Transient Kinetics of a cGMP-dependent cGMP-specific Phosphodiesterase from Dictyostelium discoideum

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ABSTRACT

Chemotactic stimulation of Dictyostelium discoideum cells induces a fast transient increase of cGMP levels which reach a peak at 10 s. Prestimulation levels are recovered in ~30 s, which is achieved mainly by the action of a guanosine 3',5'-monophosphate cGMP-specific phosphodiesterase. This enzyme is activated about fourfold by low cGMP concentrations. The phosphodiesterase has two distinct cGMP-binding sites: a catalytic site and an activator site. cAMP does not bind to either site; inosine 3',5'-monophosphate (cIMP) binds only to the catalytic site, whereas 8-bromoguanosine 3',5'-monophosphate (c-b'-GMP) preferentially binds to the activator site. For detailed kinetic measurements we have used [3H] cIMP as the substrate and c-b'-GMP as the activator. c-b'-GMP activated the hydrolysis of [3H]cIMP by reducing the $K_m$ whereas the $V_{max}$ was not altered.

The hydrolysis of [3H]cIMP was measured at 5-s intervals by using a new method for the separation of 5'-nucleotides from cyclic nucleotides. The hydrolysis of [3H]cIMP by nonactivated enzyme or by preactivated enzyme was linear with time, which indicates that a steady state is reached at the catalytic site within 5 s after addition of the substrate. In contrast, the hydrolysis of [3H]cIMP immediately after activation by 0.1 μM c-b'-GMP was not linear with time, but increased in a quasi-exponential manner with a time constant of 21 s. This suggests that a steady state at the activator site is only reached in 30–45 s after addition of the activator. The on-rate of activation ($k_+$) was $3 \times 10^5 M^{-1} s^{-1}$ for c-b'-GMP and $1.4 \times 10^5 M^{-1} s^{-1}$ for cGMP. The off-rate of activation ($k_-$) was 0.03 s$^{-1}$ for both c-b'-GMP and cGMP. The significance of these kinetic constants for the chemotactant-mediated cGMP response in vivo is discussed.

Chemotaxis has an important function during the life cycle of the cellular slime molds. In the vegetative stage the amoebae have to find their bacterial food. At this time the amoebae are chemotactic to folic acid and pterin (13, 14) both of which are excreted by bacteria. When the food source is exhausted the amoebae aggregate to form a multicellular slug that differentiates into a fruiting body. Cell aggregation is mediated by chemotaxis (1). The best studied species, Dictyostelium discoideum, utilizes pulsatile signals of cAMP (6); chemotactants from other species have been (partially) identified (4, 17, 19).

All chemotactants induce a similar accumulation of guanosine 3',5'-monophosphate (cGMP) in sensitive cells. This increase in cGMP is transitory, peaking at 10 s and returning to the basal level by 30 s after stimulation (8, 11, 20, 21, 26, 27). The involvement of cGMP in the transduction of chemotactic signals is further suggested by the isolation of chemotactant mutants which show an altered cGMP response (9, 15, 22). Recently, it has been shown that, in addition to the well-documented nonspecific phosphodiesterase (12), D. discoideum and other species have an intracellular phosphodiesterase specific for cGMP (2, 5, 11, 24). A mutant of D. discoideum (stm F “streamer” mutant NP368) has been isolated that does not possess the cGMP-specific phosphodiesterase (15, 22). In this mutant the increase of cGMP reaches higher levels than in wild-type cells and attainment of the peak is delayed until ~20 s. Prestimulation levels are not recovered within 2 min. These mutant cells respond to chemotactic signals for a longer period than wild-type cells. In vivo cGMP levels are mainly degraded by the cGMP-specific phosphodiesterase despite the presence of a 100–1,000-fold excess.

Abbreviations used in this paper: c-b'-GMP, 8-bromoguanosine 3',5'-monophosphate; cGMP, guanosine 3',5'-monophosphate; cIMP, inosine 3',5'-monophosphate.
of the non-specific enzyme in a homogenate of *D. discoideum* (22). Clearly there must be compartmentation of phosphodiesterase activity.

