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Nitric oxide-releasing compounds inhibit *Dictyostelium discoideum* aggregation without altering cGMP production

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The effects of nitric oxide-releasing compounds on *Dictyostelium discoideum* cell development and guanylyl cyclase activity were studied. The addition of SNP (sodium nitroprusside) or SIN-1 (3-morpholino-syndnonimine) to starved cells inhibited their differentiation and aggregation in a concentration-dependent manner. In contrast to mammalian systems, SNP did not significantly affect guanylyl cyclase activity in cell lysates of *D. discoideum*, nor did it stimulate cGMP production in intact cells. The results suggest that the inhibitory effects of NO on *D. discoideum* cell aggregation are through a mechanism independent of an effect on guanylyl cyclase activity.

Dictyostelium discoideum; Nitric oxide; Aggregation; Guanylyl cyclase

1. INTRODUCTION

In aggregating cells of *Dictyostelium discoideum*, adenylyl cyclase is responsible for the production of the chemotactic signal, extracellular cAMP pulses [1,2]. Adenylyl cyclase also plays a prominent role in the phenomena of signal relay. This refers to the synthesis and release of cAMP into the medium in response to cell stimulation by a pulse of cAMP [1,2]. Cells also respond to such stimulation with an activation of their guanylyl cyclase but the subsequently produced cGMP is retained within the cell [1,2]. Based on the phenotype of mutants defective in cGMP phosphodiesterase [3], the changes in intracellular concentrations of cGMP are believed to be important in the regulation of the chemotactic response. These mutants show prolonged periods of chemotactic movement, which correspond with prolonged periods of increased intracellular cGMP [3]. The regulation of guanylyl cyclase and the mechanism(s) of its activation in *D. discoideum* are poorly understood. Both soluble and membrane-associated guanylyl cyclase activities have been reported in these cells [4,5]. However, little progress has yet been made towards the purification and characterization of the enzyme, most likely because of its relative instability in broken cell preparations.

It has been well documented that soluble guanylyl cyclase in higher eukaryotes can be activated by nitric oxide (NO), which itself can be produced by a number of vertebrate cells [6,7]. NO or NO-releasing compounds increase the production of cGMP both in vitro and in vivo [6–8]. This action is generally believed to be the mechanism by which NO and NO-releasing compounds produce their wide variety of biological effects in mammalian systems [6]. Whether *D. discoideum* cells produce NO, and if NO could stimulate their guanylyl cyclase activity, is currently unknown. Our previous studies suggest that NO does alter another metabolic event in these cells. We have demonstrated that NO-releasing compounds activate an ADP-ribosyltransferase activity that modifies a 41 kDa cytosolic protein [9]. However, cGMP or its analogues cannot stimulate the ADP-ribosylation of this protein, suggesting that guanylyl cyclase activation may not be required for this ADP-ribosylation to occur. In the present study, the effects of NO-releasing compounds on *D. discoideum* cell development and guanylyl cyclase activity are investigated. The results indicate that NO can inhibit cell aggregation and that this effect appears to occur in the absence of any changes in guanylyl cyclase activity.

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2. MATERIALS AND METHODS

2.1. *Cell culture conditions*

D. discoideum strain AX-2 was grown at 21°C to a density of 3×10^6 cells/ml in HL-5 medium [10]. Starvation was initiated by washing cells twice in 20 mM phosphate buffer, pH 6.4, and resuspending them at 10^7 cells/ml [11]. Differentiation to aggregation competence occurred in spinner suspension, and the developmental state of cells was determined microscopically as previously described [12]. Cells were considered aggregation competent when most (>75%) of the cells displayed an elongated morphology and formed end-to-end contacts [12]. This usually occurred in control cells after 4–5 h of starvation. Aggregation was considered to be inhibited if cells did not show signs of aggregation competence within 8 h of starvation.

2.2. *Guanylyl cyclase assay*

Cells were washed with 10 mM Tris-HCl, pH 8.0, and resuspended at 2.5×10^8 cells/ml in buffer containing 2 mM HEPES, pH 7.4, 70 mM sucrose, 220 mM D-mannitol, 1 mM EDTA, 1 mM EGTA, and 0.5 mM albumin. Cells were lysed at 4°C by passage through a 5.0 μm polycarbonate filter [13]. Guanylyl cyclase activity was measured as described with minor modifications [14]. For measurement of the Mg^{2+}-dependent activity, we used a reaction mixture (0.1 ml) containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM GTP, 2–3 μCi [α-^{32}P]GTP, and 20 μl cell lysate. The Mn^{2+}-dependent activity was assayed as described above except that 2 mM $MnCl_2$ was included in the reaction mixture. The mixture was incubated at 21°C for 2 min and the reaction was terminated by adding 0.1 ml of a solution containing 2% of SDS, 1.4 mM cGMP and 30,000 cpm [8-^{3}H]cGMP, and boiling for 1 min. [^{32}P]cGMP formed was isolated by sequential chromatography on Dowex-1 and neutral alumina, and quantified by liquid scintillation counting. The recovery of cGMP ranged from 40% to 55%. Enzyme activity was linear with incubation time for up to 3 min, and with increasing amounts of homogenate up to 30 μl. The preparation and assay of soluble guanylyl cyclase from rat liver were as described [15].

