1. Introduction

In a suitable environment spores of the Dictyostelia [1] germinate yielding small amoebae. Each amoebae feeds on bacteria and divides. Following consumption of the food supply amoebae aggregate forming a slug where cells differentiate into spores and stalk. The orientation of cells towards a food source or an aggregation center is guided by gradients of concentration of chemotactic molecules. In D. discoideum vegetative amoebae are attracted by folic acid [2] and aggregative amoebae by cAMP [3].

The sensory reception of a cAMP signal involves binding of cAMP to cell-surface bound receptors [4-7] and analysis of the signal in terms of changes of concentration over distance [8]. The sensory system transmits the signal to pseudopod formation into the direction of the attractant within 10 s [9].

Recently we have shown that, at physiological concentrations, cAMP induces an increase in the level of cGMP that precedes pseudopod formation [10]. cGMP peaks are high (up to 10-fold with 5 X 10^-7 M cAMP) and brief (pre-stimulation level is reached in about 25-30 s after addition of cAMP).

Several characteristics in common between attractant mediated chemotaxis and attractant mediated cGMP accumulation in D. discoideum and the cAMP insensitive species D. lacteum [11] indicate that cGMP may be involved in the transmission of the chemotactic signal to pseudopod formation during aggregation [10,12].

In the present study we examined the specificity of cAMP mediated cGMP accumulation in D. discoideum in comparison to the specificity of cAMP mediated chemotaxis by using various cAMP and AMP derivatives, the release of cGMP to the incubation medium following addition of cAMP to sensitive cells of D. discoideum, the existence of a cGMP phosphodiesterase in a homogenate of D. discoideum and the effect of folic acid addition to vegetative amoebae of D. discoideum on the content of cGMP. Finally the possible function of cGMP during the processing of a chemotactic signal is discussed.

2. Materials and methods

2.1. Organisms

D. discoideum, NC-4(H), was grown in association with Escherichia coli on a solid medium [13]. The cells were harvested, freed of bacteria by centrifugation [13] and suspended in 10 mM phosphate buffer, pH 6.0, at a density of 10^7 cells/ml. Starvation was induced by shaking [14].

2.2. Assay of cGMP

cAMP mediated cGMP accumulation was determined as described [10]. After 4 h shaken cells of
D. discoideum were centrifuged, washed three times in cold phosphate buffer and adjusted to 2 × 10⁸ cells/ml. This cell suspension had air bubbled through for 10 min at room temperature. After bubbling, 100 µl samples were pipetted into conic Eppendorf tubes before 20 µl cAMP (or a derivative), also dissolved in phosphate buffer, were added while shaking was continued. At the time indicated 200 µl ethanol–HCl (60 vol. ethanol – 1 vol. 11 N HCl) were pipetted into the tubes. Ethanol–HCl extracts, containing 1500 cpm [8-³H]cGMP (20 Ci/mmol), were centrifuged (8000 × g for 2 min) after standing at 0°C for 15 min. After centrifugation the pellet was washed once with 200 µl ice-cold ethanol–HCl and the pooled supernatants dried at 65°C. After drying, samples were dissolved in 500 µl water, extracted twice with water saturated ether and the aqueous phase dried overnight at 65°C and then dissolved in 300 µl 50 mM Tris–HCl buffer, pH 7.5, containing 4 mM EDTA. cGMP recovery was then calculated (from 70% to 60%) and the cGMP content measured by the radioimmunoassay of Steiner et al. [15] using antibody and [8-³H] cGMP supplied by Amersham. Standard and unknowns were assayed in duplicate. The high specificity of the antibody for cGMP allows cGMP measurement without further sample purification. Neither cAMP showed cross-reactivity with the antibody nor did the various cAMP and AMP derivatives. Authenticity of the determination of cGMP was confirmed by the destruction of the reactivity with the antibody by prior treatment with cyclic nucleotide phosphodiesterase (Boehringer).

2.3. Assay of cGMP in the medium
After 4 h shaking the cells were prepared as described above and triggered with cAMP (final concentration 5 × 10⁻⁸ M). At the time indicated, starting with 5 s, 100 µl of the cell suspension were pipetted into a plastic tube that contained 30 µl HCl 0.8 N and 3 drops of immersion oil (Merck) on top of the acid. Immediately after pipetting the cell suspension (the tube was standing in the centrifuge) the sample was centrifuged (8000 × g for 15 s in an Eppendorf microcentrifuge) and 75 µl of the supernatant pipetted into a tube containing 200 µl ethanol–HCl (to which 1500 cpm [8-³H] cGMP had been added). Under these conditions cells sediment immediately and the pH of the medium is lowered to 2–3 stopping phosphodiesterase activity. Ethanol–HCl samples were treated as described above and the content of cGMP assayed. The time at which centrifugation started was chosen for plotting extracellular cGMP content. The volume of the cells (about 10 µl taking 5 × 10⁻¹⁰ ml/cell) was taken into account to estimate the total amount of cGMP released.

