Nonequilibrium Kinetics of a Cyclic GMP-binding Protein in *Dictyostelium discoideum*

PETER J. M. VAN HAASTERT, HANS VAN WALSUM, and FRANK J. PASVEER

Cell Biology and Morphogenesis Unit, Zoological Laboratory, University of Leiden, 2311 GP Leiden, The Netherlands. Dr. Pasveer’s present address is the Computation Unit, University of Technology, Delft, The Netherlands.

**ABSTRACT** Chemoattractants added to cells of the cellular slime mold *Dictyostelium discoideum* induce a transient elevation of cyclic GMP levels, with a maximum at 10 s and a recovery of basal levels at ~25 s after stimulation. We analyzed the kinetics of an intracellular cGMP binding protein in vitro and in vivo. The cyclic GMP binding protein in vitro at 0°C can be described by its kinetic constants $k_1 = 2.5 \times 10^8$ M$^{-1}$ s$^{-1}$, $k_{-1} = 3.5 \times 10^{-3}$ s$^{-1}$, $K_d = 1.4 \times 10^{-9}$ M, and 3,000 binding sites/cell. In computer simulation experiments the occupancy of the cGMP binding protein was calculated under nonequilibrium conditions by making use of the kinetic constants of the binding protein and of the shape of the cGMP accumulations. These experiments show that under nonequilibrium conditions the affinity of the binding protein for cGMP is determined by the rate constant of association ($k_1$) and not by the dissociation constant ($K_d$). Experiments in vivo were performed by stimulation of aggregative cells with the chemoattractant cAMP, which results in a transient cGMP accumulation. At different times after stimulation with various cAMP concentrations, the cells were homogenized and immediately thereafter the number of binding proteins which were not occupied with native cGMP were determined. The results of these experiments in vivo are in good agreement with the results of the computer experiments. This may indicate that:

(a) The cGMP binding protein in vivo at 22°C can be described by its kinetic constants: $k_1 = 4 \times 10^8$ M$^{-1}$ s$^{-1}$ and $k_{-1} = 6 \times 10^{-3}$ s$^{-1}$.

(b) Binding of cGMP to its binding protein is transient with a maximum at about 20-30 s after chemotactic stimulation, followed by a decay to basal levels, with a half-life of ~2 min.

(c) The cGMP binding proteins get half maximally occupied at a cGMP accumulation of $[cGMP]_{10} = 2 \times 10^{-8}$ M, which corresponds to an extracellular stimulation of aggregative cells by $10^{-10}$ M cAMP.

(d) Since the mean basal cGMP concentration is $2 \times 10^{-7}$ M, the small increase of cGMP cannot be detected accurately. Therefore the absence of a measurable cGMP accumulation does not argue against a cGMP function.

(e) There may exist two compartments of cGMP: one contains almost all the cGMP of unstimulated cells, and the other contains cGMP binding proteins and the cGMP which accumulates after chemotactic stimulation.

(f) From the kinetics of binding, the cellular responses to the chemoattractant can be divided into two classes: responses which can be mediated by this binding protein (such as light scattering, proton extrusion, PDE induction, and chemotaxis) and responses which cannot be (solely) mediated by this binding protein such as relay, refractoriness, phospholipid methylation, and protein methylation.
Pulse-wise addition of cAMP to *D. discoideum* cells evokes several responses. In postvegetative cells, cAMP induces the following: a decrease in light absorption in cell suspensions (12), a small, but significant excretion of cAMP, which is involved in the relay of the signal (8), chemotaxis (4), induction of phosphodiesterase (17, 18), and under some conditions, (23) acceleration of development to the aggregative phase (5, 11). In aggregative cells, cAMP induces the following: light scattering (12), relay (8, 14), chemotaxis (20), cGMP accumulation (25, 42), excretion of protons (22), protein methylation (28), and phospholipid methylation (1, 28). The cAMP signal enters the cell via cell surface receptors (15, 16, 21).

All known chemoattractants induce a transient elevation of cGMP levels: cAMP in aggregative cells of *D. discoideum* (25, 42); folic acid in vegetative cells of *D. discoideum* (31, 42) and *D. lacteum*, *D. minutum*, and *Polysphondylium violaceum* (Kakebeeke, personal communication); and partly purified, active cGMP levels: cAMP in aggregative cells of *D. discoideum* (12), relay (8, 14), chemotaxis (20), cGMP accumulation (12), and phospholipid methylation (1, 28). The cAMP signal enters the cell via cell surface receptors (15, 16, 21).

