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INHIBITION OF RECEPTOR-STIMULATED GUANYLYL CYCLASE BY INTRACELLULAR CALCIUM IONS IN *DICTYOSTELIUM* CELLS

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In *Dictyostelium discoideum* extracellular cAMP stimulates guanylyl cyclase and phospholipase C; the latter enzyme produces Ins(1,4,5)P_3 which releases Ca^{2+} from internal stores. The following data indicate that intracellular Ca^{2+} ions inhibit guanylyl cyclase activity. 1) *In vitro*, Ca^{2+} inhibits guanylyl cyclase with IC_{50}=41 nM Ca^{2+} and Hill-coefficient of 2.1. 2) Extracellular Ca^{2+} does not affect basal cGMP levels of intact cells. In electro-permeabilized cells, however, cGMP levels are reduced by 85% within 45 s after addition of 10^{-6} M Ca^{2+} to the medium; halfmaximal reduction occurs at 200 nM extracellular Ca^{2+}. 3) Receptor-stimulated activation of guanylyl cyclase in electro-permeabilized cells is also inhibited by extracellular Ca^{2+} with half-maximal effect at 200 nM Ca^{2+}. 4) In several mutants an inverse correlation exists between receptor-stimulated Ins(1,4,5)P_3 production and cGMP formation. We conclude that receptor-stimulated cytosolic Ca^{2+} elevation is a negative regulator of receptor-stimulated guanylyl cyclase.

The cellular slime mold *D.discoideum* uses extracellular cAMP for cell-cell communication during chemotaxis and differentiation (1-3). cAMP binds to surface receptors, activates G-proteins and stimulates several second messenger systems, including adenylyl cyclase, guanylyl cyclase and phospholipase C. The produced cAMP is secreted in the medium where it can diffuse and activate neighboring cells. The produced cGMP remains largely intracellular where it activates cGMP receptors or is degraded by a cGMP-stimulated cGMP-phosphodiesterase (4). The produced Ins(1,4,5)P_3 (5,6) liberates Ca^{2+} ions from non-mitochondrial stores (7).

The activation of adenylyl cyclase and phospholipase C are most likely mediated by GTP-binding regulatory proteins (8); the mechanism by which guanylyl cyclase is activated is less well understood. Earlier experiments with saponin treated cells revealed stimulation of guanylyl cyclase activity by Ca^{2+} ions (9-11). Recently, a Mg^{2+}-dependent guanylyl cyclase

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activity was identified in *D.discoideum* membranes that is strongly inhibited by Ca^{2+} (12,13), suggesting that *in vivo* guanylyl cyclase activity may be inhibited by Ca^{2+} ions rather than stimulated. We have analyzed the regulation of guanylyl cyclase by surface receptors and intracellular Ca^{2+} in electro-permeabelized cells and conclude that *in vivo* intracellular Ca^{2+} inhibits guanylyl cyclase in *D.discoideum*.

MATERIALS AND METHODS

Materials [^{3}H]cGMP and cGMP antiserum were obtained from Amersham.
Cells and culture conditions *D.discoideum* cells (strain NC4) were grown on plates as described (5). Cells were harvested at the log-phase, washed three times with 10 mM KH_2PO_4/Na_2HPO_4, pH 6.5 (phosphate buffer), resuspended in this buffer to a density of 10^7 cells/ml, and starved for 4 hours.
Electro-permeabilization Cells were washed three times in buffer A (20 mM HEPES, 1.5 mM $MgCl_2$, pH 7.0) resuspended in this buffer to a density of 10^8 cells/ml and electroporated by two 7 kV pulses discharged as described (5). Cells were immediately incubated in Ca^{2+}/EGTA buffers with 5.9 mM EGTA and different concentrations of $CaCl_2$, which were calculated using a $K_D=1.85x10^8$ for the Ca^{2+}/EGTA equilibrium constant at pH 7.0 (15).
cGMP response Cells were stimulated with 0.1 μM cAMP and lysed at times indicated in the figure by the addition of 3.5% (vol/vol) perchloric acid. The cGMP content was measured in the neutralized extract by radioimmunoassay as described (14).
Guanylyl cyclase assay (13) Cells were washed three times in 40 mM HEPES, pH 7.0, resuspended to 10^8 cells/ml in 40 mM HEPES, 3 mM $MgCl_2$, 50μM GTPγS, 11.8 mM EGTA and different concentrations of $CaCl_2$, and lysed by rapid filtration through a 5μm Nuclepore filter. At 30 s after lysis, the guanylyl cyclase reaction was started by mixing equal volumes of lysate and a mixture of 10 mM dithiothreitol and 0.6 mM GTP. The reaction was terminated with perchloric acid at 0, 30 and 60 s, and cGMP was measured in the neutralized extracts by radioimmunoassay (13).

