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Evidence for a Messenger Function of Cyclic GMP During Phosphodiesterase Induction in *Dictyostelium discoideum*

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Chemotactic stimulation of vegetative or aggregative *Dictyostelium discoideum* cells induced a transient elevation of cyclic GMP levels. The addition of chemoattractants to postvegetative cells by pulsing induced phosphodiesterase activity. The following lines of evidence suggest a messenger function for cyclic GMP in the induction of phosphodiesterase: (i) Folic acid and cyclic AMP increased cyclic GMP levels and induced phosphodiesterase activity. (ii) Cyclic AMP induced both cyclic GMP accumulation and phosphodiesterase activity by binding to a rate receptor. (iii) The effects of chemical modification of cyclic AMP or folic acid on cyclic GMP accumulation and phosphodiesterase induction were closely correlated. (iv) A close correlation existed between the increase of cyclic GMP levels and the amount of phosphodiesterase induced, independent of the type of chemoattractant by which this cyclic GMP accumulation was produced. (v) Computer simulation of cyclic GMP binding to intracellular cyclic GMP-binding proteins indicates that half-maximal occupation by cyclic GMP required the same chemoattractant concentration as did half-maximal phosphodiesterase induction.

In the presence of nutrients, amoebae of the cellular slime mold *Dictyostelium discoideum* grow as single cells. When the food supply is exhausted, cells pass through a starvation phase, aggregate, and form a fruiting body consisting of stalk cells and spores. Vegetative cells react chemotactically to folic acid (21), which probably acts as a food-seeking device. Aggregation-competent cells react chemotactically to cyclic AMP (cAMP) (13), which is excreted in pulses by neighboring cells (26). Stimulation of vegetative cells with folic acid (20, 32) or of aggregative cells with cAMP (18, 32) results in a fast transient elevation of cyclic GMP (cGMP) levels.

During the transition period from the vegetative to the aggregative phase, amoebae undergo drastic changes. The activity of adenylate cyclase (9), membrane-bound phosphodiesterase (PDE) (23), and extracellular PDE inhibitor (15) increases; also, the number of cAMP receptors (5, 6, 14, 17) and contact sites A (3) is higher during the aggregative phase. The addition of pulses of cAMP to postvegetative cells (approximately 1 h after removal of bacteria) decreases the length of the interphase (10, 11) and induces an earlier increase of PDE activity, cAMP receptors, and contact sites A (4, 10, 11, 25). Also, the addition of pulses of folic acid to postvegetative cells reduces the length of the interphase (31) and induces PDE activity (2).

Based on the differential activity of several cAMP derivatives, we suggested that the cAMP receptor for chemotaxis (16) and cGMP accumulation (20) in the aggregative phase and PDE induction in the postvegetative phase (29) are identical. The cAMP receptor seems to be a rate receptor (29), which means that the activity of the receptor is proportional to the frequency of occupation and not to the fraction of receptors occupied (24, 28).

This characteristic of the rate receptor explains why a fast-dissociating cAMP derivative induces more PDE than cAMP can in postvegetative cells and why such a derivative can increase cGMP levels in these cells although cAMP cannot induce measurably higher cGMP levels (29).

In this paper we present several lines of evidence for a messenger function for cGMP during induction of cyclic nucleotide PDE (EC 3.1.4.17).

MATERIALS AND METHODS

Chemicals. Pterin, xanthopterin, aminopterin, pterin-6-carboxylic acid, isoxanthopterin, leucopterin, and

