MULTIPLE DEGRADATION PATHWAYS OF CHEMOATTRACTANT MEDIATED CYCLIC GMP ACCUMULATION IN DICTYOSTELIUM

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Chemoattractants induce a transient accumulation of cGMP levels in Dictyostelium. Intracellular cGMP levels reach a peak at 10 s and prestimulated cGMP levels are recovered at about 30 s. Intracellular and extracellular cGMP levels were detected simultaneously after stimulation of D. lacteum cells with monapterin and of D. discoideum cells with cAMP. In both species about 20% of the intracellularly accumulated cGMP was secreted. All slime mold species investigated so far contain an intracellular phosphodiesterase specific for cGMP. A mutant of D. discoideum which does not contain this cGMP-specific enzyme shows a strongly retarded decline of intracellular cGMP levels. Secretion of cGMP is in this mutant not sufficient to explain the decline of cGMP levels which indicates the involvement of nonspecific phosphodiesterase in intracellular cGMP regulation. These results show multiple degradation pathways of intracellularly accumulated cGMP. In wild-type cells about 20% is secreted, 10–20% is hydrolyzed intracellularly by non-specific phosphodiesterase, while the majority (60–70%) is hydrolyzed intracellularly by a cGMP-specific phosphodiesterase. The relationships of intracellular regulation of cGMP and cAMP levels are discussed.

Introduction

Chemotaxis is essential during the whole life cycle of the cellular slime molds. In the vegetative stage, amoebae find their food source via a chemotactic response to folic acid and pterins [1,2] which are excreted by bacteria. When the food supply is exhausted, the amoebae aggregate to form a multicellular slug which differentiates into a fruiting body. Cell aggregation is mediated by chemotaxis to a compound, called acrasin, which is excreted by the aggregation center. The acrasin of the best studied slime mold species, Dictyostelium discoideum, has been identified as cAMP [3]. Recently, we identified the acrasin of D. lacteum as a pterin derivative; monapterin is a commercially available substitute of the acrasin with equipotent chemotactic activity [4].

Since 1977, evidence has been accumulating for the involvement of cGMP in the transduction of chemotactic signals [5–11]. All chemoattractants induce a similar transient increase in cGMP levels which reaches a peak at 10 s after stimulation with a recovery of basal levels at about 30 s after stimulation.

Recently, a group of mutants of D. discoideum (stim F ‘streamer’ mutants) have been isolated which show both an altered chemotactic behavior and an altered cGMP response [11]. Maximal cGMP levels are reached at about 20 s, and pretimated levels are recovered 2–3 min after chemotactic stimulation. These observations suggest that removal of intracellular cGMP is important for normal chemotactic behavior.

Dictyostelium cells contain at least two classes
of cyclic nucleotide phosphodiesterase activity. One class specifically hydrolyzes cGMP and is characterized by cGMP stimulation and intracellular localization [7,12–14]. Almost all slime mold species contain similar levels of this cGMP-specific phosphodiesterase activity. A second class of enzymes hydrolyzes cAMP about 3-times faster than cGMP, both measured at subsaturating concentrations [15,16]. This enzyme is referred to as nonspecific phosphodiesterase. In species where cAMP acts as chemoattractant (e.g., D. discoideum) this enzyme is present in excess with about 100-fold higher activity than the cGMP-specific phosphodiesterase [14], and is localized extracellularly, on the cell surface and intracellularly. Some species which do not use cAMP as chemoattractant (e.g., D. lacteum) have very low levels of the nonspecific phosphodiesterase (about 10-fold less than the cGMP-phosphodiesterase), and the enzyme is localized only intracellularly (unpublished observations).

In this study we investigated the removal of chemoattractant-mediated intracellular cGMP accumulation which appears to be a complex of secretion, intracellular hydrolysis by nonspecific phosphodiesterase and intracellular hydrolysis by cGMP-specific phosphodiesterase.