Experiments on the coupling between extracellular cAMP and the intracellular cGMP accumulation (20) have shown that the cAMP signal is detected within 2 s. The maximal cGMP concentration is always reached at 10 s after stimulation. This does not depend on the length of the stimulus (0.5 s or >10 min) or on the concentration of the stimulus. Adaptation is involved in the cAMP-mediated cGMP response. Cells respond to increases of the cAMP concentration, and accommodate within a few seconds to the new concentration by an adaptation process. Cells "de-adapt" after removal of the stimulus with first order kinetics with a half-life of 1.5 min (23). Adaptation is also involved in the detection mechanisms of chemotactic signals (21).

To understand the function and action of cGMP during chemosensory transduction we started to investigate the proteins directly involved in its action: a cGMP-binding protein (26), guanylate cyclase, and a cGMP-phosphodiesterase. Changes of cGMP levels after chemotactic stimulation are fast (seconds). Therefore, it might well be that the interactions between cGMP and target proteins never reach a steady state. This is indeed has been shown recently for the cGMP-binding protein of *D. discoideum* (26). The cGMP-phosphodiesterase is activated about fourfold by concentrations of cGMP within the range of chemotactic stimulation (2, 27). Activation of the enzyme by cGMP may require some time so that the cGMP synthesized by activated guanylate cyclase has at least a short period to accumulate. After cGMP is degraded, the activated phosphodiesterase will gradually recover its low activity form. If cells are stimulated during this recovery period the synthesized cGMP may not accumulate, because it is immediately hydrolyzed by the still activated phosphodiesterase. Therefore, activation of the phosphodiesterase by cGMP might be involved in the dynamics of the cGMP response, as well as in the adaptation process.

In this paper we determined the activity of the cGMP-phosphodiesterase in vitro on a time scale that is proportional to the rate of cGMP fluctuations in vivo (seconds). This revealed that at the catalytic site of the enzyme a steady state is reached in vitro within 5 s after addition of the substrate. In contrast, activation of the enzyme by cGMP in vitro was more slow; a steady state is reached only after 30-45 s. The consequences of these different activation rates for the function of the enzyme in vivo are discussed.

**MATERIALS AND METHODS**

Materials: cGMP, cyclic inosine 3',5'-monophosphate (cIMP), and 8-bromoguanosine 3',5'-monophosphate (cBMP) were purchased from Boehringer Mannheim Biochemicals (Mannheim, Federal Republic of Germany); reversed phase packing materials (Bondapak C18/Porasil B, 37-75 μm) was obtained from Waters Associates (Millipore Corp., Milford MA), [8-3H]cGMP (0.35 TBq/mmol) and [8-3H]cAMP (0.95 TBq/mmol) were from American Corp. (Buckinghamshire, Great Britain). [8-3H]cIMP was synthesized from [8-3H]cAMP according to the method of Caminell et al. (3), with the exception that pyruvate acetate was replaced by ammonium acetate for the purification (25). Immediately before use [8-3H]cIMP was purified further. 25 μl was diluted to 400 μl with 10 mM phosphate buffer, pH 7.0, and applied to a reversed phase column (6 ID x 18 mm). The column was washed at atmospheric pressure with 600 μl 1% methanol 10 mM phosphate buffer, pH 7.0. The radiochemical purity was >99.9%. cGMP-phosphodiesterase was partially purified from *D. discoideum* NC-4 (H) as described previously (2).

**RESULTS**

Previous results (2, 24) revealed that the cGMP-specific phosphodiesterase from *D. discoideum* contains two different cGMP-binding sites, which may be represented by:

\[
E + cGMP \xrightarrow{k_1} E \sim cGMP
\]

\[
cGMP \xrightarrow{k_{-1}} 5'GMP\quad \xrightarrow{k_{-n}} GMP
\]

The enzyme *E* with low catalytic activity is activated by cGMP to an enzyme-cGMP-complex (*E* ~ cGMP) with high catalytic activity; *k₁* is the on-rate of activation, *k₋₁* is the off-rate of activation, *V* is the *V*ₚ, and a prime denotes the activated species. It would be convenient if the activation site and the catalytic site could be regulated and measured independently. This requires a radioactive substrate that does not activate, and a cGMP-derivative that is a potent activator but does not bind to the catalytic site. Previous experiments (2, 24) suggested that [8-3H]cIMP and c-b'-GMP are suitable candidates for, respectively, the substrate and the activator.

