

Stimulation of Inositide Degradation in Clumping *Stigmatella aurantiaca*

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Numerous external signals which activate inositol phospholipid hydrolysis in eukaryotes are known; probably all of these signals are transduced by G proteins. So far, neither signal-transducing G protein nor receptor-regulated phospholipase C has been found in prokaryotes. However, a group of bacteria, the myxobacteria, displays cellular and tissue-like differentiation; therefore, it appeared that a search for the various activities involved in a signal-activated phosphatidylinositol cycle might be rewarding. Here, we report that in *Stigmatella aurantiaca*, under conditions which promote clumping, inositol phospholipid synthesis and degradation were stimulated with the resulting formation of inositol phosphate and inositol bisphosphate. The turnover was Ca^{2+} dependent and was increased by fluoride ions. Membrane preparations from these cells showed a phospholipase C activity which increased with the stage of incubation and which was stimulated by GTP γ S, suggesting G protein dependency. To what extent this system in a prokaryotic cell shares properties of the phosphatidylinositol cycle in eukaryotes remains unexamined.

Signal-stimulated transmembrane processes leading to the formation of secondary messengers such as cyclic AMP or inositol phosphates (3, 12) are present in all eukaryotic cells, but they have not yet been found in prokaryotes.

However, some bacteria display unusual features, including multicellular development. In this respect, myxobacteria appear of particular interest as organisms able to elaborate complex developmental and vegetative interlocking cycles. The myxobacteria are gram-negative, gliding bacteria, which carry out developmental sequences. The two most-studied species are *Mycococcus xanthus* (10, 16) and *Stigmatella aurantiaca* (24, 25). *S. aurantiaca* has evolved a kind of multicellular life cycle affording a unique opportunity among bacteria for the study of cell-cell interactions leading to aggregation. This step is followed by a subsequent cooperative morphogenesis which presents some analogy to the cycles of two cellular slime molds (20, 30). When they are starved on an agar surface, individual cells move toward aggregation centers before constructing complex structures and fruiting bodies in which cells differentiate into myxospores (25). As previously shown in our laboratory (6), *S. aurantiaca* is also one of the few gram-negative, aerobic bacteria containing inositol phospholipids. In growing cells, up to 25% (0.65 ± 0.1 nmol/ 10^8 cells) of the total phospholipids were inositol lipids. Phosphatidylinositol (PI) and PI phosphate represented only 0.5 and 0.05%, respectively.

Therefore, we addressed the possibility that a PI cycle operates during part or all of the developmental sequences induced by starvation.

MATERIALS AND METHODS

Strains. *S. aurantiaca* DW 4 ATCC 33878 and DW 135, a slowly clumping mutant, were isolated by D. White, who kindly gave us these strains. The culture and starving conditions used to induce clumping were as described elsewhere (13, 21, 22).

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Inositol phospholipid synthesis. Mid-log-phase cells were harvested by centrifugation at $9,000 \times g$ for 10 min and were washed once at room temperature with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-2-ethanesulfonic acid; pH 7.2). The cells were resuspended to 5×10^8 cells per ml in the same buffer containing 3.4 mM CaCl_2 or 10 mM NaF and 3.4 mM CaCl_2 or 0.5 mM EGTA [ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid]. The suspensions were shaken at 30°C in a rotary shaker at 130 rpm.

At different stages of clumping, 2.5 μCi of *myo*-[2- ^3H]inositol (specific activity, 19.6 Ci/mmol; Amersham Laboratory) per ml was added. After a further 3 min, incubations were terminated by perchloric acid (final concentration, 0.5 N). Lipids were then extracted (4); the lipidic phase was washed once with upper phase obtained by mixing CHCl_3 - CH_3OH -water containing 1 mM inositol (2:2:1.8 [vol/vol/vol]). Each chloroform phase was evaporated under vacuum; total lipids were dissolved in liquid scintillation fluid and counted.

Inositol phosphate formation. The water-soluble (upper) phases were neutralized with NaOH and applied to a 1-ml column of Dowex 1 ($\times 8$; formate form; Sigma). The radioactive inositol phosphates were eluted sequentially as described elsewhere (2). [^3H]inositol phosphate fractions were pooled before counting. The following three standard inositol phosphates (Amersham) were utilized: *D*-*myo*-[2- ^3H]inositol-1-phosphate, *D*-*myo*-[2- ^3H]inositol-1,4-bisphosphate, and *D*-*myo*-[2- ^3H]inositol-1,4,5-trisphosphate.