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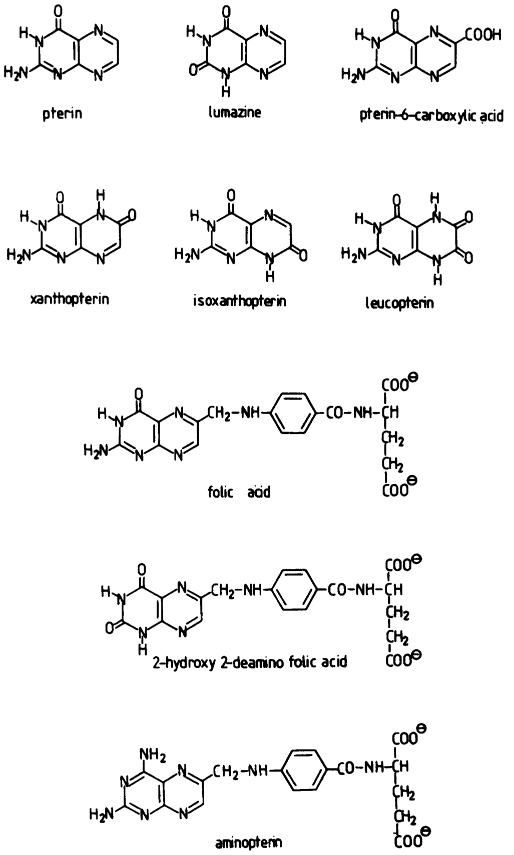

FIG. 1. Conformation of pterin and folic acid derivatives. The aromatic hydroxy functions are in keto-enol tautomerism and probably in the keto conformation.

lumazine were purchased from Sigma Chemical Co.; folic acid was from British Drug House; [8-^{3}H]cAMP and the cGMP radioimmunoassay kit were from Amersham Corp. The cAMP derivatives were a gift from B. Jastorff (7) and 2-hydroxy-2-deaminofolic acid was a gift from P. Kakebeeke (8).

Organisms. *D. discoideum* NC-4(H) was grown on SM agar in association with *Escherichia coli* B/r and harvested as previously described (12). Cells were freed from bacteria by repeated centrifugation and starved by being shaken in 10 mM sodium potassium phosphate buffer, pH 6.0, in a spinner suspension at 22°C.

PDE induction. PDE induction was measured as described previously (29). Cells starved for 1 h were washed twice in 10 mM phosphate buffer, pH 7.0, and suspended at a density of 10^7 cells per ml. Twelve pulses of chemoattractant were added to 100-μl cell suspensions at 5-min intervals. At 15 min after the addition of the last pulse, cells were homogenized by being frozen and thawed under agitation. PDE activity was determined by a previously described procedure (29). The PDE induction (I) is defined as $I = (A - B)/B$, where A is PDE activity after pulsation with a chemoattractant and B is PDE activity after pulsation with 10 mM phosphate buffer, pH 7.0.

cGMP levels. cGMP concentrations were determined by a modification (29) of the method of Mato et al. (18). Cells starved for 1 h in a spinner suspension were collected by centrifugation, washed twice with 10 mM phosphate buffer, pH 6.0, and suspended in the same buffer at a density of 10^8 cells per ml. Cell suspensions (100 μl) were stimulated with 20 μl of chemoattractant under vigorous agitation at 22°C. After 10 s of stimulation, 100 μl of cold perchloric acid (3.5%, vol/vol) was added, and samples were placed on ice. Suspensions were neutralized with 50 μl of potassium bicarbonate (50% saturated solution at 22°C) and centrifuged. The cGMP content in 100 μl of the supernatant was determined by radioimmunoassay.

Computer simulations. The binding of cGMP to its binding protein can be described by the differential equation

$$db/dt = [K_1(\mathrm{cGMP} - R_0 b)(1 - b)] - K_{-1} b \quad [1]$$

where b is the fraction of the binding proteins which are occupied with cGMP, cGMP is the total cGMP concentration, R_0 is the total binding protein concentration, K_1 is the rate constant of association, and K_{-1} is the rate constant of dissociation.