2.4. Folic acid mediated cGMP accumulation
The cells had air bubbled through for 10 min without previous starvation by shaking. A final density of 10⁸ cells/ml was used instead of 2 × 10⁸ cells/ml due to the higher basal cGMP content in vegetative amoebae [10]. After bubbling, 100 µl samples were triggered with folic acid (Sigma) as described above and the cGMP content assayed.

2.5. Assay of phosphodiesterase activity
cGMP and cAMP phosphodiesterase activity was assayed according to the procedure of Thompson et al. [16]. Reaction mixtures contained 10 mM phosphate buffer, pH 7.0, 0.5 mM MgCl₂ and 1.0 × 10⁻⁷ M [8-³H]cGMP (50 000 cpm) or 0.6 × 10⁻⁷ M [8-³H]cAMP (40 000 cpm) in total vol. 400 µl. The cells of D. discoideum were starved by shaking during 5 h. After shaking the cells were homogenized in 10 mM phosphate buffer, pH 7.0, at a density of 10⁸ cells/ml by freezing at –20°C and thawing at 0°C twice under agitation. Reactions were started by addition of 100 µl homogenate or 48 000 X g supernatant (centrifuged at 4°C for 60 min in a SS-34 Sorvall rotor) containing 50–100 µg protein and terminated by heating in a boiling bath for 2 min followed by incubation in an ice-cold bath for 5 min. Following treatment with snake venom (Sigma, Ophiophagus hannah), unreacted substrate was removed by the addition of 1 ml AG-1-X2 (Bio-Rad) slurry (1 vol. resin + 2 vol. water) at pH 5.0 and centrifugation (8000 × g for 2 min). 0.5 ml of the supernatant was counted. cGMP blank values were about 1000 cpm and cAMP blank values about 500 cpm. Cyclic nucleotide hydrolysis was linear with respect to time for at least 20 min when incubated at 24°C.

Protein content was measured by the method of Lowry et al. [17]. Figures usually show values from a typical experiment repeated two to four times.
3. Results

3.1. Specificity of cAMP mediated cGMP accumulations

Figure 1 compares changes in the content of cGMP of *D. discoideum* cells in response to cAMP, AMPSMe and cUMP. cAMP, AMPSMe and cUMP increased the cGMP content within 4 s and gave a peak at 10 s after which cGMP levels declined very fast reaching the pre-stimulation level within 20–30 s. cAMP increased 10-fold the content of cGMP at $5 \times 10^{-7}$ M (final concentration). AMPSMe gave a similar increase at $5 \times 10^{-4}$ M and cUMP increased 6-fold the cGMP content at $5 \times 10^{-5}$ M. AMPSMe and cUMP were without effect on cGMP at $5 \times 10^{-7}$ M (fig. 1). All cAMP and AMP derivatives listed in table 1 increased, at the concentration mentioned in column 2, the content of cGMP. Control experiments using $5 \times 10^{-7}$ M cAMP were run in parallel with each derivative. The increase in the content of cGMP induced by each derivative was calculated taking the increase obtained with $5 \times 10^{-7}$ M cAMP as 100. The concentration of cAMP which gives, for each derivative, a similar increase in the content of cGMP is listed in column 3. A figure showing the concentration-response relationship of *D. discoideum* cGMP to cAMP has been previously published [10]. All the derivatives tested increased the cGMP content with similar kinetics to those shown in fig.1. At a concentration 100-fold lower than that mentioned in table 1 all the derivatives were without effect on the cGMP level.

Table 1

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concentration$^a$ (M)</th>
<th>cAMP$^b$ (M)</th>
<th>Nucleotide/cAMP ratio$^c$</th>
<th>Nucleotide/cAMP ratio$^d$</th>
</tr>
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<tbody>
<tr>
<td>5'-CH$_2$-cAMP</td>
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<td>1</td>
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<tr>
<td>5'-NH-cAMP</td>
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<td>$5 \times 10^{-8}$</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>cAMPSh</td>
<td>$5 \times 10^{-7}$</td>
<td>$5 \times 10^{-8}$</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>AMPMe</td>
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<td>$10^{-7}$</td>
<td>$10^2$</td>
<td>$10^3$</td>
</tr>
<tr>
<td>5'-NH$_2$-3'-AMP</td>
<td>$10^{-4}$</td>
<td>$5 \times 10^{-7}$</td>
<td>$2 \times 10^3$</td>
<td>$10^3$</td>
</tr>
<tr>
<td>AMPSMe</td>
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<td>$5 \times 10^{-7}$</td>
<td>$10^3$</td>
<td>$10^4$</td>
</tr>
<tr>
<td>cUMP</td>
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<tr>
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<td>$5 \times 10^{-8}$</td>
<td>$10^3$</td>
<td>$10^4$</td>
</tr>
</tbody>
</table>