RESULTS AND DISCUSSION

The activity of Mg^{2+}-dependent guanylyl cyclase in the presence of different Ca^{2+} concentrations is shown in figure 1. Enzyme activity was inhibited completely by micromolar Ca^{2+} concentrations; half-maximal inhibition was observed at about 41 nM. A Hill plot of these data yields a Hill coefficient of 2.1, indicating that inhibition of guanylyl cyclase by Ca^{2+} is positive cooperative. Guanylyl cyclase of rod outer segments is also inhibited by Ca^{2+} in a cooperative manner (16).

Dictyostelium cells can be effectively permeabilized by electroporation (5,17). The conditions used produce very small holes which allow the transport of molecules smaller than about 300 Daltons. Thus, cells do not leak proteins or nucleotides such as ATP or GTP (5,18). Electro-permeabilized cells in EGTA show a strong increase of cGMP levels upon stimulation with cAMP (figure 2). Addition of 10^{-6} M Ca^{2+} to electro-permeabilized cells leads to a decrease of basal cGMP levels and subsequent cAMP stimulation induces only a

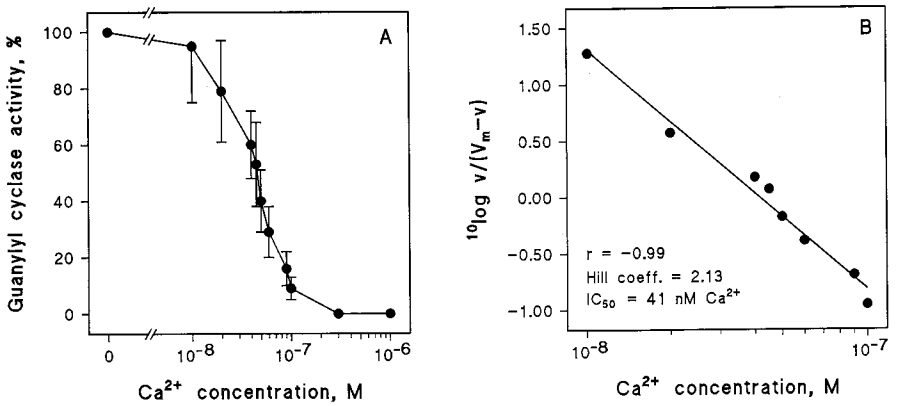

Figure 1. The regulation of guanylyl cyclase by Ca^{2+} *in vitro*.
A, Guanylyl cyclase activity was measured in a cell-free preparation at different free Ca^{2+} concentrations; half maximal inhibition occurred at 41 nM Ca^{2+}. B, Hill plot of the same data; the Hill coefficient is n=2.1.

small cGMP response. Basal and cAMP-stimulated cGMP levels were measured at different extracellular Ca^{2+} concentrations in electro-permeabilized cells, showing that both are equally inhibited with IC_{50} = 200 nM Ca^{2+} (figure 3). Extracellular Ca^{2+} had no effect on