**Steady State Kinetics**

The hydrolysis of different concentrations of cIMP and cGMP is shown in an Eady-Hofstee plot in Fig. 1. The hydrolysis of cGMP has the typical concave curve as shown before (2). The hydrolysis of cIMP by nonactivated enzyme *E* (filled symbols) follows normal Michaelis-Menten kinetics with an apparent *V*ₚ, *V*ₚ = 1,200 pmol min⁻¹ mg protein⁻¹, and an apparent *Kₐ*ₚ, *Kₐₚ = 28 μM. The hydrolysis of cIMP in the presence of activator c-b'-GMP (open symbols) also follows normal Michaelis-Menten kinetics with the same *V*ₚ, but with a lower apparent *Kₐ*ₚ (*Kₐₚ = 6.5 μM). These data show that cIMP is a good substrate without activating properties. Furthermore, activation by c-b'-GMP implies a reduction of *Kₐ*ₚ whereas *V*ₚ remains the same.

The activation of the hydrolysis of [8-3H]cIMP by different concentrations of cGMP or c-b'-GMP is shown in Fig. 2. In this figure the intercept on the abscissa equals - *K*ᵦ⁻¹ (see Appendix). The activation constant (*Kₐ*) for c-b'-GMP (0.07 μM) closely corresponds to the activation constant for cGMP (0.12 μM), which suggests that c-b'-GMP can be used as activator. However, the intercepts on the ordinate, which represent the maximal fold activation, are not identical: cGMP activates about threefold, and c-b'-GMP about fivefold. The microscopic reaction mechanism (see Appendix, *α* in mechanism II or III) might explain why c-b'-GMP consistently (2, 24) shows more activation than cGMP.

**Phosphodiesterase Assays:** The assay for steady state experiments (Figs. 1 and 2) was essentially identical to the procedure described previously (2). The incubation mixture for transient kinetics (Figs. 3-5) contained in a total volume of 150 μl 10 mM phosphate buffer pH 7.2, 10⁻⁷ M [8-3H]cIMP (-1 Kβq), different concentrations of cGMP or c-b'-GMP, and cGMP-phosphodiesterase (0.085 μg protein derived from 2 x 10⁶ cells). The reaction was terminated by the addition of 100 μl ice-cold 0.02 M phosphoric acid, which reduces the pH to 3.0. Then 200 μl was applied to a reversed phase column (6 ID x 11 mm).

[8-3H]cIMP was eluted with 800 μl 1% methanol 10 mM phosphate buffer pH 3.0, and subsequently [8-3H]cIMP was eluted with 1 ml 50% methanol 10 mM phosphate buffer pH 3.0. The blank of the assay was determined by adding the termination buffer to the substrate before the addition of enzyme; the blank was -0.1% (20 cpm) of the total radioactivity (20,000 cpm). Details on this separation procedure including conditions for cAMP and cGMP separation will be published elsewhere (25).
with substrate and activator (c-b'-GMP). Apparently, activation of the enzyme is a relatively slow process approaching a new steady state only after 30–45 s.

Two methods may be used to determine the reaction constants $k_1$ and $k_{-1}$; both methods depend on the determination of the relaxation time $\tau$ (see Appendix). In the intersection method (Fig. 3A) $\tau$ equals the time coordinate of the intersection point of the two lines that represent respectively the activity of the nonactivated enzyme (open symbols), and the activity of the transiently activated enzyme under steady state conditions (dashed line). The experiment of Fig. 3A yielded $\tau = 22$ s. In the logarithmic method (Fig. 3B) the data points before the attainment of a new steady state are transformed by making use of Eq. 12 (see Appendix). The observation that this procedure yielded a straight line through the origin (Fig. 3B) confirms the assumption that activation of the enzyme is a single quasi-exponential process that is adequately described by the mass law of action. According to Eq. 12 the slope in Fig. 3B equals $\tau^{-1}$ which yields $\tau = 21$ s.

In Fig. 4 the relaxation times for different concentrations of c-b'-GMP were determined by the intersection method. Since $\tau^{-1} = k_1 [c-b'-GMP] + k_{-1}$, a plot of $\tau^{-1}$ versus the concentration of c-b'-GMP should yield a straight line from which the rate constants can be easily determined (Fig. 5; $k_1 = 3 \times 10^5$ M$^{-1}$ s$^{-1}$, $k_{-1} = 0.03$ s$^{-1}$, $K_a = 0.1$ $\mu$M).