Materials and Methods

Culture conditions. D. discoideum NC-4H was grown in association with Escherichia coli B/r on a 0.33% glucose/peptone medium; D. lacteum and D. discoideum NP 368 were grown on a 0.1% lactose/peptone medium. Cells were harvested in the late-log phase in 10 mM sodium/potassium phosphate buffer, pH 6.5, and bacteria were removed by repeated centrifugations at 100 × g for 4 min. Cells were starved on buffered, nonnutrient agar plates at a density of 1.5-10⁶ cells/cm². Cells were collected at the aggregation competent stage, washed twice and suspended in phosphate buffer at a density of 10⁸ cells/ml. Air was bubbled through the suspensions for at least 10 min.

cGMP-stimulation. Cell suspensions were divided into 100-μl samples. D. discoideum strains were stimulated at 0 s with 20 μl of 10⁻⁷ M cAMP, 5 mM dithiothreitol (final concentrations) and D. lacteum cells with 20 μl of 10⁻⁵ M monapterin (final concentration). At the times indicated in the figures, samples were centrifuged for 3 s in an Eppendorf microcentrifuge. The centrifuge was slowed down by hand. The supernatant was added to 100 μl perchloric acid (3.5%, v/v) and to the pellet of both species were added 120 μl phosphate buffer, 10⁻⁷ M cAMP, 5 mM dithiothreitol (for D. discoideum) or 120 μl phosphate buffer, 10⁻⁵ M monapterin (for D. lacteum). The time period between the onset of centrifugation and the addition of perchloric acid to the pellet lasts approx. 20 s. Lysates were neutralized by the addition of 50 μl KHCO₃ (50% saturated solution at 20°C), shaken to allow CO₂ to escape, and centrifuged for 2 min at 8000 × g. cGMP levels in the supernatant were determined radioimmunologically. Results shown are the means of three experiments. The standard deviation is about 10% at a cGMP concentration of 1 pmol/10⁷ cells.

Extracellular cGMP hydrolysis. 20 μl of 5·10⁻⁸ M [³H]cGMP (final concentration) were added to 100-μl cell suspensions. At the times indicated in the figures, 100 μl perchloric acid were added and lysates were neutralized as described above. 100 μl of the supernatant were incubated with 50 μg snake venom for 30 min at 30°C. Then 1 ml ion exchanger (1 part Dowex AG1-X2 and 2 parts H₂O, pH 5.0) was added. Samples were shaken during 2 min, centrifuged and the radioactivity in 500 μl of the supernatant was determined.

Materials. cAMP was obtained from Boehringer; dithiothreitol and snake venom (Ophiophagus hannah) were obtained from Sigma; monapterin (6-(1-threo-1,2,3-trihydroxypropyl)pterin) was from Fluka AG, Buchs, Switzerland; [⁸⁻¹H]cGMP (0.55 TBq/mmol) and the cGMP radioimmunoassay kit were from the Radiochemical Centre (Amersham).

Results

D. lacteum cells do not have extracellular cGMP-hydrolyzing activity, do not react chemotactically to cAMP, but are chemotactically sensitive to pterins. Therefore, D. lacteum could be a convenient species to investigate secretion of cGMP. Monapterin induces an about 3-fold increase in intracellular cGMP levels (Fig. 1). Secre-
Monapterin-mediated cGMP response in aggregative *D. lacteum* cells. (A) Hydrolysis of extracellular $[^3]H$cGMP. (B) *D. lacteum* cells were stimulated with $10^{-5}$ M monapterin and intracellular (●) and extracellular (○) cGMP levels were measured.

Dithiothreitol is an inhibitor of extracellular phosphodiesterase activity of *D. discoideum* cells [17]. It is not known whether dithiothreitol influences signal transduction other than by inhibition of phosphodiesterase activity. Stimulation of *D. discoideum* wild-type cells (NC-4H) with cAMP in the presence of dithiothreitol results in the same transient cGMP accumulation as without dithiothreitol, except that basal levels are not completely recovered (Fig. 2). Separation between intracellular and extracellular cGMP levels reveals that this is due to secretion of cGMP. As in *D. lacteum*, secretion of cGMP is small (20%) if compared to intracellular degradation.