The cGMP concentration was generated as a peak with a triangular shape, of which the basal cGMP levels equal zero and are reached at 0 and ≥25 s, and of which the top cGMP level, $\Delta[\mathrm{cGMP}]_{10}$, is reached at 10 s (P. J. M. Van Haastert, J. Van Walsum, and F. A. Pasveer, J. Cell Biol., in press). Binding of cGMP to its binding proteins was computed with an

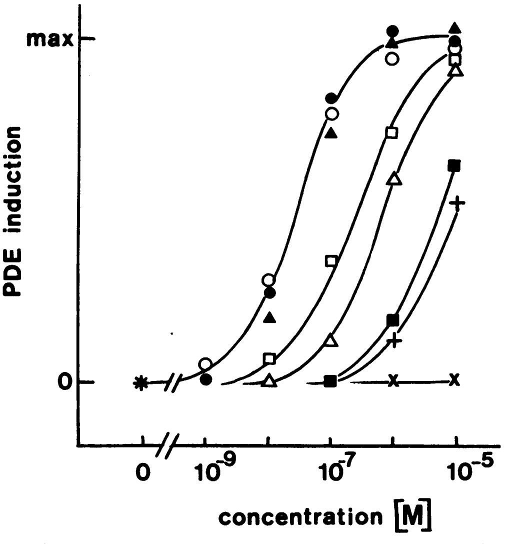

FIG. 2. PDE induction by pterins. Postvegetative cells were stimulated by 12 pulses at the indicated concentration. At 15 min after the addition of the last pulse, cells were homogenized and assayed for PDE activity. Data are from several experiments and normalized to folic acid, which had a maximum of 4 to 6 with a mean of 5.5. Symbols: ●, folic acid; ○, pterin; ▲, xanthopterin; □, aminopterin; △, pterin-6-carboxylic acid; ■, isoxanthopterin; +, deaminofolic acid; x, leucopterin; *, lumazine.

TABLE 1. Concentrations of folic acid and its derivatives required for half-maximal responses

Chemoattractant	Half-maximal concn[a]			Normalized concn[b]		
	Chem	PDE	cGMP	Chem	PDE	cGMP
Folic acid	10^{-6}–10^{-7}	3×10^{-8}	5×10^{-6}	1	1	1
Aminopterin	10^{-4}–10^{-5}	2.5×10^{-7}	7.5×10^{-5}	100	10	15
2-Hydroxy-2-deaminofolic acid	$>10^{-4}$	1×10^{-5}	$>1 \times 10^{-3}$	>100	300	>200
Pterin	10^{-6}–10^{-7}	3×10^{-8}	1×10^{-6}	1	1	0.2
Xanthopterin	10^{-5}–10^{-6}	5×10^{-8}	1×10^{-5}	10	2	2
Isoxanthopterin	10^{-3}–10^{-4}	5×10^{-6}	$>1 \times 10^{-3}$	1,000	200	>200
Leucopterin	$>10^{-3}$	$>1 \times 10^{-3}$	$>1 \times 10^{-3}$	>1,000	>10,000	>200
Pterin-6-carboxylic acid	10^{-5}–10^{-6}	7×10^{-7}	7.5×10^{-5}	10	20	15
Lumazine	$>10^{-3}$	$>1 \times 10^{-3}$	$>1 \times 10^{-3}$	>1,000	>10,000	>200

[a] Chem, Range at which 50% of cell populations showed a positive chemotactic response; PDE, concentration at which half-maximal PDE induction (2.75) was achieved (data taken from Fig. 2); cGMP, concentration at which half-maximal cGMP accumulation was achieved (increase of 4.5 pmol/10^7 cells).

[b] The data in each column have been normalized against the data for folic acid in the first three columns.

IBM 370 by solving for b in equation 1 with a method described previously (Van Haastert et al., in press). Pulsation experiments were simulated by the generation of 12 cGMP accumulations at 5-min intervals. Occupancy of the binding protein was recorded as the integral of b after 60 min and calculated with cGMP peaks of different magnitudes. The constants in equation 1 were derived from experiments in vivo (Van Haastert et al., in press): $K_1 = 4 \times 10^6\ M^{-1}\ s^{-1}$, $K_{-1} = 6 \times 10^{-3}\ s^{-1}$, and $R_0 = 10^{-8}$ M.

