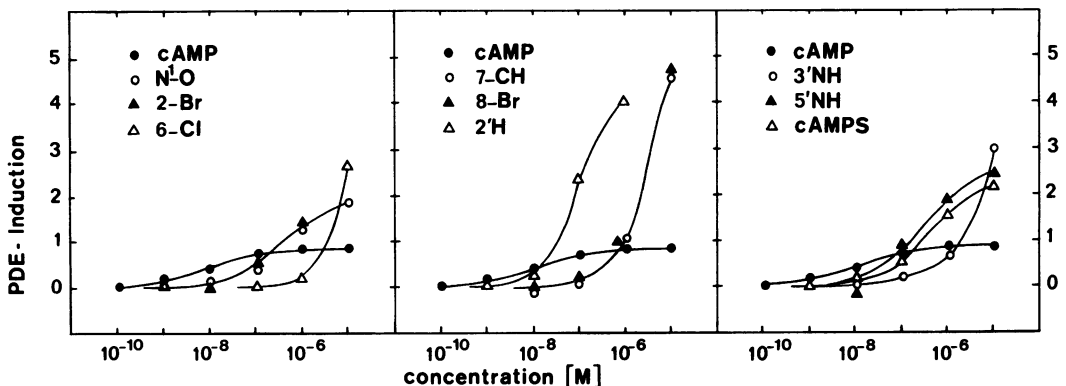FIG. 2. *Structure of cyclic AMP and cyclic AMP derivatives.*

FIG. 3. *PDE induction by cyclic AMP and cyclic AMP derivatives. For the expression of PDE induction see the legend of Fig. 1. Abbreviations: cAMP, adenosine 3',5'-monophosphate; N^1-O,N^1-oxide-adenosine 3',5'-monophosphate; 2-Br, 2-bromoadenosine 3',5'-monophosphate; 6-Cl, 6-chloropurine ribonucleoside 3',5'-monophosphate; 7-CH, 7-deazaadenosine 3',5'-monophosphate; 8-Br, 8-bromoadenosine 3',5'-monophosphate; 2'-H, 2'-deoxyadenosine 3',5'-monophosphate; 3'-NH, 3'-deoxy-3'-aminoadenosine 3',5'-monophosphate; 5'-NH, 5'-deoxy-5'-aminoadenosine 3',5'-monophosphate; cAMPS, adenosine 3',5'-monophosphorothioate.*
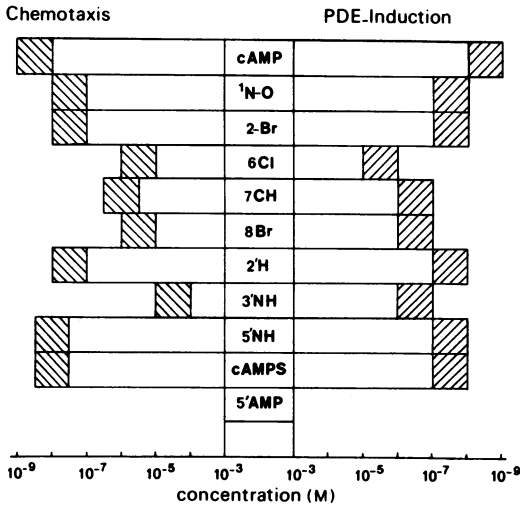
Chemotaxis   PDE-Induction

