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Chemoattractant-stimulated calcium influx in *Dictyostelium discoideum* does not depend on cGMP

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Abstract

Chemoattractant stimulation of *Dictyostelium* cells leads to the opening of calcium channels in the plasma membrane, causing extracellular calcium to flux into the cell. The genetically uncharacterised mutants *stmF* and *KI8* show strongly altered chemoattractant-stimulated cGMP responses. The aberrant calcium influx in these strains has provided evidence that the chemoattractant-stimulated calcium influx is potentiated by cGMP. We have tested this hypothesis in genetically defined mutants by measuring the calcium influx in a strain that lacks intracellular cGMP due to the disruption of two guanylyl cyclases, and in a strain with increased cGMP levels caused by the disruption of two cGMP-degrading phosphodiesterases. The results reveal that the calcium influx stimulated by cAMP or folic acid is essentially identical in these strains. We conclude that cGMP is not involved in chemoattractant-stimulated calcium influx.

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Keywords: Calcium influx; Cyclic GMP; Streamer F; *Dictyostelium*

1. Introduction

The eukaryotic cellular slime mold *Dictyostelium discoideum* is a widely used organism to study complicated processes like chemotaxis and cell signalling. Vegetative *Dictyostelium* cells are chemotactically sensitive to folic acid, which is secreted by their natural food source, bacteria. Upon starvation, a highly reproducible developmental program is initiated. Chemoattractant sensitivity is shifted from folic acid to cAMP, which is periodically secreted by starving cells. Cells moving towards the cAMP source develop into a multicellular slug that can culminate into a fruiting body, consisting of a stalk with a small spore head on top.

The *Dictyostelium* genome encodes four cAMP receptors [1–3] and at least one unidentified folic acid receptor. Most responses mediated by the chemoattractant receptors are dependent on the dissociation of receptor-coupled G-proteins, which consist of a $G\alpha$ and a $G\beta\gamma$ subunit. *Dictyostelium* cells express a single $G\beta$ and $G\gamma$ subunit and at least eleven distinct $G\alpha$ subunits [4–6]. Disruption of $G\beta$ or $G\alpha_2$ abolishes all G-protein dependent responses of the cAMP

receptors, while disruption of $G\beta$ or $G\alpha_4$ destroys all G-protein dependent responses of the folic acid receptors [7–9].

Dissociation of receptor-coupled G-proteins leads to the induction of several responses, including the activation of two guanylyl cyclases, GCA and sGC, resulting in a transient increase of cGMP levels [10,11]. Two targets of cGMP have been identified, GbpC and GbpD [12,13]. These enzymes regulate the phosphorylation of myosin and attachment of myosin to the cytoskeleton. Hydrolysis of cGMP to GMP by phosphodiesterases terminates the cGMP response. *Dictyostelium* expresses three cGMP-degrading phosphodiesterases, GbpA, GbpB and PDE3 [14,15]. Of these three enzymes, GbpA contributes the major fraction to the total phosphodiesterase activity.

Chemoattractant stimulation of *Dictyostelium* cells also leads to a calcium response [16,17]. About 6 s after addition of cAMP or folic acid, the calcium influx from the extracellular medium transiently rises and declines to the basal influx rate around 30 s after stimulation. The observation that starved $g\beta^-$ cells still show a significant cAMP-stimulated calcium influx suggests that the calcium influx response is largely G-protein independent [18]. The response of $g\beta^-$ cells is somewhat smaller than the response of wild-type cells, which was attributed to the reduced expression of cAMP receptors. Enhancing the number of receptors by expression of either cAR1, cAR2 or cAR3 proportionally increased the

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stimulated calcium influx. Recently, evidence has been presented that there is not one, but two pathways leading to the activation of a shared calcium influx mechanism [19]. During the vegetative stage and early development, the chemoattractant-induced calcium influx is found to be completely G-protein dependent, since disruption of the G β subunit abolishes all stimulated calcium influx during this phase. Unlike vegetative cells, 8 h starved cells lacking G β still show a significant cAMP-stimulated calcium influx, suggesting that upon development, a largely G-protein-independent pathway has replaced the G-protein-dependent pathway of vegetative cells.