In Fig. 2 it was shown that activation by cGMP and by c-b'-GMP are not identical. Therefore, the relaxation times for

**Transient Kinetics**

The objective of this study is to measure the activity and fluctuations of activity of this enzyme on a time scale that is important for its function in vivo. Chemotactic stimulation of *D. discoideum* cells induces an increase of cGMP levels within 2 s; a maximal concentration is reached at 10 s, and prestimulation levels are recovered at ~30 s after stimulation (8, 11, 20, 27). This requires the analysis of phosphodiesterase activity during the first 30 s of the incubation; this was possible with a new separation procedure for cyclic nucleotide and 5'-nucleotides (25).

The accumulation of product is linear with time after the nonactivated enzyme was mixed with the substrate (Fig. 3A). The same was true if the enzyme preactivated by c-b'-GMP was mixed with the substrate (Fig. 3A). This indicates that within 5 s after the onset of the enzymatic reaction a steady state is reached at the catalytic site of either the activated or nonactivated enzyme. In contrast, product accumulation is not linear with time after the nonactivated enzyme was mixed with substrate and activator (c-b'-GMP). Apparently, activation of the enzyme is a relatively slow process approaching a new steady state only after 30–45 s.

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**Transient Kinetics of cGMP-PDE**

The enzyme was rapidly mixed with 0.1 $\mu$M $[^3H]$cIMP. The reactions were terminated at the times indicated, and the reaction mixture was chromatographed on small reversed phase columns. (A) O, $[^3H]$IMP formed after mixing nonactivated enzyme with $[^3H]$cIMP; O, the enzyme was preincubated with 0.5 $\mu$M c-b'-GMP for 5 min. At $t = 0$ the activated enzyme was mixed with $[^3H]$cIMP and 0.5 $\mu$M c-b'-GMP; +, the enzyme was not preactivated by c-b'-GMP. However, activation was started at $t = 0$ by mixing the nonactivated enzyme with $[^3H]$cIMP and 0.1 $\mu$M c-b'-GMP. A blank of 25 cpm was subtracted from all data. (B) The data from the transiently activated enzyme in Fig. 3A (+ symbols) are linearized by using Eq. 12 (see Appendix). $\psi$ and $\phi$ are respectively the slope ($\psi = 23.89$ cpm/s) and the intercept on the ordinate ($\phi = -400$ cpm) of the dashed line in Fig. 3A; $t$ is the time in seconds, and $P(t)$ is the radioactivity formed at time $t$ (+ symbols in Fig. 3A). The data shown are the means of triplicate determinations of a single experiment. Two duplicate experiments gave similar results.
Figure 4  Transient activation of the hydrolysis of [1H]cIMP by different concentrations of c-b'-GMP. At t = 0 s the nonactivated cGMP-phosphodiesterase was mixed with 0.1 μM [1H]cIMP and different concentrations of c-b'-GMP. At the times indicated the reactions were terminated, and the reaction product was purified by reversed phase chromatography. The concentrations of c-b'-GMP are: 0 μM; O, 0.02 μM; ▲, 0.05 μM; ▲, 0.08 μM; ●, 0.12 μM; □, 0.16 μM; +, 0.20 μM. The data shown are the means of duplicate determination of a single experiment. Two duplicate experiments gave similar results.

FIGURE 5 Determination of the rate constants $k_1$ and $k_{-1}$ of the cGMP-phosphodiesterase. The relaxation times ($r$) were measured for different concentrations of c-b'-GMP (O) or cGMP (●) by the intersection method (see Fig. 4). According to the equation $r = k_1 [\text{activator}] + k_{-1}$, the slope of the curve equals $k_1$, and the intercept on the ordinate equals $k_{-1}$. The data shown are the means of three determinations.

different cGMP concentrations were also determined by the intersection method. This is less accurate than for c-b'-GMP, since cGMP is a less potent activator resulting in the intersection of two curves with a small angle. Nevertheless, the result is quite satisfactory, yielding a straight line for cGMP (Fig. 5).

The macroscopic kinetic parameters of the cGMP-dependent cGMP-specific phosphodiesterase in vitro for the hydrolysis on a seconds time scale are summarized in Table I.