To be able to understand the function(s) of the cGMP binding proteins, it is necessary to determine the rate constants of association and dissociation and to combine these with the kinetics of cGMP accumulation after chemotactic stimulation. The dissociation constants are ~ 10^{-9} M (32, 33). The mean cGMP concentration in unstimulated vegetative cells of *D. discoideum* seems not to be associated with cGMP binding proteins which have been shown in several organisms are often not associated with protein kinase activity (see reference 10). Also, the cGMP binding activity in homogenates of *D. discoideum* cells seems not to be associated with cGMP dependent protein kinase activity (33, 35) and can be divided into at least three fractions with different molecular weights (33). The dissociation constants are ~ 10^{-9} M (32, 33). The mean cGMP concentration in unstimulated *D. discoideum* cells is ~ 2 x 10^{-7} M (25) which is two orders of magnitude above the dissociation constant. At 2 x 10^{-7} M, 99% of the cGMP binding proteins should be occupied. This suggests that intracellular compartmentalization of cGMP and cGMP binding proteins or differences in the magnitude of the dissociation constant between the in vitro and in vivo states.

The increase of cGMP levels is only transient (24, 25, 26, 31, 40, 42), by which equilibrium of binding will not be reached.

To be able to understand the function(s) of the cGMP binding proteins it is necessary to determine the rate constants of association and dissociation and to combine these with the shape of the curve of cGMP accumulation after chemotactic stimulation.

In this paper we describe the determination of the rate constant of association (k_{+}) and the rate constant of dissociation (k_{-}) in vitro. Computer simulations of the cGMP binding in vivo were performed by making use of these kinetic data and the shape of the cGMP peak after chemotactic stimulation (25, 31). The predictions of the computer simulations are in close agreement with the results of cGMP binding experiments in vivo. The consequence for a possible function of cGMP will be discussed.

**MATERIALS AND METHODS**

**Culture Conditions**

*Dictyostelium discoideum*, NC-4(H), was grown on SM-agar in association with *Escherichia coli* B/r (19). Cells were harvested in 10 mM sodium-potassium phosphate buffer, pH 6.0 and were freed from bacteria by repeated washing and centrifugation at 100 g for 4 min. Cells were starved by shaking in 10 mM phosphate buffer 6.0 at a density of 10^{7} cells/ml at 22°C.

**Homogenate**

Cells were starved for 2 h, washed twice in 5 mM Tris/HCl, pH 7.5, and suspended at a density of 2 x 10^{9} cells/ml in Tris/HCl, pH 7.5. Cells were homogenized at 0°C by sonic disruption with a Branson B-12 sonifier with microtip for 3 times 5 s at 50 W. The homogenate was centrifuged at 0°C for 10 min at 30,000 g and the supernatant was centrifuged for 1 h at 48,000 g. The 48,000 g supernatant was used for the binding experiments in vitro which are slightly modified (39) from the procedure described by Mato et al. (32).

**Rate Constant of Association**

The incubation mixture at 0°C contained 50 mM phosphate buffer pH 6.5, 3 mM MgSO_4, 2 mM DTT (Sigma Chemical Co., St. Louis, MO), different concentrations of [3H] cGMP (15 Ci/mmol, Amersham Corp., Arlington Heights, IL) and 48,000 g supernatant in a total volume of 1,750 µl. The incubation was started by the addition of 700 µl of 48,000 g supernatant.

To intervals, 200-µl samples were filtered over Millipore filters (diameter 24 mm, pore size 0.45 µm, Millipore Corp., Bedford, MA). In all experiments, filters were washed twice with 4 ml of 50 mM phosphate buffer pH 6.5 and transferred to 4 ml of Instagel (Packard Instrument Co., Downers Grove, IL); radioactivity was determined with an LKB 1215 rack beta liquid scintillation counter (LKB Instruments, Inc., Rockville, MD). Aspecific binding was determined by including 10^{-12} M cGMP in the incubation mixture.