2.3. *Cyclic GMP measurements*

After 3.5–4 h of starvation, cells were washed and resuspended at 2×10^7 cells/ml in 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, and divided into four groups: no addition, addition of 10^{-7} M cAMP, or 0.2 mM SNP, or both. At the times indicated, an aliquot of cells was mixed with TCA (final 5%), and immediately frozen in dry-ice. The frozen samples were then thawed at room temperature and centrifuged. Supernatants were extracted 10 times with 2 vols. of water-saturated ether, and lyophilized. Cyclic GMP was measured in duplicate by a radioimmunoassay.

2.4. *Materials*

[α-^{32}P]GTP was purchased from ICN. The cGMP RIA kit was obtained from Amersham. Polycarbonate filters were from Nucleopore, and neutral alumina was from Calbiochem. All other chemicals were from Sigma.

3. RESULTS AND DISCUSSION

The effects of different concentrations of the NO-releasing compound, sodium nitroprusside (SNP), on the development of aggregation competence were examined. Cells were starved in spinner suspension for 2 h, at which time SNP was added. Concentrations of SNP less than or equal to 0.1 mM did not visibly alter the time-course of cell aggregation compared to untreated cells. In both cases, cells were generally aggregation competent by 4–5 h of starvation. In contrast, the addition of 0.2–0.5 mM SNP to cells delayed the expression of aggregation competence by approximately 2 h, while higher concentrations (e.g. 1–5 mM SNP) completely inhibited cell differentiation and aggregation. The effects of 1 mM SNP were reversible. Cells that had been treated with SNP for 6–8 h were transferred to buffer devoid of SNP. After an additional 8–12 h, the cells had fully recovered and had formed aggregates. $K_4Fe(CN)_6$, a compound that is structurally similar to SNP but devoid of the ability to release NO, had no effect on cell aggregation at concentrations up to 5 mM. Another NO releasing compound, 3-morpholino-syndnonimine (SIN-1), also affected aggregation but higher concentrations than SNP were required. SIN-1 at 1 mM did not alter aggregation, but 10 mM led to a delay of aggregation of several hours, while 30 mM was required for total inhibition. Together, these observations indicate that NO is the inhibitor of cell aggregation.

We next assessed the effects of 1 mM SNP on cells that had been starved for increasing periods of time prior to this addition. The addition of SNP at the time starvation was initiated, or within the first hour of starvation, produced only a delay (approximately 1 h) in the development of aggregation competence. When SNP was added at 2 h of starvation, cell differentiation was not observed during the 7–8 h period of the experiment. The addition of SNP to cells that had been starved 3–4 h, and which displayed early signs of aggregation competence, again were only delayed 1–2 h in their aggregation program. Once the majority of the cells had achieved aggregation competence, the addition of SNP appeared to have little or no effect on their development. Thus, SNP could inhibit cell aggregation in a dose-dependent manner and seemed to be most effective when cells had been starved for 2 h but had not yet begun to display the morphology characteristic of aggregation competence. It is of interest to note that it is at this time that cells become responsive to the addition of exogenously supplied cAMP pulses [16]. In response to such pulses, cells advance their differentiation to aggregation competence [16,17].

In higher eukaryotes, it is generally believed that the effects of NO and of NO-releasing compounds are the result of a stimulation of the soluble guanylyl cyclase and the subsequent increase in intracellular cGMP accumulation [6]. Given the probable role of cGMP in *D. discoideum* aggregation [1–3] and the fact that NO-releasing compounds inhibit that process, we examined the effects of SNP on guanylyl cyclase activity (Fig. 1).

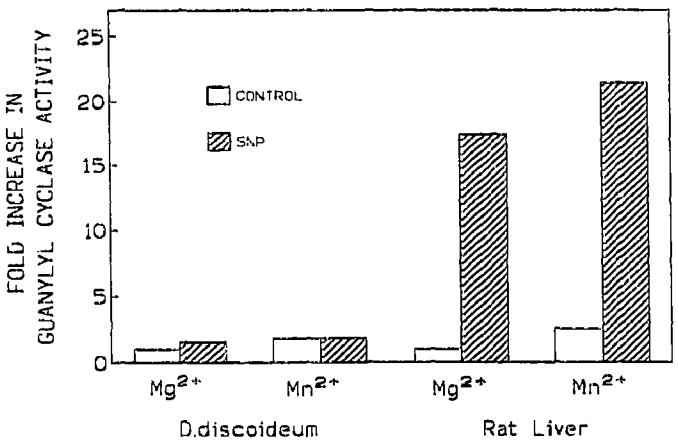

Fig. 1. Effects of SNP on guanylyl cyclase activity in *D. discoideum* cell lysates and rat liver cytosol. Guanylyl cyclase activity was measured in the presence or absence of 0.2 mM SNP as described in the text. Mg^{2+}-dependent activity of untreated samples in each case is considered as basal activity with a value of 1. The data are expressed as fold increase above this level. The data are representative of three experiments.