To discriminate between intracellular degradation by nonspecific phosphodiesterase and cGMP-specific phosphodiesterase, we have used a mutant of *D. discoideum* which lacks the cGMP-specific phosphodiesterase (mutant NP 368) [18]. Intra- and extracellular cGMP levels after stimulation of this mutant with cAMP in the presence of dithiothreitol are shown in Fig. 3. Also, in this mutant about 20% of the intracellularly accumulated cGMP is secreted. Secretion alone is not sufficient to explain the decrease in intracellular cGMP levels as is shown by the decrease in the sum of intra- and extracellular cGMP levels. This indicates intracellular hydrolysis of cGMP by nonspecific phosphodiesterase with 50% hydrolysis.

Secretion of cGMP is small but significant. The amount secreted (0.1 pmol/10^7 cells) is approx. 20% of the intracellularly accumulated cGMP levels (0.5 pmol/10^7 cells). *D. lacteum* is a delicate slime mold species and cells are easily damaged in suspensions. The small extracellular cGMP-hydrolyzing activity found in Fig. 1 is probably due to the release of intracellular cGMP phosphodiesterase, since about 10% of the cells were broken after the 10 min aeration period before stimulation. The increase in extracellular cGMP occurs within 1 min, and is more than 1% of the intracellularly accumulated cGMP. Therefore, the increase in extracellular cGMP appears to be due to secretion.

Dithiothreitol is an inhibitor of extracellular phosphodiesterase activity of *D. discoideum* cells.
after 70–90 s. In wild-type cells 50% of the intracellularly accumulated cGMP is hydrolyzed after about 15 s (Fig. 2) by cGMP-specific phosphodiesterase and nonspecific phosphodiesterase, indicating that the cGMP-specific phosphodiesterase contributes mainly to intracellular hydrolysis of cGMP.

**Discussion**

Chemotactic stimulation of *Dictyostelium* cells induces a rapid increase in intracellular cGMP levels which reach a peak after about 10 s. The accumulated cGMP rapidly declines to prestimulated levels within 30 s. In the present study we have shown that intracellular cGMP is removed by a complex of three mechanisms: in wild-type cells about 20% is secreted, 10–20% is hydrolyzed intracellularly by nonspecific phosphodiesterase, while the majority (60–70%) is hydrolyzed intracellularly by a cGMP-specific phosphodiesterase.

Recent results [19–21] suggest that intracellular cGMP is not limited to one compartment. This allows for a fourth regulation mechanism of cGMP levels: intracellular translocation between compartments. This might be especially important if the targets of cGMP, such as cGMP receptors [21], are also compartmentalized. Furthermore, are the three degradation pathways described above localized in one compartment, or, e.g., is intracellular cGMP isolated in a vesicle before secretion? Unfortunately, translocation fluxes of intracellular cGMP on a seconds time scale are difficult to investigate.

In *D. discoideum* cAMP induces not only chemotaxis and cGMP accumulation, but also the synthesis and secretion of cAMP. The possible relationship between cAMP and cGMP regulation deserves some comment. Dinauer et al. [22] have investigated the relationship between cAMP secretion and synthesis during cAMP stimulation. They showed that a great part of the newly synthesized cAMP was degraded intracellularly with a time constant of 1.73 min⁻¹, which implies 50% degradation after 25 s. Taking into account that at low substrate concentrations nonspecific phosphodiesterase hydrolyzes cAMP about 3-times faster than cGMP [16], this value closely correlates with 70–90 s for 50% degradation of cGMP by nonspecific phosphodiesterase found in strain NP 368. Dinauer et al. [22] have also shown that the rate of cAMP secretion is directly proportional to the intracellular cAMP concentration. Fig. 3 shows that the rate of cGMP secretion is not proportional to the intracellular cGMP concentration: the secretion rate is maximal between 30 and 75 s, while the cGMP concentration is maximal between 10 and 15 s. The rate of cGMP secretion more closely approaches the time curve of cAMP secretion which reaches a maximum at about 2 min after the onset of cAMP stimulation [22]. These considerations raise the question as to whether cGMP secretion and hydrolysis by nonspecific phosphodiesterase occur via components that are involved in the cAMP relay mechanism. This might be evolutionarily relevant, since the
more primitive species *D. lacteum* does show chemoattractant-mediated cGMP secretion (Fig. 1) while cAMP levels are not altered [8].

The present data showing multiple degradation pathways of intracellular cGMP may stress the importance of a refined regulation of intracellular cGMP levels during chemosensory transduction in *Dictyostelium*.

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