RESULTS AND DISCUSSION

The addition of folic acid, pterin, and their derivatives (Fig. 1) to postvegetative cells (cells starved for 1 h) resulted in different dose-response curves for PDE induction (Fig. 2), which ran parallel and seemed, to the extent it was measured, to reach approximately the same maximal response. The addition of these chemoattractants to postvegetative cells resulted in a set of dose-response curves for cGMP accumulation at 10 s which were similar in shape and sequence to the curves for PDE induction (data not shown). The concentrations which resulted in half-maximal PDE induction (2.75) and half-maximal cGMP accumulation (5 pmol/10^7 cells, $\Delta[\text{cGMP}]_{10} = 0.9\ \mu M$) and the threshold concentration for chemotaxis in these postvegetative cells are listed in Table 1. PDE induction, cGMP accumulation, and chemotaxis showed similar sensitivity to chemical modification of the folic acid or pterin molecule. This similar specificity points to an identical receptor for these three processes. Also, with cAMP, the signals for chemotaxis, cGMP accumulation, and PDE in-

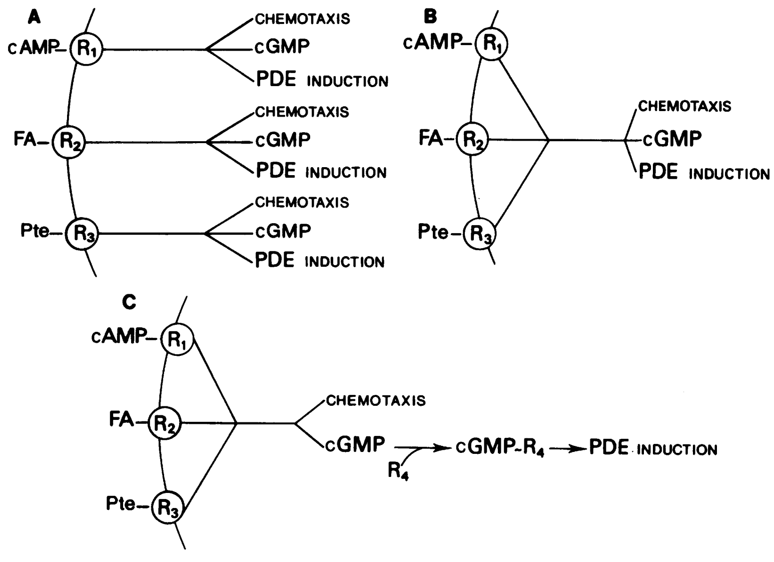

FIG. 3. Three possible transduction pathways: schemes I (A), II (B), and III (C). FA, Folic acid, Pte, pterin; R, receptor.

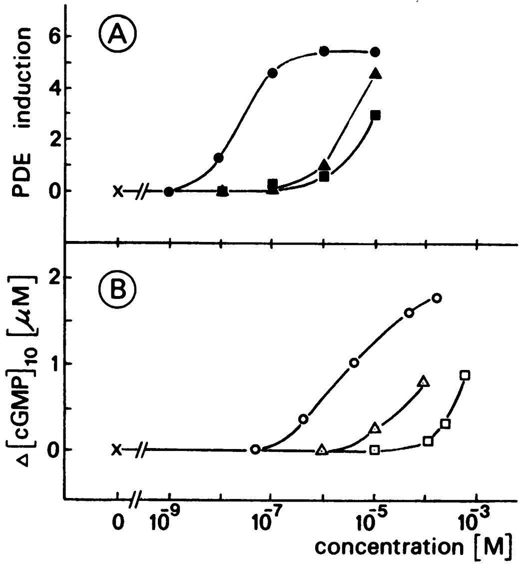

FIG. 4. Chemoattractant-mediated PDE induction and cGMP accumulation. (A) PDE induction. Postvegetative cells were stimulated with 12 pulses of folic acid (●), 7-CH-cAMP (▲), or 3′-NH-cAMP (■). PDE was assayed in a homogenate made from the cells 15 min after the last pulse was added. Data are taken from Fig. 2 for folic acid and reference 29 for 3′-NH-cAMP and 7-CH-cAMP. (B) cGMP accumulation. Postvegetative cells were stimulated with folic acid (○), 7-CH-cAMP (△), or 3′-NH-cAMP (□) and, after 10 s, lysed with perchloric acid. The lysate was neutralized with potassium bicarbonate, and cGMP was measured radioimmunologically in the supernatant. 3′-NH-cAMP and 7-CH-cAMP were not added at higher concentrations because they would compete with cGMP in binding to the antibody.