$^a$ Concentration of the cAMP agonist at which cGMP accumulation was stimulated

$^b$ Concentration of cAMP giving a similar increase in the cGMP content

$^c$ Relative cGMP accumulation – see text for explanation

$^d$ Relative chemotaxis – see text for explanation
chemotaxis for the various derivatives. A regression analysis of the data from fig.2 gives a straight line with a correlation coefficient of 0.826 and \( p > 0.99 \% \). These results indicate that a linear relationship exists between the specificity of cAMP mediated chemotaxis and cAMP mediated cGMP accumulation.

3.2. Effect of cAMP stimulation on the cGMP content in the amoebal medium

Figure 3 compares the change in the content of cGMP of \( D. discoideum \) in response to cAMP (5 \( \times \) 10\(^{-8} \) M) in the whole homogenate and in the amoebal medium. As shown in fig.3 cAMP-induced cGMP release accounts for less than 5% of the total cGMP increase.

3.3. Effect of folic acid stimulation on the cGMP content in the amoeba of \( D. discoideum \)

Figure 4 shows the change in the content of cGMP of \( D. discoideum \) in response to folic acid (5 \( \times \) 10\(^{-6} \) M and 5 \( \times \) 10\(^{-5} \) M final concentration). As shown in fig.4 folic acid induced, within 2 s, an increase in the content of cGMP. cGMP concentration reached its peak at 8–10 s and recovered pre-stimulation level at 20–30 s. Similar results have been recently obtained by Wurster et al. [21]. Folic acid mediated cGMP accumulation is concentration dependent (fig.4) with a concentration for half-maximal stimulation (\( K_a \)) of about 5 \( \times \) 10\(^{-6} \) M (fig.5).

3.4. cAMP and cGMP phosphodiesterase activity in \( D. discoideum \)

In \( D. discoideum \) the 48,000 X g supernatant hydrolyzed cAMP slightly faster than cGMP at a
Fig. 5. Folic acid concentration dependence of folic acid mediated cGMP accumulation. Cells were stimulated with various folic acid concentrations as described in Materials and methods. Samples were taken at 0 s, 4 s, 6 s, 8 s, 10 s, 15 s, 20 s and 30 s after folic acid stimulation. cGMP peaks were observed between 8–10 s. The maximal cGMP increase with respect to basal concentration was then calculated and plotted taking the increase obtained at 1.6 × 10^{-4} M folic acid as 100. Abscissa: molar concentration of folic acid.

substrate concentration of, respectively, 0.6 × 10^{-7} M and 1.0 × 10^{-7} M (fig. 6a). In the presence of 0.5 mM dithiothreitol, a cAMP phosphodiesterase inhibitor in D. discoideum [22], cGMP was hydrolyzed about 3-times faster than cAMP, that is, while 0.5 mM dithiothreitol inhibited cAMP hydrolysis for about 80% cGMP hydrolysis was only inhibited for about 45% (fig. 6). cGMP hydrolysis was optimal between pH 7.0 and 8.0 either in the presence or absence of dithiothreitol (data not shown). The plot of phosphodiesterase activity versus cGMP concentration in the presence of 2 mM dithiothreitol gave a concentration for half-maximal activity of about 3 μM cGMP (fig. 6b). Hill plot of the enzyme activity gave a slope of 1.7 indicating positive cooperativity-like kinetics (fig. 6b). cGMP phosphodiesterase activity was not affected by the presence of either theophylline or caffeine at 2 mM concentration (data not shown).

4. Discussion

The data presented demonstrate a close correlation between the chemotactic activity of various cAMP agonists and their effect on cGMP accumulation. The same cAMP receptor appears to be involved in both processes. Folic acid attracts vegetative amoebae of D. discoideum [2] and also stimulates cGMP accumulation. The K_a for folic acid mediated cGMP accumulation is in the same range as its chemotactic activity. Previously we have shown that cAMP mediated cGMP accumulation also occurs at physiological concentrations for chemotaxis, that the K_a for this response is
in the same range as the dissociation constant of the cell-surface bound cAMP receptor and that cGMP elevation precedes pseudopod formation [10]. Contrary to cAMP [23,24], cGMP function and hydrolysis seems to be mainly intracellular. Both cAMP and cGMP phosphodiesterase are present intracellularly. Which form of phosphodiesterase plays the most important role in controlling the cGMP content in intact cells is not known. These results indicate a role of cGMP during chemotaxis in D. discoideum. cAMP and fructose acid not only induce chemotaxis [2,3] but also cell differentiation [25–27] and, in the case of cAMP, adenylate cylinder activation [28]. More information is required to know which of these biological effects are regulated by cGMP.

References