**Computer Simulations of Transient Kinetics In Vivo**

Chemotactic stimulation of *D. discoideum* cells induces a transient accumulation of cGMP levels that reach a peak at 10 s; prestimulation levels are recovered within ~30 s after stimulation. To understand the functioning of the cGMP-phosphodiesterase in vivo we have to combine the pace of intracellular fluctuations of cGMP levels with the transient kinetics of the cGMP-phosphodiesterase. Fluctuations of cGMP levels are slow if compared with the time required for the attainment of a steady state at the catalytic site. This suggests that the catalytic activity of the two enzyme species in vivo is adequately described by a Michaelis-Menten equation. In contrast, fluctuations of intracellular cGMP levels are fast if compared with the time required for the attainment of a steady state at the activator site. During the transient cGMP accumulation in vivo a steady state will not be reached at the activator site of the enzyme. Therefore, the proportioning of the two enzyme species, $E$ and $E \sim cGMP$, should be described by a differential equation (see Appendix, Eq. 2).

$$\frac{dh}{dt} = k_1[cGMP](1 - h) - k_{-1}h$$

where $h$ is the fraction of enzyme with high activity, and $[cGMP]$ is the intracellular cGMP concentration after chemotactic stimulation. Recently, we obtained a similar equation for the kinetics of a cGMP-binding protein of *D. discoideum* in vivo (23).

Calculations on the activity of the cGMP-phosphodiesterase during and after the transient cGMP accumulation in vivo are shown in Fig. 6. Phosphodiesterase activity is maximal at ~20 s after the onset of the cGMP accumulation, and declines thereafter to prestimulated activity with a half-life of ~30 s (Fig. 6 B). The peak concentration of cGMP required for half-maximal activation of phosphodiesterase activity is ~1 μM; this is produced by stimulation of aggregative *D. discoideum* cells with ~10⁻⁸ M cAMP (8).

### Table I

Macroscopic Kinetic Properties of the cCMP-Phosphodiesterase from *D. discoideum*

| Parameter | Units | cGMP | cIMP | c-b'-GMP |
|-----------|-------|------|------|---------|
| $V$ | pmol min⁻¹ (mg protein)⁻¹ | 1,200 (1) | 1,200 (1) | ~750* |
| $K_m$ | μM | ND | 28 (1) | ND |
| $K_v$ | μM | 5.4 (1) | 6.5 (1) | ~50 |
| $k_1$ | μM | 0.12 (2) | ~9 | 0.07 (2) |
| $k_{-1}$ | μM | 0.21 (5) | ~9 | 0.10 (5) |
| $k_1$ | (μM)⁻¹s⁻¹ | 0.14 (5) | ~9 | 0.30 (5) |
| $k_{-1}$ | s⁻¹ | 0.03 (5) | ~9 | 0.03 (5) |

Numbers between parentheses indicate the figures from which the data were derived. ND, not detectable because cGMP and c-b'-GMP activate the enzyme.

* Estimated from the rate of hydrolysis at ~10⁻⁸ M and the apparent $K_v$ of 150 μM. Data is from Bulgakov and Van Haastert (2).

* Estimate from the concentration of c-b'-GMP required to induce 50% inhibition of the hydrolysis of cGMP by the activated enzyme. Data is from Bulgakov and Van Haastert (2).

* cIMP does not activate the enzyme.
The transient cGMP accumulation was presented as a triangle with a top concentration at t = 10 s ([cGMP]₀), and basal levels before 0 s and after 25 s. (B) Calculations for different values of kᵋ; [cGMP]₀ = 1 μM; kᵋ = 0.03 s⁻¹. (C) Calculations for different values of kᵋ; [cGMP]₀ = 1 μM; kᵋ = 0.03 s⁻¹. (D) Calculations for different values of kᵋ; [cGMP]₀ = 1 μM; kᵋ = 0.14 (μM)⁻¹s⁻¹.

**DISCUSSION**

The cGMP-dependent cGMP-specific phosphodiesterase has two binding sites for cGMP: an activator site and a catalytic site. Binding of cGMP to the activator site increases the affinity (Kₐ) of the catalytic site, while the hydrolytic activity of the catalytic site (V) is unaltered. The enzyme and cGMP reach a steady state at the catalytic site within 5 s after the addition of a constant substrate concentration. In contrast, a steady state is approached at the activator site only at 30–45 s after the addition of an activator. The combination of these kinetic data of the phosphodiesterase with the chemoattractant-mediated transient cGMP accumulation in vivo suggests that the cGMP-phosphodiesterase in vivo is transiently activated after chemotactic stimulation (Fig. 6). This may indicate that activation of the cGMP-phosphodiesterase by cGMP has an important function during chemosensory transduction.