duction were detected by the same cAMP receptor (29). Recent results suggest that folic acid and pterin are detected by different receptors (30), which led to scheme I (Fig. 3).

The production of cGMP and the induction of PDE in postvegetative cells by 3′-deoxy-3′-amino cAMP (3′-NH-cAMP), 7-deazo-cAMP(7-CH-cAMP), and folic acid is shown in Fig. 4. The observation that these cAMP derivatives induced a strong cGMP accumulation in postvegetative cells whereas cAMP itself did not was predicted by the rate characteristics of the cAMP receptor (29). The half-maximal increase of cGMP levels occurred at 100-fold-higher concentrations than did the half-maximal increase of PDE induction, and this 100-fold difference was independent of the stimulus (folic acid, pterine, or cAMP derivatives) (Fig. 4 and Table 1). The similar effect with different stimuli indicates that the signals converge to one pathway (Fig. 3, scheme II).

Which mechanism can explain the fact that PDE induction already takes place at a 100-fold-lower stimulus concentration than does intracellular cGMP accumulation? The difference in sensitivity can be explained by two mechanisms. (i) The presence of spare receptors (1); not all cell surface receptors have to be occupied for maximal transduction of the signal. (ii) Occupation of the cell surface receptor leads to the production of a second messenger; only small amounts of this messenger are needed for complete transduction of the signal (27). In aggregative cells cAMP induced various responses. The demonstration that these various responses have different sensitivities to cAMP (Table 1 in Van Haastert et al., in press, and Fig. 5 in P. J. M. Van Haastert and T. M. Konijn, Mol. Cell. Endocrinol., in press) makes the hypothesis of spare receptors unlikely. Therefore, we searched for an intracellular messenger which functions at very low concentrations of the extracellular signals (cAMP, folic acid, or pterin). Recently we showed that an intracellular, cGMP-binding protein may have such properties (Van Haastert et al., in press).

The dose-response curves (Fig. 4B) can be described by the equation:

$$\Delta[\mathrm{cGMP}]_{10} = 1.8 \times 10^{-6}\,(X/X + Y) \qquad (2)$$

where $\Delta[\mathrm{cGMP}]_{10}$ is the increase in cGMP concentration 10 s after stimulation, X is the concentration of chemoattractant, and Y is the concentration of chemoattractant yielding half-maximal (0.9 μM) cGMP accumulation (folic

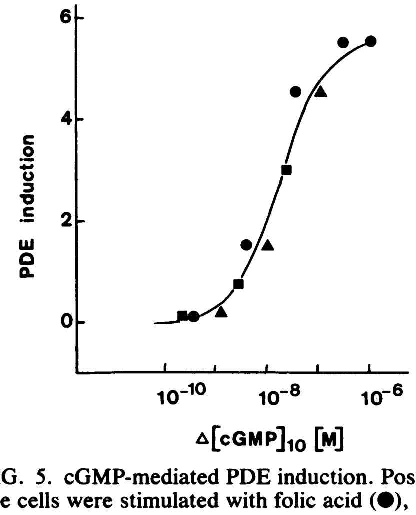

FIG. 5. cGMP-mediated PDE induction. Postvegetative cells were stimulated with folic acid (●), 7-CH-cAMP (▲), and 3′-NH-cAMP (■). cGMP accumulation and PDE induction were determined as described in the legend to Fig. 4. The cGMP accumulation evoked by a chemoattractant at a certain concentration is represented against the PDE induction evoked by the same chemoattractant at the same concentration (see text).