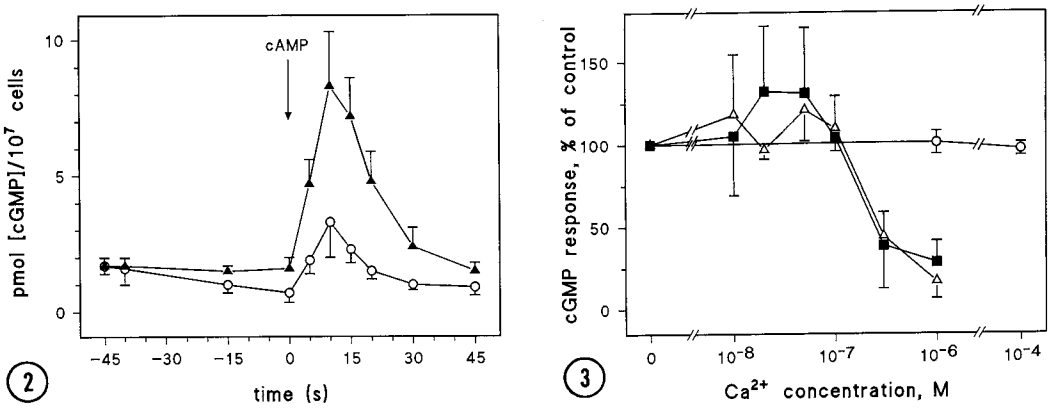

Figure 2. Ca^{2+} regulation of the cAMP-induced cGMP response in permeabilized cells.
Cells were electro-permeabilized and preincubated for 45 s with 5.9 mM EGTA (▲) or 5.9 mM EGTA with 1 μM free Ca^{2+} (o). Cells were then stimulated at t=0 with 0.1 μM cAMP, lysed at the times indicated and cGMP levels were measured.

Figure 3. The regulation of basal and stimulated cGMP levels by Ca^{2+}. Electro-permeabilized cells were incubated for 45 s at different free Ca^{2+} concentrations and stimulated with 0.1 μM cAMP. cGMP levels were measured just before (■) and 10 s after stimulation (▵). Basal cGMP levels were also measured in non-permeabilized cells (o). Data are presented as means and standard deviations relative to the control without Ca^{2+}. The control levels in pmol/10^7 cells were: 0.67 ± 0.26 pmol for (■), 10.0 ± 1.9 pmol for (▵), and 0.71 ± 0.13 pmol for (o).

TABLE I. The regulation of cGMP and Ins(1,4,5)P_3 in *D.discoideum* cells

Condition	cGMP	Ins(1,4,5)P_3	Ref.
Response of mutant *fgd*C to cAMP	increased	reduced	19
Basal levels in mutant Dd-RAS-THR12	normal	increased	21, 23
Response of wild-type cells to 8-CPT-cAMP	increased	reduced	24

8-CPT-cAMP, 8-p-chlorophenylthioadenosine 3',5'-cyclic monophosphate.

basal cGMP levels of intact *D. discoideum* cells (figure 3), suggesting that the inhibition by Ca^{2+} in electro-permeabilized cells was due to changes of the intracellular Ca^{2+} concentration.

It has been proven difficult to measure cytosolic Ca^{2+} concentrations in *Dictyostelium* cells. Cytosolic Ca^{2+} concentrations are likely to be regulated partly by Ins(1,4,5)P_3. To establish a possible regulation of guanylyl cyclase by Ca^{2+} *in vivo*, we have collected data on receptor-mediated formation of both cGMP and Ins(1,4,5)P_3 in intact cells for a variety of mutants (Table I). The cAMP mediated activation of phospholipase C was lost in mutant *fgd*C and the cGMP response was slightly larger than in wild-type cells (19). Transformants overexpressing a mutated ras gene (Dd-RAS-THR^{12}) showed an increased formation of Ins(1,4,5)P_3 (20) due to the enhanced conversion of phosphatidylinositol to phosphatidyl-inositolphosphate (21). This effect was associated with an increased activity of a protein kinase C-like enzyme (22). Thus, it is expected that in mutant Dd-RAS-THR^{12} both Ins(1,4,5)P_3, Ca^{2+} and PKC activities are increased. The cGMP response is diminished in this transformant (23). Finally, in wild-type cells the partial antagonist 8-p-chloro-phenylthioadenosine 3',5'-cyclic monophosphate induced a decrease of Ins(1,4,5)P_3 levels, whereas a very strong cGMP response was induced (24).