FIG. 4. *Diagram of the threshold concentrations for chemotaxis and PDE induction. Threshold concentrations for chemotaxis in aggregative cells were determined with the small-population assay (18). The concentration at which 50% of the populations react positively is approximately in the middle of the concentration range indicated. Data are only slightly different from data published previously (19, 20, 26, 28). The threshold concentration for PDE induction has been taken from Fig. 3. For abbreviations, see the legend of Fig. 3.*

concentration resulted in the induction of more PDE activity than the addition of a cyclic AMP derivative with a low threshold concentration; e.g., PDE induction by 8-bromo-cyclic AMP only occurred above 10^{-7} M, which is two orders of magnitude higher than the threshold concentration of cyclic AMP for PDE induction. However, at 10^{-5} M, 8-bromo-cyclic AMP induced more PDE than cyclic AMP did (Fig. 3). This could be explained by a better stability against hydrolysis by PDE. However, cyclic AMP pulses at 0.1 and 10 μM have different half-lives (the apparent K_m of PDE is about 1 μM) but give approximately the same PDE induction. Second, 8-bromo-cyclic AMP can give more PDE induction than adenosine 3′,5′-monophosphorothioate, although hydrolysis of the former is much faster than that of the latter (23, 36).

If the dose-response curves cannot be explained by differences of degradation of the doses, they may depend on the mechanism by which the cyclic AMP receptor transduces the signal.

Activation of a receptor by a ligand can be explained by two distinct models as follows. According to the occupation theory, the activity of the receptor is proportional to the fraction of occupied receptors; according to the rate theory

(34), the activity is proportional to the frequency of ligand-receptor combinations. These two theories predict two distinct sets of dose-response curves for the ligand and ligand derivatives (38) (Fig. 5). A derivative missing a hydrogen bond interaction with the receptor needs a higher concentration for association (Fig. 5). At high concentrations all receptors are occupied. The receptor-cyclic AMP derivative bond will be less tight than with the natural ligand, by which dissociation of the complex may occur faster. Due to this faster dissociation, more associations per unit of time can take place (Fig. 5).

The experimental data measured for the induction of PDE by cyclic AMP and cyclic AMP derivatives are not in agreement with the theoretical curve for the occupation receptor but more close to the rate receptor curves (Fig. 5). Stringent precautions should be taken when the dose-response curves are used to discriminate between rate receptors and occupation receptors because several assumptions have been made to derive the equation represented in Fig. 5 (38). Stimulation of aggregative cells with cyclic AMP and vegetative cells with folic acid results in a brief transient elevation of the cyclic GMP levels (29, 39). Stimulation of vegetative cells with cyclic AMP does not result in an elevation of cyclic GMP levels (29), probably due to a lack of sufficient cyclic AMP receptors. The rate theory predicts that cyclic GMP levels can be increased in vegetative cells by stimulation with a cyclic AMP derivative, but only if a high concentration of a derivative with a very low affinity is applied. This is shown in Fig. 6. A hydrogen bond interaction at the 3′ position of 3′-deoxy-3′-amino-cyclic AMP with the cyclic AMP receptor cannot take place. This may result in a fast dissociation of the 3′-deoxy-3′-amino-cyclic AMP–re-
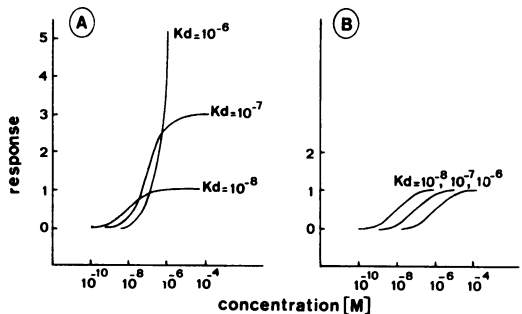

FIG. 5. *Theoretical dose-response curves of a rate receptor and an occupation receptor for a ligand with $K_d = 10^{-8}$ M and ligand derivatives with $K_d = 10^{-7}$ M and 10^{-6} M. (A) Rate receptor, (B) occupation receptor. Figure reproduced with permission from reference 38.*

ceptor complex. At the high concentrations used (Fig. 6), all cyclic AMP receptors are occupied with cyclic AMP or 3'-deoxy-3'-amino-cyclic AMP. Due to the fast dissociation of 3'-deoxy-3'-amino-cyclic AMP, sufficient associations per unit time take place, resulting in a significant increase of the cyclic GMP content of vegetative cells. These results and the data in Fig. 3 and 5 give good evidence for the hypothesis that the chemotactic receptor is a rate receptor.