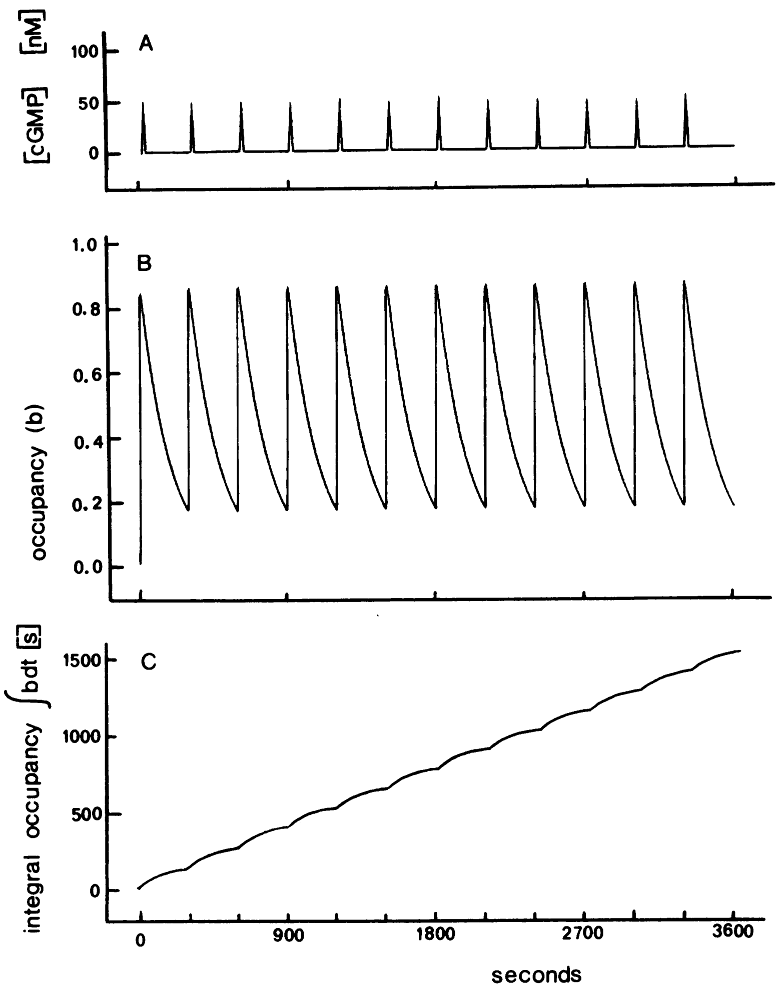

FIG. 6. Computer simulation of pulse experiments. Experiments were simulated by the generation of 12 cGMP peaks at 5-min intervals (A). The occupancy of a cGMP receptor (B) was computed by solving for b in equation 1 by a previously described method (Van Haastert et al., in press). The computation scheme was extended with the integration of b to record the integral of receptor occupancy (C). Integrations are in steps of 2 ms in the presence of cGMP and 10 ms in its absence. Data are plotted for each second. Parameters: $K_1 = 4 \times 10^6$ M^{-1} s^{-1}, $K_{-1} = 6 \times 10^{-3}$ s^{-1}, $R_0 = 10^{-8}$ M, $\Delta[\text{cGMP}]_{10} = 5 \times 10^{-8}$ M.

acid, $Y = 5 \times 10^{-6}$ M; 7-CH-cAMP, $Y = 1.6 \times 10^{-4}$ M; 3'-NH-cAMP, $Y = 8 \times 10^{-4}$ M; Fig. 4B).