**Rate Constant of Dissociation**

The incubation mixture at 0°C contained 50 mM phosphate buffer pH 6.5, 3 mM MgSO_4, 2 mM DTT, 5 x 10^{-5} M [8-3H]-cGMP (15 Ci/mmol) and 48,000 g supernatant in a total volume of 1,500 µl. The incubation was started by addition of 600 µl of 48,000 g supernatant. About 5 min after the addition of 48,000 g supernatant, equilibrium between association and dissociation was almost reached and further association between [8-3H]-cGMP and the binding protein was prevented either by a 50-fold dilution of the incubation mixture in 50 mM phosphate buffer, 3 mM MgSO_4, 2 mM DTT with or without 10^{-7} M unlabeled cGMP, or by the addition of 15 µl of unlabeled cGMP (10^{-9} M final concentration) to the incubation mixture. Dissociation of [H]-cGMP from the complex was followed by filtration of 10-ml samples at different times after dilution or by filtration of 200-µl samples at different times after the addition of excess unlabeled cGMP.

**In Vivo Experiments**

Cyclic GMP levels are temporarily elevated after chemotactic stimulation. This cGMP may be temporarily bound to cGMP receptors. The number of receptors that were not occupied with native cGMP was determined by fast homogenization and immediate incubation with [H]-cGMP. The homogenate should be incubated long enough with [H]-cGMP to reach equilibrium but as short as possible since the native cGMP binding protein complex will dissociate. 2 x 10^{-8} M [H]-cGMP is sufficient to reach 95% of the equilibrium binding within 1 min and low enough to keep relative aspecific binding small. The partial dissociation of the native complexes will be taken into account during the interpretation of the results. The cGMP content of the cell will compete with [H]-cGMP for binding to the protein. To keep this competition constant, cells were homogenized after the cGMP accumulation has returned to basal levels and in the presence of DTT at 0°C which inhibits the hydrolysis of native cGMP.

The procedure is as follows: Cells were starved for 5 h, centrifuged, washed twice, and suspended in 10 mM phosphate buffer, pH 6.0, at a density of 2 x 10^{6} cells/ml. Air was bubbled through the suspension for at least 10 min. At t = 0 s, cell suspensions (500 µl each) were stimulated at 22°C with 100 µl of different cAMP concentrations. At t = 10 s, a 100-µl sample was withdrawn and added to 100 µl of 3.5% (vol/vol) perchloric acid for the determination of the cGMP content (for procedure, see reference 38). 7 s before homogenization was started, 10 µl of DTT (2 mM final concentration) was added and, 2 s later, tubes were placed in ice. Cells were homogenized in ice by sonication for 6 s. 10, 12, and 14 s after homogenization was started, 100-µl homogenates were added to tubes (at 0°C) containing 50 mM phosphate buffer pH 6.5, 3 mM MgSO_4, 2 mM DTT, 2 x 10^{-8} M [8-3H]-cGMP (final concentrations) in a total volume of 250 µl. 70, 72, and 74 s after the beginning of the homogenization, 200-µl samples were filtrated. Cells were stimulated with different cAMP concentrations and homogenized at different times after stimulation.
RESULTS

Binding of cyclic GMP to a receptor can be given as (see reference 3)

\[
c\text{GMP} + F \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} B
\]

(1)

where \( F \) is the free receptor, \( B \) is the occupied receptor, \( k_1 \) is the rate constant of association, and \( k_{-1} \) is the rate constant of dissociation.

The increase of the fraction of receptors occupied \((b)\) is given by

\[
\frac{db}{dt} = k_1[c\text{GMP}](1 - b) - k_{-1}b
\]

(2)

Association

The binding of cGMP to the binding protein before and during the establishment of equilibrium is shown in Fig. 1A. Assuming that there are far less cGMP binding proteins than cGMP molecules, Eq. 2 is solved yielding:

\[
\ln \left( \frac{1 - b}{x} \right) = (k_1[c\text{GMP}] + k_{-1})t
\]

(3)

where \( x \) is the fraction of receptors occupied at equilibrium. Representation of the data of Fig. 1A as the left part of Eq. 3 vs. time gives straight lines (Fig. 1B) with a slope \((\alpha)\) which equals

\[
\alpha = k_1[c\text{GMP}] + k_{-1}
\]

(4)

Presentation of \( \alpha \) vs. the cGMP concentration results in a straight line (Fig. 1C) with a slope equal to \( k_1 \), an intercept on the ordinate equal to \(-K_d\). The experiment shown in Fig. 1 gives the following constants: \( k_1 = 1.8 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \), \( k_{-1} = 2.3 \times 10^{-3} \text{ s}^{-1} \), and \( K_d = 1.4 \times 10^{-9} \text{ M} \).