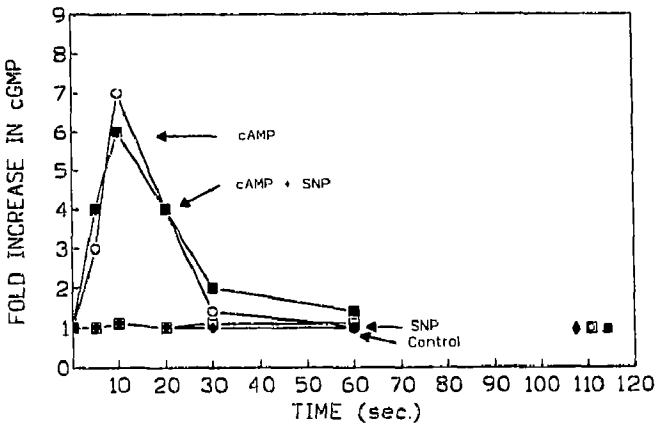

Fig. 2. Effects of cAMP and SNP on cGMP production in *D. discoideum* cells. Cells were starved for 3.5–4 h and treated with 10^{-7} M cAMP (○), 0.2 mM SNP (□), or both cAMP and SNP (■). Control cells (◆) received no treatment. Cyclic GMP levels were measured as described in the text. The data are expressed as the fold increase in cGMP production at the time after treatment compared with that at time 0. The levels indicated by the symbols at the right of the diagram represent those seen after approximately 2, 5, 10, 15, 20 and 30 min. The data are representative of five experiments.

As has been reported in mammalian systems, the addition of SNP to rat liver cytosol greatly stimulates guanylyl cyclase activity [15]. As shown in Fig. 1, SNP addition to rat liver cytosol resulted in a 17–18-fold increase in the Mg^{2+}-dependent activity and an 8–9-fold increase in the Mn^{2+}-dependent activity. *D. discoideum* also displays both types of activities, the latter being 1.8–2-fold greater than the Mg^{2+}-dependent activity. However, in contrast to the rat liver system, neither activity was significantly altered by the addition of SNP. Concentrations of SNP between 0.2–1 mM produced only a slight (1.5-fold) increase in the Mg^{2+}-dependent enzyme and no changes in the Mn^{2+}-dependent activity. The experiment shown in the figure used cells that had developed aggregation competence, but similar results were obtained using preparations from growing and from 3–4 h starved cells. We did attempt to fractionate lysates into crude membrane and cytosolic fractions prior to SNP addition, but the enzyme rapidly lost activity during the procedure.

The above data would indicate that SNP does not affect guanylyl cyclase in *D. discoideum*. To confirm this finding and to eliminate possible artefacts due to the instability of the enzyme in cell lysates, we determined the effects of SNP treatment on cGMP production of intact cells (Fig. 2). Treatment of cells with SNP had no effect on cGMP production, either within the first min after addition, or after longer periods up to 30 min. Concentrations of SNP that inhibit (1 mM) or delay (0.5 mM) the development of aggregation competence were tested and yielded the same results. A number of different laboratories have shown that a pulse of submicromolar concentrations of cAMP results in a transient increase in intracellular cGMP concentration within 10 s of stimulation [1,2,18]. Therefore, we tested the possibility that SNP inhibits cell aggregation by inhibiting cAMP-induced cGMP production. As shown in this figure, both SNP-treated and untreated cells responded to a pulse of 10^{-7} M cAMP by increasing their cGMP production.

The results reported in this study indicate that the NO-releasing compounds, SNP and SIN-1, inhibit cell aggregation. This inhibition does not appear to be mediated by a stimulation of cGMP production, the more commonly observed effect of this compound in other systems. SNP also did not interfere with the ability of cAMP to stimulate cGMP production. This leads us to consider alternative mechanisms for the action of SNP in this system. In a previous manuscript, we have reported that SNP and SIN-1, added to cell lysates, stimulate the ADP-ribosylation of a 41 kDa protein, the nature of which is currently unknown [9]. SNP stimulates the ADP-ribosylation of a protein of similar molecular mass in human platelets and various rat tissue [19]. It is well known that NO or SNP can inhibit the aggregation and adhesion of platelets [6]. It is generally assumed that NO elicits its physiological effects by stimulating the soluble guanylyl cyclase in those cells [6]. The role of the NO-stimulated ADP-ribosylation has remained obscure. As reported in this present study, no obvious effect of SNP on guanylyl cyclase in *D. discoideum* was observed. Thus, the inhibitory effects of SNP on cell aggregation in our system, and perhaps platelets as well, may reflect changes in ADP-ribosylation. It is of interest to note that the relative potency of SNP and SIN-1 on *D. discoideum* aggregation are mirrored by their same relative effectiveness in stimulating p41 ADP-ribosylation [9]. Preliminary evidence also suggest that intact *D. discoideum* cells may respond to SNP treatment by enhancing the ADP-ribosylation of p41 [9]. Continued efforts are directed toward identifying p41 and investigating a potential role for its ADP-ribosylation in vivo.

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