Phospholipase C activity in clumping cells. Cells from different stages of clumping were sonicated in ice for a total of 2 min with 30-s bursts of a sonicator probe and an intervening 30 s (MSE disintegrator [10 μm peak to peak]) directly in HEPES- Ca^{2+} solution. The membranes were sedimented at $18,000 \times g$ for 20 min and washed once with the same solution. To prepare the substrate, the cells were incubated for 30 min in the presence of 3.4 mM Ca^{2+} and [^3H]inositol as previously described. The chloroform phase was evaporated, and total lipids were dissolved in HEPES- Ca^{2+} solution by agitation. Membranes (5 μg of membrane protein) were incubated on a rotary shaker at 30°C in the presence of GTP γ S [guanosine-5-*O*-(3-thiotriphosphate)] or GTP γ S and GDP (final volume, 0.4

ml of Ca^{2+} -HEPES solution containing lipids from 10^8 *S. aurantiaca* cells [50,000 cpm]). The reactions were terminated by the addition of perchloric acid (final concentration, 0.5 N). The lipids were extracted, and the water-soluble phase of each incubation mixture was loaded onto an anion-exchange column and eluted as previously described (2). Phospholipase activity was assessed by using lipids extracted from *Stigmatella* cells incubated in the presence of $[\text{U-}^{14}\text{C}]$ acetate. Diglyceride formation was authenticated by chromatography (19) after incubation with the membrane fraction (data not shown).

Chemicals were from Sigma unless otherwise stated.

RESULTS AND DISCUSSION

Washed *S. aurantiaca* cells (strain DW 4[21]) suspended in a HEPES- Ca^{2+} solution associated rapidly (13, 22). Within a few minutes, cells became adhesive and clumps could be visualized. When they were transferred onto an agar surface, these clumps developed with the formation of numerous fruiting bodies. Clumping, as measured by a decrease in optical density, was completely inhibited by the addition of EGTA in excess relative to Ca^{2+} or by the omission of Ca^{2+} . Conversely, 10 mM fluoride ions accelerated clumping. F^- stimulates G protein-transduced signals (7, 12); therefore, it was included in some experiments. From these suspensions of cells submitted to 3-min pulses of tritiated inositol added at different stages of incubation, two labelled fractions could be obtained after CHCl_3 - CH_3OH extraction (4).

Analysis of the liposoluble fraction (Fig. 1a) showed that under standard conditions, net synthesis of inositol lipids was increasing at the onset of a series of events leading to the formation of cohesive clumps; after about 20 min of incubation, net synthesis was slowing down. EGTA inhibited this Ca^{2+} -dependent synthesis, while fluoride ions stimulated both the initial increase and the connected decrease in synthesis.

These data were obtained from pulse-labelling experiments. The results might therefore be explained by one of the following hypotheses: inositol uptake was rate limiting and varied with the state of starvation, or the synthesis was activated and then the rate of synthesis apparently declined because inositol lipid degradation was also activated by a signal somehow related to cell-cell interaction or to starvation.

Inositol uptake as a rate-limiting factor was ruled out by measurement of its intracellular concentration (data not shown). The second hypothesis was then tested. Hydrosoluble inositol-1-phosphate (IP_1) and inositol-1,4-bisphosphate (IP_2) were isolated through chromatography (2) of the hydrophilic fraction obtained after CHCl_3 - CH_3OH extraction; Fig. 1b shows that increased accumulation of IP_1 and IP_2 correlated with an apparent decrease in inositol lipid synthesis after 15 to 18 min of incubation. This accumulation was also stimulated by fluoride ions between 15 to 18 min and 20 to 23 min and required calcium ions after 15 min of incubation. Formation of inositol phosphates from inositol lipids has been ascribed to phospholipase C (19, 26); therefore, we searched for such an activity.