Mutant analysis has provided strong evidence for a potentiation of the chemoattractant-stimulated calcium influx by the stimulated cGMP response. Mutant streamer F (*stmF*) displays an enhanced and prolonged cGMP response due to a strongly reduced activity of the phosphodiesterase GbpA [15,20]. Stimulated calcium entry in *stmF* is also enhanced in both magnitude and duration [21]. Further evidence for a possible relation between these two responses was obtained using mutant KI8 that lacks a cAMP-stimulated cGMP response [22,23]. The cAMP-stimulated calcium influx of KI8 cells is not absent, but significantly reduced. On the basis of these observations, it has been proposed that cGMP potentiates the chemoattractant-stimulated influx of calcium ions [23].

The mutation responsible for the specific phenotype in the chemically obtained mutants *stmF* and KI8 has not yet been identified. Therefore, it cannot be excluded that the altered phenotypic characteristics of these mutants for cGMP and calcium influx are not related to each other, but result from multiple, independent mutations. Recently, most proteins that are directly involved in the cGMP transduction pathway have been identified, which has yielded several new knockout cell strains [10–13]. A *gca*[−]/*sgc*[−] mutant has been constructed in which both guanylyl cyclase genes have been disrupted [24]. In a similar fashion, a *gbpA*[−]*B*[−] mutant has been constructed with a disruption of two cGMP-degrading phosphodiesterase genes [13]. Mutant *gca*[−]/*sgc*[−] is unable to synthesise any amount of cGMP, while mutant *gbpA*[−]*B*[−] shows elevated basal cGMP levels and an enhanced cGMP response. Using these cell strains, we have tested the hypothesis that cGMP enhances the chemoattractant-stimulated calcium influx. Our data reveal that the folic acid and cAMP-stimulated calcium influx in the mutant strains is essentially identical to the influx response of wild-type cells. We conclude that the chemoattractant-stimulated calcium entry is not affected by the cytosolic cGMP concentration.

2. Materials and methods

2.1. Materials

⁴⁵CaCl₂ (23.2 mCi/mg Ca²⁺; 1 Ci=37 GBq) and [³H] cAMP (50 Ci/mmol) were obtained from Amersham Phar-

macia Biotech. cAMP was from Boehringer Mannheim. For the cGMP concentration determination, we used the cGMP [³H] Biotrak assay kit from Amersham.

2.2. Strains and culture conditions

Used cell strains AX3, *gca*[−]/*sgc*[−], *sGC*^{oe}, *gbpA*[−]*B*[−] were grown axenically in shaken culture on HG5 medium (contains per litre: 14.3 g oxoid pepton, 7.15 g bacto yeast extract, 1.36 g Na₂HPO₄·12H₂O, 0.49 g KH₂PO₄, 10.0 g glucose) to a density of 2–5 × 10⁶ cells/ml. For starvation, cells were harvested by centrifuging 3 min at 300 × g, washed once with 10 mM KH₂PO₄/Na₂HPO₄, pH 6.5 (PB), resuspended to a density of 10⁷ cells/ml in PB and incubated 5 h on a rotary shaker at 160 rpm and 21 °C.

2.3. Calcium influx assay

The calcium influx was determined as described previously [23]. In brief, 100 µl cell suspension (10⁸ cells/ml) was added to an equal volume of an uptake mixture containing 10 µM CaCl₂ and 25 pCi ⁴⁵Ca. For stimulated samples, the uptake mixture was supplemented with either 10 µM cAMP or 10 µM folic acid. The uptake of ⁴⁵Ca was terminated by adding 800 µl quenching solution containing 12.5 mM CaCl₂. Cells were immediately spun down for 4 s at 14000 × g, and the supernatant was aspirated. After resuspension of the cells in 100 µl H₂O and addition of scintillation liquid, the radioactivity was determined using a liquid scintillation counter.

2.4. cAMP binding assay and cGMP response

cAMP binding to cell surface receptors was determined by adding 10 µl of 100 mM DTT, 40 pM [³H] cAMP and 900 µl 90% saturated ammoniumsulfate to an 80-µl suspension of starved cells (10⁸ cells/ml). Cells were incubated 5 min on ice, supplemented with 10 µl 10 mg/ml BSA and centrifuged for 2 min at 14000 × g. Supernatant was discarded and the pellet was resuspended in 100 µl 100 mM acetic acid. Radioactivity was determined after addition of scintillation liquid.

The cGMP response assay was performed as previously described [25], and cGMP concentrations were determined using the Biotrak [³H] cGMP assay system.