An attempt was made to show activation of cGMP-phosphodiesterase activity in vivo by stimulation of cells with cAMP, rapid homogenization followed immediately by the detection of cGMP-phosphodiesterase activity during 10 s. This was not successful, due to the presence of a 100–1,000-fold excess of nonspecific phosphodiesterase activity in homogenates of aggregative D. discoideum cells. The nonspecific phosphodiesterase can be inhibited by dithiothreitol and excess cAMP, which does not inhibit the cGMP-phosphodiesterase. However, the nonspecific phosphodiesterase became sensitive to these inhibitors only a few minutes after preparation of the homogenate (unpublished observations). Therefore, it was not possible to detect specifically the activity of the cGMP-phosphodiesterase within a few seconds after disruption of the cells.

Recently it was shown that adaptation is involved in the transduction of chemotactic signals (18, 20). Cells that are stimulated at 0 s do not respond again with a cGMP accumulation to an identical stimulus applied at 30 s. At 30 s after chemotactic stimulation the cGMP concentration has returned to basal levels, but the cGMP-phosphodiesterase is still partially activated (Fig. 6B). Cells that are restimulated at 30 s might again start to synthesize cGMP, but this cGMP might not accumulate, because the phosphodiesterase is still activated. Thus, activation of cGMP-phosphodiesterase might be responsible for the adaptation process. This hypothesis demands that the rate of “de-adaptation” is equal to the rate at which the activated phosphodiesterase decays to the low activity form (k⁻₁). The rate constant of deadaptation is 7 × 10⁻³ s⁻¹ (20), whereas the kᵋ = 3 × 10⁻² s⁻¹; thus at 100 s after chemotactic stimulation the cGMP-phosphodiesterase has returned to the low activity form, while cells are still adapted by 50%. This leads to the conclusion that the cGMP-phosphodiesterase is not responsible for the adaptation process and, consequently, that adaptation is localized at or before the guanylate cyclase.

In wild-type D. discoideum cells the cGMP concentration always reaches a peak at 10 s after stimulation, and basal levels are recovered in ~30 s. A mutant has been isolated that does not have the cGMP-phosphodiesterase and that shows an altered cGMP response (15, 22): cGMP reaches higher levels after stimulation, the attainment of the peak is delayed to 20 s, and prestimulated levels are recovered in only 2–3 min. These mutant cells show an altered chemotactic response during cell aggregation that does not terminate after 100 s as in wild-type cells, but which can last as long as 500 s (15). These observations suggest that the cGMP-phosphodiesterase is involved in the dynamics of the cGMP response and in the dynamics of chemotaxis.

The cGMP response is the result of modulation of guanylate cyclase and cGMP-phosphodiesterase activity. Mato and Malchow (10) have shown that chemotactic stimulation induces an activation of guanylate cyclase in vivo. This activated guanylate cyclase is not stable in vitro, but decays to the prestimulated activity with a half-life of ~20–30 s. The cGMP response may be described qualitatively by the following events: chemotactic stimulation of D. discoideum cells leads to a fast activation of guanylate cyclase activity, cGMP levels will accumulate, since phosphodiesterase activity is low during the first few seconds (see Fig. 6B). As time proceeds guanylate cyclase activity decreases, whereas phosphodiesterase activity
increases. At some moment cGMP levels will not increase further. After longer times (10-30 s) guanylate cyclase activity drops to prestimulated levels, whereas cGMP-phosphodiesterase activity is maximally active; this results in a decrease of the cGMP concentration to prestimulated levels. After a still longer period the cGMP-phosphodiesterase decays to prestimulated levels. In this description the decay rate of the guanylate cyclase and the on-rate of the cGMP-phosphodiesterase determine the dynamics of the cGMP response. A quantitative description can be given only if the exact modulation of guanylate cyclase activity after chemotactic stimulation in vivo is known. This requires the detection of enzyme activity at different times, but within 30 s after chemotactic stimulation. These experiments are in progress.