Dissociation

The rate constant of dissociation \((k_{-1})\) can be calculated from experiments in which association between \(^3\text{H}-\text{cGMP}\) and the binding protein is prevented after equilibrium is reached. If new associations of \(^3\text{H}-\text{cGMP}\) do not take place, the following equation can be derived:

\[
\frac{-\ln b}{y} = k_{-1}t
\]

(5)

where \( y \) is the fraction of binding proteins occupied with \(^3\text{H}-\text{cGMP}\) immediately after dilution or addition of an excess of unlabeled cGMP. In Fig. 2A the dissociation of \(^3\text{H}-\text{cGMP}\) from the binding protein is shown after the addition of an excess of unlabeled cGMP. New associations between \(^3\text{H}-\text{cGMP}\) and the protein are negligible after addition of excess of unlabeled cGMP. Representation of the data of Fig. 2A as the left part of equation (5) vs. time results in a straight line (Fig. 2B) with a slope \( k_{-1} = 4 \times 10^{-3} \text{ s}^{-1} \). A 100-fold dilution in buffer with or without \(10^{-5} \text{ M} \) cGMP gave identical results, indicating the absence of cooperativity at the rate constant of dissociation (6, 7).

In all experiments the filters were washed with buffer which requires about 10–15 s. Dissociation of the complex is sufficiently slow to allow this washing procedure.

Dissociation Constant

Determination of the dissociation constant with a Scatchard plot (36) is only allowed if equilibrium is reached. As can be seen from Eq. 3 and 4, this is determined by the time constant \( \alpha \), which depends on both rate constants and the cGMP concentration. By making use of the rate constants determined in Figs. 1 and 2, 95% of the equilibrium value is reached at \(10^{-9} \text{ M} \) cGMP after \(~10\) min and at \(10^{-9} \text{ M} \) cGMP after \(~20\) min. A Scatchard plot of a 1-h incubation is shown in Fig. 3, yielding a dissociation constant of \( K_d = 1.4 \times 10^{-9} \text{ M} \) and 50 fmol bound/homogenate of 10⁷ cells (equal to 3,000 binding sites/cell = \(10^{-9} \text{ M} \) mean intracellular concentration). If the incubation time is 10 min, the number of binding proteins will be overestimated and the magnitude of the dissociation constant underestimated (reference 2 and unpublished observations). The rate constants derived from several experiments in vitro at \(0^\circ\text{C} \) (Figs. 1, 2, and 3) are \( k_1 = 2.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \), \( k_{-1} = 3.5 \times 10^{-3}\text{ s}^{-1} \), \( K_d = 1.4 \times 10^{-9} \text{ M} \), and 3,000 binding sites/cell.

Computer Simulations

The basal cGMP level is \(~1\) pmol/10⁷ cells (25) which corresponds to a mean concentration of \(~2 \times 10^{-7} \text{ M} \) (taking a cell diameter of 10 \(\mu\)m). If this cGMP and the cGMP binding proteins are in the same compartment, equilibrium of binding will be reached in unstimulated cells, resulting in an occupancy of this binding protein \((K_d = 1.4 \times 10^{-9} \text{ M}) \) of \(~99.3\%\). Since this is unlikely, either the \(K_d\) in vivo is much higher (\(K_d\) might be overestimated and the magnitude of the dissociation constant underestimated (reference 2 and unpublished observations). The rate constants derived from several experiments in vitro at \(0^\circ\text{C} \) (Figs. 1, 2, and 3) are \( k_1 = 2.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \), \( k_{-1} = 3.5 \times 10^{-3}\text{ s}^{-1} \), \( K_d = 1.4 \times 10^{-9} \text{ M} \), and 3,000 binding sites/cell.