The membrane fraction of sonicated cells effectively hydrolyzed endogenous inositol lipids and added pure PI but not phosphatidylethanolamine or phosphatidylglycerol, liberating inositol phosphate and inositol from endogenous as well as from exogenous substrates prepared as described in Materials and Methods. After 30 min of incubation, enzymatic activity was enhanced up to five times in sonicated membranes prepared from DW 4 cells undergoing clumping compared with similar preparations from growing DW 4 cells. With the slowly clumping mutant, phospholipase activity was increased only

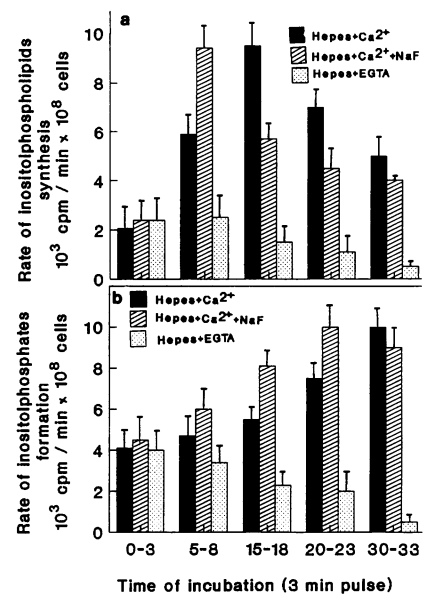


FIG. 1. (a) Rate of inositol phospholipid synthesis. Mid-log-phase cells were harvested, washed once with HEPES buffer, and resuspended in the same buffer containing CaCl_2 , NaF and CaCl_2 , or EGTA. At different stages of clumping, *myo*- $[\text{2-}^3\text{H}]$ inositol was added. After a further 3 min, total lipids were then extracted (4) and counted. Mean values of three independent experiments from which data for panels a and b were simultaneously obtained are given. Error bars represent standard errors of the means. (b) Rate of inositol phosphate formation. The radioactive inositol phosphates were eluted sequentially as described elsewhere (3). $[\text{3H}]$ inositol phosphate fractions were pooled before counting. More than 95% of the total counts represented were from IP_1 . When elution was performed on extracts obtained after 30 min of continuous labelling, labelled IP_1 represented about two of three of the total counts, and IP_2 represented about one of three.

three times, and it took up to 60 min to reach the maximum (Fig. 2). Phospholipase C activity in membranes from growing cells is increased by adding $\text{GTP}\gamma\text{S}$; this increase is reversed by GDP (Fig. 3). In neutrophil plasma membranes, phospholipase C activity is not GTP dependent when Ca^{2+} concentration is 1 mM (8). Stimulation and reversion were observed in vitro, even in the presence of 3.4 mM Ca^{2+} .

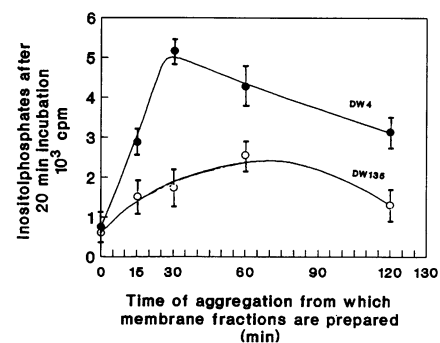


FIG. 2. Variation of phospholipase C activity in aggregating cells. $[\text{3H}]$ inositol lipids were incubated with unlabelled membranes from *S. aurantiaca* DW 4 or DW 135. After 20 min, lipids were extracted, and the water-soluble phase of each incubation mixture was loaded onto an anion-exchange column and eluted as previously described (3).

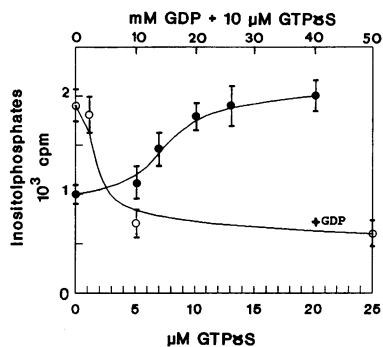


FIG. 3. Phospholipase C activity in the presence of GTP γ S. [3 H]inositol lipids were incubated with membranes in the presence of GTP γ S (●) or GTP γ S and GDP (○). Inositol phosphates were analyzed as described in the legend to Fig. 2. The results are the means of three independent experiments. Error bars represent the standard errors of the means.