The cGMP accumulation at low doses of these compounds is calculated by equation 2 and expressed versus the PDE induction produced by the same concentration of chemoattractant in Fig. 5. If cGMP functioned as a messenger, then the implication (Fig. 5 and the model of Strickland and Loeb [27]) is that a cGMP receptor should be present which has the necessary kinetics of association and dissociation to be able to mediate these low and short-lived cGMP accumulations. Recently we investigated the nonequilibrium kinetics of an intracellular cGMP-binding protein in vitro and in vivo (Van Haastert et al., in press), which revealed that the binding of cGMP to the protein in vivo follows the law of mass action (equation 1) and that the in vivo parameters are $K_1 = 4 \times 10^6$ M^{-1} s^{-1}; $K_{-1} = 6 \times 10^{-3}$ s^{-1}; $R_0 = 10^{-8}$ M.

Assuming that the PDE induction is proportional to the total amount of information which has entered the cell, the amount of PDE induced should be proportional to the mean of the binding protein concentration occupied with cGMP during the 12 pulses: mathematically, this is the integral of occupied receptors after 12 successive accumulations of cGMP levels (Fig. 6).

Twelve increases of cGMP levels at 5 min of the magnitude $\Delta[\text{cGMP}]_{10} = 20$ nM causes 50% of the maximal attainable integral of binding

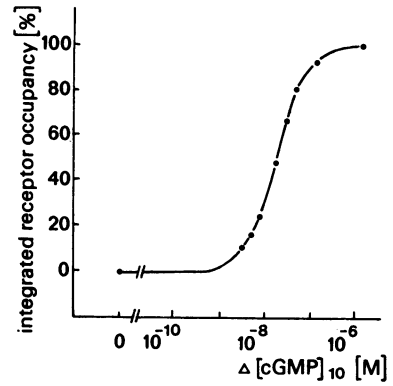

FIG. 7. Dose-response curve of the top of the cGMP peak versus the integral of receptor occupancy. The cGMP concentration at the top of the 12 identical cGMP peaks is presented on the abcissa, and the integral of receptor occupancy after 1 h is presented on the ordinate. The integral of receptor occupancy produced by 12 cyclic GMP peaks with an increase of $\Delta[\text{cGMP}]_{10} = 2 \times 10^{-6}$ M (10 pmol of cGMP per 10^7 cells) is set at 100%. Computation was made as described in the legend to Fig. 6 and in Van Haastert et al., in press. Parameter values $K_1 = 4 \times 10^6\ \text{M}^{-1}\ \text{s}^{-1}$, $K_{-1} = 6 \times 10^{-3}\ \text{s}^{-1}$, $R_0 = 10^{-8}$ M.

protein occupancy (Fig. 7). This relation between the cGMP accumulation and the occupancy of the cGMP-binding protein (Fig. 7) is very close to the correlation between cGMP accumulation and the magnitude of PDE induction (Fig. 5). Thus, cGMP in combination with the cGMP-binding protein has exactly the necessary sensitivity to transduce the chemotactic signals, which suggests the transduction pathway of scheme III (Fig. 3).

Although the correlations (Fig. 5 and 7) are based on in vivo experiments, they do not exclude the possibility of another unknown messenger being present to transduce the signal. This messenger, however, should have the same kinetic properties as the cGMP system. Scheme III is further supported by cyclic nucleotide localization studies done with immunofluorescent techniques (19, 22). Whereas cAMP stains homogeneously over the cell, cGMP stains predominantly in the nucleus, which suggests a function for cGMP in the nucleus.

In summary, several lines of evidence indicate that cGMP has a messenger function between activation of a cell surface receptor by a chemoattractant and the induction of PDE. (i) Folic acid and cAMP increase cGMP levels (18, 20, 32) and induce PDE (2, 10, 11, 31). (ii) cAMP induces PDE and causes cGMP accumulation, both depending on a rate receptor (29). (iii) A rough correlation exists between the effect of chemical modification of folic acid (Table 1) or cAMP (10, 29) on cGMP accumulation and PDE induction. (iv) A dose-response correlation exists between the increase in cGMP levels and the amount of PDE induced independent of the nature of the chemoattractant by which this cGMP accumulation is produced (Fig. 4 and 5). (v) An intracellular cGMP-binding protein is present which will be occupied for 50% of maximum at 12 cGMP accumulations which result in half-maximal PDE induction (Fig. 6 and 7).

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