FIGURE 1 Determination of the rate constants by detection of binding before equilibrium is reached. (A) Binding is followed after mixing homogenate and different concentrations of \(^8\text{H}-\text{cGMP}\). (○), \(10^{-6} \text{ M} \); (■), \(6 \times 10^{-9} \text{ M} \); (△), \(10^{-9} \text{ M} \). Aspecific binding was determined by including \(10^{-5} \text{ M} \) cGMP in the incubation mixture and are for: (○), 110 cpm; (□), 80 cpm; and (△), 40 cpm. At 15 min, equilibrium was reached. (B) Representation of the data of A. From all data the appropriate blanks are subtracted and divided by the cpm bound under equilibrium conditions (15-min incubation). This yields b/x. Symbols as in A. (C) Representation of the data of B. The slope (a) of the curves in B is expressed vs. the cGMP concentrations, yielding a slope equal to \( k_1 \), an intercept on the ordinate equal to \( k_{-1} \), and an intercept on the abscissa equal to \(-K_d\).
vitro than in vivo) or basal cGMP and binding proteins are in different compartments. In the computer simulation experiments, we assume that basal cGMP and cGMP binding proteins are in different compartments and that cGMP accumulates only in the compartment of the cGMP binding proteins. We also assume that the cGMP peak has a triangle-like shape with a maximum at 10 s and a recovery of basal levels (0 M in this compartment) at 25 s after chemotactic stimulation (25) (Fig. 5 D). Intracellular occupation of the cGMP binding proteins is then given by the following differential equation:

\[ \frac{db}{dt} = k_1([cGMP] - [R]_b)(1 - b) - k_{-1}b \]  

(6)

where \([R]_b\) is the total binding protein concentration (10\(^{-8}\) M) and [cGMP] is the total cGMP concentration, given by the triangle of the cGMP accumulation and decline.

The fraction of binding proteins occupied by cGMP (b) was calculated with a PDP 11-45 computer by integration of Eq. 6 with 2-ms steps; negative free cGMP concentrations were excluded.

Binding of cGMP to its binding protein reaches a maximum at \(\sim 20\) s after initiation of the cGMP peak, whereby dissociation of the complex becomes noticeable.

The fraction of binding proteins occupied with cGMP depends strongly on the magnitude of the cGMP accumulation (Fig. 4 A). Variation of the rate constant of association \((k_1)\) at fixed cGMP accumulation (Fig. 4 B) results in identical curves as variation of the cGMP accumulation at fixed values of \(k_1\) (Fig. 4 A). This is evident from Eq. 6 where \(k_1\) and [cGMP] are multiplied. The magnitude of \(k_1\) determines the sensitivity of the receptor; it determines the level to which cGMP must rise to reach a 50% occupation of the binding proteins.

The rate constant of dissociation \((k_{-1})\) has minor effects on the maximum of binding protein occupancy but pronounced effects on the length of the period the binding-protein-cGMP complex exists (Fig. 4 C). Since cGMP itself disappears faster than the complex, such a binding protein may be conceived as the most simple form of memory for which \(k_{-1}\) determines the length of the information storage.

The dissociation constant \((K_d = k_{-1}/k_1)\) does not give much information on receptor occupancy if the conditions are out of equilibrium. In Fig. 5, binding of cGMP to three binding proteins with identical dissociation constants is shown. Due to their differences in rate constants, the sensitivity and duration of binding are completely different.

Fig. 4 A shows that cGMP levels have to rise only to a small extent \((3 \times 10^{-8} \text{ M})\) to reach half-maximal receptor occupancy. This increase would be comparable with a cGMP accumulation in whole cells from 1.0 to 1.1 pmol/10\(^7\) cells. In aggregative cells, this is evoked by stimulation with \(\sim 10^{-10} \text{ M CAMP}\).

**In Vivo Experiments**

The foregoing results suggest that, in vivo, the cGMP binding proteins get occupied at very small increments of cGMP levels, and that occupancy is transient with a maximum at \(\sim 20-25\) s after chemotactic stimulation and with a half-life of \(\sim 3-4\) min. An attempt was made to show this in vivo by stimulation of aggregative cells with CAMP, rapid disruption of the cells, and fast determination of the number of binding proteins that can still be occupied with \(^3\text{H}\)-cGMP.