APPENDIX: MATHEMATIC DESCRIPTION OF TRANSIENT KINETICS OF cGMP PHOSPHODIESTERASE

Reaction Mechanism

The cGMP-dependent cGMP-specific phosphodiesterase from D. discoideum has two cGMP binding sites, one for activation and one for hydrolysis. The macroscopic reaction mechanism may be described as mechanism I.

\[
E + A \overset{k_1}{\rightleftharpoons} E'A
\]

\[
S \overset{K_m}{\rightleftharpoons} P
\]

In this equation, E is the enzyme with low activity, A is the activator, S is the substrate, P is the product, and V is the \( V_{max} \); a prime denotes the activated species.

A microscopic reaction mechanism is required to derive a rate equation for the hydrolysis of cGMP. The reaction mechanism for nonessential activated enzymes has been presented as mechanism II (see page 227 of reference 16).

\[
E + S \overset{K_a}{\rightleftharpoons} ES \overset{k_o}{\rightarrow} E + P
\]

\[
A \overset{\alpha K_a}{\rightleftharpoons} EAS \overset{\beta K_o}{\rightarrow} EA + P
\]

Activation of the enzyme in the absence of substrate is given for this mechanism by \( E + A \equiv E'A \). However, we have observed that several physical manipulations of the enzyme may lead to (partial) activation in the absence of the activator. Such treatments are brief warming of the enzyme at 35°C or incubation at alkaline pH (2). This suggests that activation of the enzyme proceeds via a more complex mechanism:

\[
E \overset{K}{\rightarrow} E'
\]

\[
E \overset{K}{\rightarrow} E'
\]

\[
A \overset{\alpha K_a}{\rightleftharpoons} EAS
\]

Thus, the activator stabilizes the existence of the activated enzyme species. Combining this activation mechanism with the substrate leads to mechanism III, which is probably the most simple microscopic reaction mechanism of the cGMP-phosphodiesterase. This mechanism can be written in simplified notation as

\[
E + A \overset{k_1}{\rightleftharpoons} E'A
\]

\[
1 - h \overset{a}{\rightarrow} k_{-1} h,
\]

The steady state rate equation for mechanism III can be derived with the King-Altman method (7, 16); however, the equation is of little practical value, because it is not possible to obtain experimental data for the high number of microscopic rate constants involved in this mechanism.

Analysis of cGMP phosphodiesterase activity reveals that a steady state at the activator site is only reached after ~30-45 s. Therefore, a rate equation describing presteady state kinetics has also to be derived; to our knowledge such an equation cannot be solved for mechanisms II and III.

The aim of this study is to derive a mathematical description applicable to the in vivo situation in which cGMP levels change on a seconds time scale. It would be sufficient to obtain macroscopic rate constants that are correct on a seconds time scale. The approach to use mechanism I implies that apparent rate constants are obtained, and that the description is probably not correct on a millisecond time scale.

Assumptions

The following assumptions are made to derive mathematical equations: (a) The cGMP-phosphodiesterase exists in only two conformations with low and high catalytic activity. (b) The enzyme is activated because of a decrease of apparent \( K_m \) rather than an increase of apparent \( V \) (see Fig. 1). (c) The substrate does not activate the enzyme (cIMP; see Fig. 1 and references 2 and 24). (d) The activator of the enzyme does not compete with the substrate for the catalytic site (c-b-R-GMP at concentrations below 1 \( \mu \)M and cGMP at concentrations below 0.1 \( \mu \)M; see references 2 and 24). (e) The substrate concentration remains constant. (The experiments were done in such a way that not more than 15% of the substrate was hydrolyzed.) (f) The activator concentration remains constant. (The enzyme concentration is probably much smaller than the activator concentration, and the activator is not significantly degraded during the incubation.) (g) A steady state is always reached between substrate and catalytic site (see Fig. 3). (h) Activation of the enzyme is described by the mass law of action with the simple equation:

\[
E + A \overset{k_1}{\rightleftharpoons} E'A
\]

where \( h \) is the fraction of enzyme in \( E'A \) and \( a \) is the concentration of activator.
Rate Equations

These assumptions lead to the following set of equations:

$$\frac{dh}{dt} = k_1 a (1 - h) - k_{-1} h,$$

(2)

$$v = \frac{dp}{dt} = (1 - h(t))V \frac{s}{s + K_m} + h(t)V \frac{s}{s + K_m'}$$

(3)

where \( p \) is the concentration of the product, \( V \) is the apparent \( V_{\text{max}} \) of the enzyme, \( s \) is the substrate concentration, \( K_m \) is the apparent \( K_m \) of enzyme \( E \), and \( K_m' \) is the apparent \( K_m \) of \( E'A' \).