Figure 7 shows the response of a rate receptor and an occupation receptor after addition and removal of cyclic AMP. Both receptors slowly reach an equilibrium response. A short time after the addition of cyclic AMP, a small fraction of the receptors are occupied, so that an occupation receptor gives only a small response. Because many receptors can still be occupied after this short time period, the response of a rate receptor is high. During the time period in which equilibrium is reached, more receptors are occupied, and less associations per unit time can take place. At equilibrium the number of associations equals the number of dissociations. After removal of cyclic AMP no associations can take place any longer, and so the response of a rate receptor declines immediately. Cyclic AMP does not dissociate immediately; therefore the response of an occupation receptor declines slowly after removal of cyclic AMP. Diffusion of cyclic AMP over the length of a cell (10 μm) only takes approximately 0.05 s if the diffusion coefficient of cyclic AMP is 10^{-5} cm^2 s^{-1} (4). This means that diffusion of a cyclic AMP molecule would be faster than detection of its location if

FIG. 7. Time-dependent signal transduction by activation of a rate receptor and an occupation receptor (after Patton [34]). At t = 0 s, 10^{-8} M cyclic AMP was added to a vigorously shaking cell suspension. At t = 2 s, cyclic AMP was removed. Conditions: $k_{-1} = 1$ s^{-1}; $k_1 = 10^8$ M^{-1} s^{-1}; $K_d = 10^{-8}$ M. k_{-1} has been estimated from data in reference 32. Solid line, Occupation receptor; dashed line, rate receptor; horizontal bar (a), diffusion time of cyclic AMP for a displacement of 10 μm.

the cell has to rely on occupation receptors. The rate of association may be sufficiently high to detect a gradient of cyclic AMP with a rate receptor.

The input signal for chemotaxis in D. discoideum has been described by a temporal gradient of cyclic AMP (7) and a spatial gradient of cyclic AMP (2, 30). Chemotaxis in bacteria depends on analysis of a temporal gradient of the chemoattractant (3, 22). Detection of temporal and spatial gradients requires a very fast transduction of the chemotactic signal. An occupation receptor would not be a suitable mediator of a rapid transduction; a rate receptor appears to be a better vehicle to transduce such a signal. The observation that other fast-responding structures, such as those involved in gustatory stimulation of insects (11) and ileum contraction by acetylcholine in guinea pigs (34), may depend on activation of rate receptors indicates that transduction of fast-changing signals to rapid-responding structures by mediating rate receptors may be of general importance.
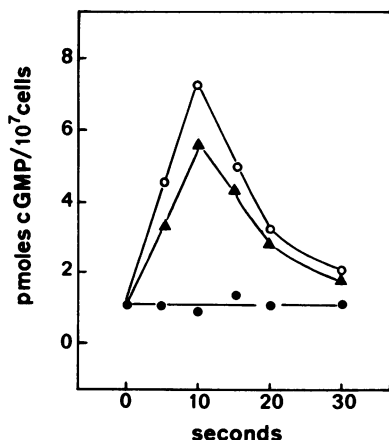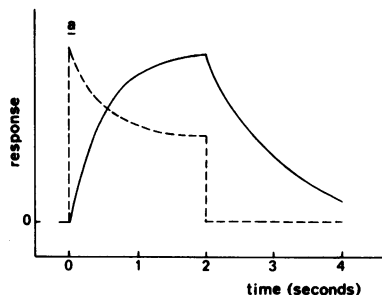
FIG. 6. Cyclic GMP accumulation in postvegetative cells after stimulation with cyclic AMP and a cyclic AMP derivative. (●) cyclic AMP, 2 × 10^{-5} M; (▲) 3'-deoxy-3'-amino-cyclic AMP, 5 × 10^{-4} M; (○) folic acid, 2 × 10^{-5} M.

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