The mathematics of these experiments are as follows: Assume that in vivo net-dissociation of the native complexes starts at \(t = a\), and that, at that moment, the fraction of binding proteins occupied with native cGMP equals \(n\), then the fraction...
of binding proteins occupied with native cGMP in vivo ($b_f$) at different times thereafter is given by:

$$\ln \frac{b_f}{n} = -k_{-1}(t_f - a)$$

(7)

where $k_{-1}$ is the rate constant of dissociation in vivo. At $t = t_f$, cells are sonicated and incubated with $^3$H-cGMP. Dissociation of the native complex proceeds further, but now with the rate constant of dissociation in vitro ($k_{-1}^{\text{in}}$). The fraction of binding proteins occupied with native cGMP ($b_f$) at the moment of filtration ($t_f + 72$) is given by:

$$\ln \frac{b_f}{n} = -k_{-1}^{\text{in}}(t_f - a) - k_{-1}^{\text{out}} \cdot 72$$

(8)

The cGMP concentration in the binding assay ($2 \times 10^{-8}$ M $^3$H-cGMP and $\sim 10^{-8}$ M native cGMP) is sufficient to label at least 95% of the binding proteins not occupied with native cGMP within 1 min. If we assume that binding proteins in nonstimulated cells are not occupied with native cGMP, then it follows that:

$$b_f' = 1 - \frac{\text{cpm} - b_l}{\text{cpm}^* - b_l}$$

(9)
FIGURE 6 Dissociation of the cGMP binding protein complex in vivo. (A) Aggregative cells were stimulated with \(10^{-7}\) M cyclic AMP at \(t = 0\) s. At the time indicated (\(t = t^*\) s), cells were homogenized by sonication for 6 s. At \(t = t^* - 7\) s, DTT was added, and at \(t = t^* - 5\) s, cells were placed in ice. Immediately after sonication homogenates were incubated in triplicate with \(^{3}\)HcGMP for 1 min, followed by filtration. (B) Determination of the rate constant of dissociation in vivo (\(k_{-1}\)). As will be shown in Fig. 7 B, stimulation with \(10^{-7}\) M cAMP leads to complete occupation of the binding protein at 30 s after stimulation, which indicates that in Eq. 8, \(n = 1\). Substitution of \(cpm^* = 1,250, bl = 110, \) and \(k_{2,1} = 3.5 \times 10^{-3}\) s\(^{-1}\) in Eq. 8 and 9 yields:

\[
-ln \frac{1250 - cpm}{886} = k_{-1} (t^* - a)
\]

where \(cpm\) is derived from A. Presentation of the left hand of this equation vs. the time of sonication (\(t^*\)) yields a line with a slope equal to the rate constant of dissociation in vivo (\(k_{-1} = 6 \times 10^{-3}\) s\(^{-1}\)), and with an intercept on the abscissa equal to the time at which net-dissociation starts (\(a = 30\) s).

FIGURE 7 Dose-response curves of the chemoattractant-mediated binding of cGMP to its intracellular binding protein in vivo. (A) Aggregative cells were stimulated with different cAMP concentrations at 0 s. At 10 s, a sample of the cell suspension was withdrawn for the determination of the cGMP levels (C)). At 33 s, 2 mM DTT was added; at 35 s, cells were placed in ice; and from 40 to 46 s cells were homogenized by sonication. Immediately after sonication, triplicate samples of the lysates were incubated with \(^{3}\)H-cGMP for 1 rain, followed by filtration. (B) Data from A were transformed by calculation of the cGMP accumulation at 10 s after stimulation with Eq. 10, and by calculation of the occupancy of the binding protein in vivo at 30 s after stimulation (\(n\)) with Eq. 8 and 9. Substitution of \(a = 30, k_{-1} = 6 \times 10^{-3}\) (both derived from Fig. 6 B), \(k_{-1} = 3.5 \times 10^{-3}\) t \(= 40, cpm^* = 1,000,\) and \(bl = 110\) in Eq. 8 and 9 yields

\[
\frac{1,000 - cpm}{890} = e^{3.31}
\]

where \(cpm\) is derived from data (O) of Fig. 7 A.