Steady State Kinetics

Eq. 2 is solved for \( \frac{dh}{dt} = 0 \) yielding

$$h = \frac{a}{a + K_a}.$$  

(4)

where \( K_a \) is the activation constant; \( K_a = k_{+1}/k_{-1} \). Substitution of Eq. 4 in Eq. 3, and taking \( s \ll K_m \) and \( s \ll K_m' \) results in

$$\frac{v}{V} = \frac{s}{K_m} \left( 1 + \frac{K_a}{a} \right) \left( \frac{V}{K_m'} - \frac{V}{K_m} \right).$$

(5)

This equation is rearranged to

$$\frac{V}{K_m'} \frac{s}{K_m} = \frac{V}{K_m} \frac{cpm^0}{cpm - cpm^0} = \left( 1 + \frac{K_a}{a} \right) \left( \frac{K_m'}{K_m'} - \frac{K_m'}{K_m} \right).$$

(6)

where \( cpm^0 \) is the radioactivity of \([\text{PH}]5'\text{IMP} \) formed in the absence of activator, and \( cpm \) in the presence of activator. The expression, \( cpm' \) (\( cpm - cpm^0 \)) versus \( a^{-1} \), should yield a straight line with an intercept on the abscissa equal to \( -K_{-1}^{-1} \) and an intercept on the ordinate equal to \( K_m' (K_m - K_m')^{-1} \).

Transient Kinetics

The solution of \( h \) in Eq. 2 yields

$$h(t) = \frac{a}{a + K_a} \left( 1 - e^{-t} \right).$$

(7)

where

$$t^{-1} = k_1 a + k_{-1}.$$  

(8)

Activation starts at \( t = 0 \). Substitution of Eq. 7 in Eq. 3, followed by integration yields

$$p(t) = \left( V \frac{s}{s + K_m} + c \right) t - c t^\frac{1}{2} \left( 1 - e^{-\frac{1}{2}} \right).$$

(9)

where

$$c = \frac{a}{a + K_a} V \left( \frac{s}{s + K_m} - \frac{s}{s + K_m'} \right).$$

(10)

For large \( t \) Eq. 9 is simplified to

$$p(t) = \left( V \frac{s}{s + K_m} + c \right) t - c t^\frac{1}{2} \left( 1 - e^{-\frac{1}{2}} \right).$$

(11)

This asymptotic function is used to determine the time constant \( \tau \), which contains the important rate constants \( k_1 \) and \( k_{-1} \). \( \tau \) can be determined in two different ways.

Intersection Method: The product accumulation curve of the nonactivated enzyme (Eq. 9, \( \chi = 0 \)) crosses the asymptote of the transiently activated enzyme at large \( t \) (Eq. 11) in a point with a time coordinate equal to \( \tau \).

Logarithmic Method: The asymptote of the transiently activated enzyme at large \( t \) (Eq. 11) has a slope \( \psi = V \frac{s}{s + K_m'} \)

\( + \chi \), and an intercept on the ordinate \( \phi \). Substitution of these parameters in Eq. 9 and rearrangement yields

$$\frac{1}{\phi} \left( \frac{p(t) - \psi t}{\psi} \right) = \frac{t}{\tau}.$$  

(12)

\( \psi \) and \( \phi \) are determined graphically; expression of the left hand of Eq. 12 versus time should yield a straight line through the origin with a slope equal to \( \tau^{-1} \). The rate constants \( k_1 \) and \( k_{-1} \) are determined graphically by the expression of \( \tau^{-1} \) versus the activator concentration \( a \). According to Eq. 8 this should yield a straight line with slope equal to \( k_1 \), intercept on the ordinate equal to \( k_{-1} \), and an intercept on the abscissa equal to \( -K_{-1}^{-1} \).

The intersection method has the advantage that \( \tau \) is determined relatively accurately, and that experiments are relatively easy to perform. However, this method does not confirm the assumption that activation is a single quasi-exponential process (assumption \( h \)). The justification of this important assumption, which, in fact, justifies the use of mechanism I, can only be provided by the logarithmic method.

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Van Haastert and Van Lookeren Campagne Transient Kinetics of cGMP-PDE 715
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