The experiments with electroporated cells in EGTA and previous data of experiments with mutant cells clearly demonstrate that receptor-mediated cGMP formation can occur in the absence of receptor-mediated stimulation of phospholipase C as well as in the absence of elevated intracellular Ca^{2+} concentrations. These results confirm experiments on the effect of Ca^{2+} on guanylyl cyclase activity *in vitro*, showing that this bivalent cation is a potent inhibitor of enzyme activity. In contrast to previous results with saponin treated cells (9-11), the present results imply that *in vivo* intracellular Ca^{2+} inhibits guanylyl cyclase.

The activity of guanylyl cyclase in membranes without Ca^{2+} and the rate of cGMP accumulation in intact cells upon stimulation with cAMP are nearly identical (both 40-60 pmol/min per equivalent of 10^7 cells). This may suggest that in unstimulated cells guanylyl

cyclase is inhibited by Ca^{2+} and that cAMP stimulation of enzyme activity is mediated by the loss of this inhibition. We could not find evidence for this hypothesis, because basal and cAMP-stimulated cGMP levels show the same sensitivity for Ca^{2+} (figure 3).

In aggregation competent cells guanylyl cyclase is activated by extracellular cAMP, whereas folic acid stimulates the enzyme in growing cells. Folic acid and cAMP are detected by different surface receptors, but share a common guanylyl cyclase (25). The activation of guanylyl cyclase by cAMP is probably mediated by the receptor cAR1, because the cyclic nucleotide specificity for binding to cAR1 is identical to the specificity for guanylyl cyclase activation (26, 27), and cAMP-stimulation of guanylyl cyclase is lost in cells with reduced expression of cAR1 (28). Besides cAR1 and guanylyl cyclase, an additional component is required for stimulation by cAMP, because cAMP cannot stimulate guanylyl cyclase in cells that overexpress cAR1 during growth; these cells do express guanylyl cyclase which can be activated by folic acid (29, and unpublished results). The missing component could be a G-protein, as mutant *fgd*A with a defective Gα2-subunit fails to show cAMP-simulated guanylyl cyclase, whereas stimulation by folic acid is unaltered (30). These observations suggest that the sensory transduction pathways from surface receptor to guanylyl cyclase may include different receptors for cAMP and folic acid, different G-proteins and a common guanylyl cyclase. In this scheme Ca^{2+} is a negative regulator of guanylyl cyclase activity *per se*, but is not involved in the activation mechanism of the enzyme.

The negative regulation of guanylyl cyclase by Ca^{2+} ions has also been described for the enzyme from bovine retinal rods (31), where the protein recoverin mediates this inhibition. Possibly the guanylyl cyclase activity in *Dictyostelium* is regulated by a similar protein, especially since the inhibition by Ca^{2+} shows the same sensitivity and cooperativity for Ca^{2+} with both enzymes. In *Paramecium*, however, the opposite is found: Ca^{2+} ions stimulate guanylyl cyclase activity (32).

In conclusion we have demonstrated that Ins(1,4,5)P_3-mediated Ca^{2+} release is a negative regulator of guanylyl cyclase activity. This suggests that Ca^{2+} and cGMP may have partially antagonistic functions in *D.discoideum*. Guanylyl cyclase and phospholipase C are activated most likely by the same surface receptor. The inhibition of guanylyl cyclase by Ca^{2+} may induce or amplify existing intracellular gradients of cGMP and Ca^{2+}. Therefore, inhibition of guanylyl cyclase by Ca^{2+} may help the cell to orient effectively in chemotactic gradients of extracellular cAMP.

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