where \(cpm\) is radioactivity bound to the lysate of stimulated cells, \(cpm^*\) is radioactivity bound to the lysate of nonstimulated cells, \(bl\) is blank: radioactivity was bound in the presence of \(10^{-5}\) M cGMP. In Fig. 6 A, cells were lysed at different times after stimulation of the cell suspension with a high cAMP concentration. Lysates prepared at 30-40 s after stimulation bind significantly less \(^{3}\)H-cGMP than lysates of nonstimulated cells, or lysates prepared at longer times after stimulation with cAMP. Stimulation with \(10^{-6}\) M 5'AMP, or addition of \(10^{-7}\) M cGMP after homogenization of nonstimulated cells, was without effect (data not shown). Fig. 6 B is a linearization of the data of Fig. 6 A using Eq. 8 and 9, which reveals that dissociation in vivo at \(22^\circ C\) is a first-order process with \(k_{-1}\) equal to \(6 \times 10^{-3}\) s\(^{-1}\); net-dissociation starts at \(30\) s after chemotactic stimulation.

In Fig. 7 A, cells were lysed at 40 s after stimulation with different cAMP concentrations. At high cAMP concentrations, still \(^{3}\)H-cGMP can be bound to the lysate, most probably due to partial dissociation of the native complexes during the 1-min period of the in vitro binding assay. cAMP has a half-maximal effect at \(10^{-10}\) M. The increase of cGMP levels at 10 s after stimulation with cAMP (Fig. 7 A) can be written as

\[
\Delta[cGMP]_{10} = 1.8 \times 10^{-8} \frac{[cAMP]}{[cAMP] + 10^{-8}}
\]

This equation is a mathematical description of the dose-response curve shown in reference 25 and in Fig. 7 A. The occupancy of the cGMP binding protein at 30 s after chemotactic stimulation (\(n\)) is calculated with Eq. 8 and 9. Presentation of \(n\) vs. \(\Delta[cGMP]_{10}\) (Fig. 7 B) reveals that the binding protein is half-maximally occupied by a cGMP accumulation of \(2 \times 10^{-8}\) M, which is consistent with the computer simulations (Fig. 4 A and B) if \(k_{1}\) in vivo at \(22^\circ C\) equals \(4 \times 10^6\) M\(^{-1}\)s\(^{-1}\).

DISCUSSION

Based on chromatographic data, Mato et al. (33) have shown three cGMP binding proteins in the soluble fraction of a
produced by 1 × 10⁻¹° M cAMP (Fig. 7). At this extracellular concentration, followed by a decay to basal levels with a half-life of ~2 minutes. Autonomous oscillations of extracellular cAMP occur at a frequency of about one pulse per 5 min (14). This suggests that each pulse of extracellular cAMP can be recognized separately by the intracellular cGMP binding protein. Determinations of the occupancy of the binding protein in vivo are in good agreement with the results of the computer simulations. This has several implications:

(a) It is likely that there exist two cGMP compartments. Evidence for this has been provided by Pan and Wedner (34) and by Mato and Steiner (29) who showed with immunofluorescence techniques that cGMP is present in the nucleus.

(b) The cGMP receptor in vivo can be described by k₁ = 4 × 10⁶ M⁻¹s⁻¹, k⁻₁ = 6 × 10⁻³ s⁻¹, cooperativity is absent and there are ~3,000 binding sites per cell which give a mean concentration of ~10⁻⁹ M.

(c) The cGMP peak after chemoattractant stimulation can be described by a peak with the shape of a triangle, and its maximum at 10 s after stimulation, independent of the concentration of chemoattractant added.

(d) Binding of cGMP to the binding proteins is transient with a maximum at about 20–30 s after chemoattractant stimulation, followed by a decay to basal levels with a half-life of ~2 min (Fig. 6).