3. Results

3.1. cGMP response

To investigate the suggested relation between the cAMP-induced cGMP accumulation and calcium influx, we have performed calcium influx assays on several mutants displaying either an enhanced or a reduced cGMP response (Fig. 1). Both guanylyl cyclase genes of the *gca*[−]/*sgc*[−] cell

Fig. 1. Time course of the cAMP-stimulated cGMP response of 5 h starved wild-type AX3 cells and four mutants. The two guanylyl cyclases were disrupted in strain *gca⁻/sgc⁻*, and two cGMP-degrading phosphodiesterase genes were disrupted in mutant *gbpA⁻B⁻*. Mutant *stmF* lacks activity of the phosphodiesterase GbpA, but is most likely not mutated in the *gbpA* gene.

strain were disrupted by homologous recombination. This mutant is unable to synthesise any amount of cGMP [24]. We also created a rescue of this mutant by transfecting the *gca⁻/sgc⁻* mutant with a vector containing the open reading frame of *sgc* under the control of an A15 promoter and A8 terminator (manuscript in preparation). The resulting strain is called sGC^{OE} and shows a cGMP response similar in kinetics as the response of wild-type cells, but with a higher maximum (Fig. 1). Finally, we have used the *gbpA⁻B⁻* strain, which has disruptions in two phosphodiesterase encoding genes [13]. This mutant shows both elevated basal

and stimulated cGMP concentrations that surpasses the cGMP response of *stmF*, which appears to lack GbpA phosphodiesterase activity [20]. Southern blotting has confirmed that only the targeted genes *gca*, *sgc*, *gbpA* and *gbpB* are disrupted and that the selection marker did not integrate at other sites into the genome [15,24].

3.2. cAMP-stimulated calcium uptake

We have determined the amount of ⁴⁵Ca uptake in the presence and absence of 10 μ M cAMP of 5 h starved *Dictyostelium* cells displaying either an enhanced or a reduced cGMP response. The results show that unstimulated AX3, *gca⁻/sgc⁻* and *gbpA⁻B⁻* cells accumulate ⁴⁵Ca linearly over time, while the calcium influx of cAMP-stimulated cells rapidly rises between 15 and 30 s after cAMP addition (Fig. 2). After 30 s, the stimulated influx rate decreases to the basal influx rate. This experiment yields no evidence for a relation between the intracellular concentrations of cGMP and the stimulated calcium influx. Both the kinetics and the magnitude of the stimulated calcium influx are very similar in the three cell strains.

To accurately quantify the cAMP-stimulated calcium uptake, we have performed multiple measurements of the amount of calcium taken up at 30 s after stimulation. At this time point, the ratio between stimulated and basal uptake of ⁴⁵Ca is maximal, allowing the most accurate comparison of the responses in the different strains. The basal calcium uptake during 30 s is about 30 pmol per 10⁷ cells for each cell strain (Fig. 3). Stimulation with cAMP results in an approximate 3-fold increase of calcium uptake in wild-type cells, which is in good accordance with earlier reported

Fig. 2. Time course of a typical calcium influx experiment of 5 h starved cells. Graphs with solid symbols represent the calcium influx of cells stimulated with 10 μ M cAMP. Open symbols represent unstimulated cells. The cAMP-stimulated calcium influx (panel D) is obtained by subtracting the unstimulated influx from the stimulated influx (AX3, diamonds; *gbpA⁻B⁻*, squares; *gca⁻/sgc⁻*, triangles).

Fig. 3. Calcium influx of cAMP-stimulated and unstimulated cells. Influx of calcium was determined at 30 s after adding cells to a mixture containing 10 μ M 45 CaCl₂ in the presence (solid bars) or absence (open bars) of 10 μ M cAMP. Error bars represent the standard deviation of at least two independent experiments with sextuplicate determinations. For comparison, the calcium influx data of *stmF* and XP55 have been included [23].

results [17,23,26]. On the basis of earlier reported data of KI8, we would expect an approximate 40% decrease of stimulated calcium influx of the *gca*⁻/*sgc*⁻ cells compared to wild-type cells [23]. However, statistical analysis of our data reveals that the response of *gca*⁻/*sgc*⁻ does not deviate significantly from the response of wild-type cells. The stimulated calcium influx of *stmF* cells has been found to be over 2-fold larger than the stimulated uptake of wild-type cells [23], which we would also expect to observe in *gbpA*^{-B-} cells. However, our results show the calcium responses of *gbpA*^{-B-} and *sGC*^{OE} not to be larger, but in fact slightly but significantly smaller than the response of wild-type cells.