Having previously published that inositol lipids from exponentially growing *S. aurantiaca* were resistant to alkaline methanolysis (6), we searched for the kind of inositol derivatives synthesized under starvation. PI was the first [3 H]inositol lipid formed, followed by PI phosphate and two derivatives which accumulated. PI is the precursor of two ceramide phosphoinositols. These ceramide derivatives, the major inositol-containing lipids in growing cells, are similar to those found by Smith and Leister in yeasts (17a, 26a). By treating purified PI extracted from cells undergoing clumping with alkaline methanol (5), 80% of the PI molecules were shown actually to be diesters, liberating glycerylphosphorylinositol (11). Under the experimental conditions used when the cells were pulse-labelled, diester [3 H]inositol PI accounts for 60 to 65% of all of the labelled inositol phospholipids. In exponentially growing cells, the diacyl PI pool represents 2% of the total inositol lipids and therefore has been overlooked.

The observed changes in the rate of inositol PI synthesis and phosphodiesterase activity were not related simply to starvation but indeed reflected the beginning of a developmental pathway, as was shown by using a slowly clumping mutant, *S. aurantiaca* DW 135 (13). When the mutant was suspended in standard solution at the same cell density, complete clumping of strain DW 135 took about 120 min instead of 30 min with strain DW 4; the highest rate of inositol phospholipid synthesis measured by pulse-labelling with [3 H]inositol was observed after 45 min, while the same rate was observed after 15 min of incubation for the normal strain (Fig. 4a). Maximum phosphodiesterase activity in membrane fractions was also delayed and reached only 50% of that observed in strain DW 4 (Fig. 4b).

Whether it is dependent on extracellular chemical signals or is related to cell-cell interaction, clumping in *S. aurantiaca* is correlated with important physiological changes which may provide insight into signal transduction leading to a distinctive developmental cycle. Complete analogy between this putative prokaryotic transduction system and the eukaryotic one cannot be assessed at present; however, most of the molecules involved in the PI cycle in eukaryotes are present in *S. aurantiaca*. (i) The cascade of clumping-related events is dependent on Ca^{2+} ions and is stimulated by F^- . (ii) Inositol phospholipids are rapidly metabolized, giving rise to the formation of inositol phosphates. (iii) During clumping, a phospholipase C activity is strongly stimulated; this activity is also increased in vitro by GTP γ S and is inhibited by GDP. (iv) Experiments performed in our group showed that a membrane-associated

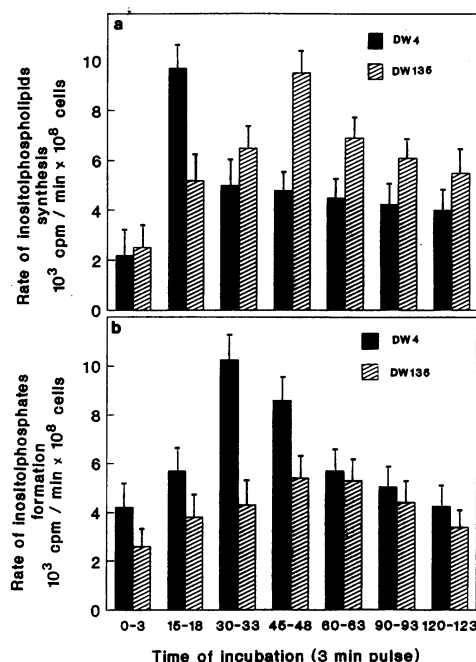


FIG. 4. (a) Rate of inositol phospholipid synthesis in DW 4 and DW 135. Experiments were performed as described in the legend to Fig. 1. Lipids were dissolved in liquid scintillation mixtures and counted. Horizontal bars represent maximum and minimum values. (b) Rate of inositol phosphate formation in DW 4 and DW 135: inositol phosphates recovered from the water-soluble phases (see Fig. 1b).

protein could be visualized after photoaffinity labelling with [32 P]GTP (9). Purification and characterization of this G protein, which has a GTPase activity, are in progress.

The signals which coordinate aggregation and cell differentiation are unknown. A few candidates have been described, including guanosine (28), light (15), and a pheromone (27). In *M. xanthus*, genetic analysis of defective mutants indicates that several signals secreted by the cells are involved (17).

All of these facts support the assumption that a G protein-transduced signal, depending on cell-cell contact or chemical compounds, initiates cellular differentiation.

Since DNA can be transferred in *S. aurantiaca* by conjugation (14), this organism appears very promising for analyzing and testing the expression and the physiological and molecular functions of various genes which have been related to PI kinase (29, 18), G proteins (1), or other proteins (23) involved in signal transduction.

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