(e) In aggregative cells, cGMP binding proteins get half-maximally occupied (at 30 s) at a cGMP accumulation produced by 1 × 10⁻¹⁰ M cAMP (Fig. 7). At this extracellular cAMP concentration, intracellular cGMP levels increase from 2.0 × 10⁻⁷ M to 2.2 × 10⁻⁷ M. This accumulation cannot be measured accurately. Therefore the absence of a detectable change of cGMP concentration cannot be used as evidence against a second messenger function of cGMP. Thus, although cGMP levels do not change measurably in vegetative cells after stimulation with cAMP, the levels may change sufficiently to cause a significant occupancy of cGMP binding proteins. On the other hand, during chemotaxis of aggregative cells towards cAMP, only a small number of the cell-surface cAMP receptors are occupied (13, 27). This results in only a small increase of cGMP levels (Δ[cGMP]₈₀ ≈ 4 × 10⁻⁸ M). The absence of

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### Table 1

**Possible Functions of the cGMP-binding Protein as Mediator of Chemoattractant-induced Cellular Responses**

| Cellular response | Source |
|-------------------|--------|
| **Mediated by the cGMP-binding protein** |        |
| PDE-induction by cAMP | Occurs in vegetative cells (18, 38) |
| PDE-induction by folic acid | Half-maximal PDE-induction occurs at 3 × 10⁻⁸ M (unpublished results), while half-maximal cGMP accumulation occurs at 10⁻⁸ M (31). |
| Light scattering by cAMP | Occurs in vegetative cells and in aggregative cells at low concentrations, 50% response at ~10⁻⁹ M (12). |
| pH response by cAMP | Occurs in aggregative cells at low concentrations, 50% response at 3 × 10⁻⁸ M (22). |
| Chemotaxis to cAMP | At threshold concentrations (10⁻⁹ M in aggregative cells, and 10⁻⁸ M in vegetative cells), only a small number of cAMP receptors are occupied (13, 27). |
| Refractoriness by cAMP | Occurs at high cAMP concentrations (25). 50% refractoriness of the cGMP accumulation occurs by stimulation with 10⁻⁸ M CAMP (unpublished results). |
| Relay by cAMP in aggregative cells | 50% response occurs at 10⁻⁷ – 10⁻⁸ M (9). |
| Phospholipid methylation by cGMP in vitro | Occurs at high cGMP concentrations, 50% activation at 8 × 10⁻⁸ M cGMP (11). |
| Protein methylation by cAMP | Occurs only at high cAMP concentrations (28). |

* The half-maximal response occurs at a concentration of chemoattractant which is equal to or below the concentration which results in half-maximal occupation of the cGMP binding proteins.

† The half-maximal response occurs at concentrations far above the concentration which induces 50% occupation of the cGMP binding protein. These responses may be mediated by cGMP, but not solely via the cGMP binding protein described in this paper. A cellular process in the second group can be involved in a process of the first group if only 1% of the second response is already sufficient to get 50% of the first group response, e.g. if protein methylation is involved in chemotaxis, 1% of the maximal increase of methylation should be sufficient to get chemotaxis.

§ This high concentration seems incompatible with a function during chemotaxis, since only very small increases of cGMP levels take place. However, if the methyltransferase works in the immediate environment of the guanylate cyclase and if diffusion of cGMP is limited, a high local cGMP concentration may be present (37). Stimulation of aggregative cells with low concentrations of cAMP should then activate methyltransferase significantly, without producing a high increase of mean cGMP levels.

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chemotaxis towards cAMP in the mutant AGIP 55 (26) cannot be explained solely by the decrease of production of cGMP, because cGMP levels can still increase up to 10^{-6} M in this mutant (26).

27. The cellular functions which are evoked by chemotactants and are mediated by this cGMP protein take place at very low accumulations of cGMP levels. In aggregative cells, 50% of the response should be evoked by extracellular cAMP at a concentration of 10^{-10} - 10^{-9} M and not at a concentration where 50% of the cell surface cAMP receptors are occupied (between 10^{-2} and 10^{-8} M). Even in vegetative cells, sufficient cAMP receptors might be present to produce enough cGMP to reach significant occupancy of the cGMP binding proteins. The cellular response which might be mediated by this binding protein and responses which can not be mediated solely by this binding protein are listed in Table 1.

The function of this cGMP binding protein is unknown. No cGMP-dependent protein kinase activity has been demonstrated in D. discoideum (33, 35).

The kinetics of binding and of the cGMP accumulation show that 50% of the cGMP binding proteins get occupied at a very low occupancy of cell surface cAMP receptors. This limits the cellular responses which can be mediated solely by this receptor to PDE-induction, light scattering, pH changes, and chemotaxis.

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