The cAMP-stimulated calcium uptake of starved *Dictyostelium* cells has been found to be dependent on the amount of expressed cell surface receptors [18]. We have therefore performed cAMP-binding assays on each mutant (data not shown). Correction of the calcium influx for the amount of cAMP binding sites does not significantly alter

the graph as shown in Fig. 3. On the basis of our data, we cannot support the hypothesis that cGMP enhances the stimulated calcium influx.

3.3. Folic acid-dependent calcium uptake

Very recently, it has been proposed that there are two pathways leading to the activation of calcium influx [19]. The folic acid-stimulated calcium influx of vegetative cells completely depends on the presence of functional G-proteins, while the cAMP-stimulated calcium influx of 8 h starved cells is largely independent of G-proteins. To determine whether the G-protein-dependent pathway of vegetative cells is dependent on cGMP, we have measured the folic acid stimulated calcium uptake of vegetative cells (Fig. 4). Folic acid induces a small but significant ($P < 0.05$) calcium influx response. The *gca*⁻/*sgc*⁻ cell strain lacking any cGMP is still able to exhibit enhanced calcium influx after folic acid stimulation. Moreover, the *gbpA*^{-B-} mutant with elevated cGMP levels does not show a calcium influx larger than observed in *gca*⁻/*sgc*⁻ cells, implicating that cGMP also does not enhance the folic acid-stimulated calcium influx response that is activated by a G-protein-dependent pathway.

4. Discussion

Chemoattractant-stimulated *Dictyostelium* cells show a transient accumulation of cytosolic calcium due to an increased calcium influx from the medium [16,23]. Based on detailed data obtained from the random mutants *stmF* and KI8, it has been proposed that cGMP potentiates the chemoattractant-stimulated calcium influx [21,27]. In our experiments, we have tested this hypothesis by using genetically defined cell strains with either enhanced or reduced cGMP levels, caused by mutations that were introduced by homologous recombination.

Fig. 4. Calcium uptake of AX3, *gbpA*^{-B-} and *gca*⁻/*sgc*⁻ cells. Influx of calcium was determined at 45 s after adding cells to a mixture containing 10 μ M 45 CaCl₂ in the presence (solid bars) or absence (open bars) of 10 μ M folic acid. Error bars represent the standard deviation of triplicate determinations.

We observed that the calcium uptake response of the *gca⁻/sgc⁻* strain devoid of any cGMP is not smaller than the response of wild-type cells. In addition, the calcium response in strains with increased cGMP levels due to overexpression of sGC or inactivation of phosphodiesterase genes is not larger than the response of wild-type cells. These similar responses were observed both in folic acid-stimulated vegetative cells and in cAMP-stimulated 5 h starved cells. We conclude that cGMP does not enhance the chemoattractant-stimulated calcium influx.

In agreement with this, genome analysis has not been able to provide evidence for a potential role for cGMP in calcium influx. The nearly completed genome of *D. discoideum* was screened extensively for cGMP binding motifs [12]. This led to the identification of four cGMP binding proteins, GbpA–D, but no evidence has yet been found that these proteins are involved in the regulation of calcium channels. Recent inspection of the genome revealed no additional cGMP binding motifs or calcium channels with cyclic nucleotide binding domains.

The reasons why mutants KI8 and especially *stmF* exhibit significantly altered calcium influx relative to its wild-type XP55 remain elusive. One explanation is that cGMP can only potentiate calcium influx in an XP55 background and not in AX3, possibly due to a mutation in a cGMP signalling pathway in AX3. However, we have no evidence for a difference in cGMP signalling between AX3 and XP55. Alternatively, the potentiation of calcium influx in *stmF* could not be due to cGMP but to the unidentified mutated gene in *stmF*. The enhanced cGMP response in *stmF* is caused by a reduced activity of GbpA phosphodiesterase, but the promoter region and open reading frame of the *gbpA* gene in *stmF* strain do not contain significant mutations [15,28]. It is therefore likely that a regulatory protein is defective in *stmF*, potentially not only affecting GbpA phosphodiesterase activity, but also other responses such as the calcium influx.

Taken together, the present results with defined cGMP mutants demonstrate that cGMP does not potentiate the calcium influx. Thus, guanylyl cyclases, strongly inhibited by calcium ions, do not initiate a complex negative feedback loop, in which cGMP inhibits its own formation by enhancing the cytosolic calcium concentrations.

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