Vol. 206, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 5, 1995 Pages 401-407

LITHIUM IN THE MATING RESPONSE AND CELL CYCLE OF *SACCHAROMYCES CEREVISIAE*

Blake E. Smith, Danton H. O'Day, and Gerald A. Proteau[1]

Department of Zoology, Erindale
College, University of Toronto, Mississauga, ON, Canada, L5L 1C6

Received November 30, 1994

Studies with Li^+, an inhibitor of phosphoinositide metabolism, demonstrated that an early response of *Saccharomyces cerevisiae* to *a*-factor pheromone was negatively affected by this cation. This was monitored by the production of ß-galactosidase from a reporter construct containing the promoter region of the yeast STE3 gene and the coding region of the *E. coli* LacZ gene. Growth and progression through the cell cycle were also affected by Li^+ and analysis of budded/unbudded ratios revealed that Li^+ caused yeast cells to arrest in G1. These data provide support for the role of inositol phosphates in the mating response and cell cycle of *Saccharomyces cerevisiae*.

The cellular events that take place during the mating process in yeast involve receptors for pheromones found on the surface of each of the haploid cell types. The α-factor and *a*-factor receptors (encoded by STE2 and STE3, respectively) belong to the seven-membrane-spanning domain class of G-protein-linked receptors (1, 2, 3). Interaction of a ligand with a receptor can activate G-proteins by promoting the exchange of GDP for GTP by the α-subunit of the G-protein (reviewed in reference 4). This in turn may lead to the activation of PLC which cleaves a number of different inositol-containing phospholipids producing DAG and inositol phosphates (Reviewed in reference 5). When PIP_2 is the substrate, IP_3 is formed which then can promote the release of Ca^{2+} from intracellular stores (6).

The role of phosphoinositides in the life cycle of *S. cerevisiae* remains unclear, yet they may serve some important functions. It is clear that phosphoinositide turnover, or least the presence of PIP_2, is required for complete progression through the cell cycle (7, 8, 9). Many

[1]To whom correspondence should be addressed. Present address: Department of Microbiology, Collège Universitaire de Saint-Boniface, University of Manitoba, 200 Avenue de la Cathédrale, Saint-Boniface, Manitoba, Canada, R2H 0H7. Fax:204-237-3240.

Abbreviations: PLC- phospholipase C; DAG- 1,2-diacylglycerol; PIP_2- phosphatidyl inositol 4,5-bisphosphate; IP_3- inositol 1,4,5-trisphosphate.

0006-291X/95 $5.00

enzymes and activities involved in the metabolism of inositol phosphates have been identified in yeast. These include phosphatidylinositol synthase (10, 11), *myo*-inositol-1-phosphate synthase (12), phosphatidylinositol 3-kinase (13), and phosphatidylinositol 4-kinase (14).

While various lines of evidence suggest inositol phosphates may serve as intracellular messengers in yeast, their role in the mating response to pheromonal stimulation has not been determined. In this study we have examined the effects of lithium, an inhibitor of inositol phosphate cycling, on cell growth, progression through the cell cycle, and the ability of *a*-factor to induce expression from a STE3-LacZ reporter gene construct. Our data suggests a role for inositol phosphate signalling in all of these processes.

Materials and Methods

a-Factor Preparation and Testing

The *a*-factor pheromone was prepared from *S. cerevisiae* DC5 cells (MATa leu2-3 leu2-112 his4 gal2 can1) by the method described by Proteau *et al.* (15). The *a*-factor preparations were assayed for biological activity through the ability of the pheromone to irreversibly arrest α-cells that carry the sst2 allele. A quantity of *a*-factor (2 µl) was spotted on a lawn of YP1 (MATα sst2-1) cells and incubated overnight at 30°C. Activity was measured by the diameter of the zone of inhibition caused by the pheromone. One unit of *a*-factor activity was defined as the amount of pheromone required to cause a zone of inhibition 1 cm in diameter.

ß-Galactosidase Assays

ß-galactosidase assays were done by a modification of the assay described by Hagen and Sprague (16). The yeast strain used was YY69 (MATα leu2-3 leu2-112 his4 gal2 can1) containing the plasmid pSL35 and was a gift of George Sprague (University of Oregon). The plasmid pSL35 contains LEU2 as a selectable marker and a STE3-LacZ reporter construct. The construct consisted of the 5' sequences of STE3 and the entire LacZ gene. ß-galactosidase activities were calculated using the equation:

$$\text{Activity} = (212.6)\frac{(OD_{420})(OD_{550})}{t(OD_{600})}$$

Where OD_{420} measures *o*-nitrophenol and light scattering, OD_{550} accounts for light scattering, and cell density is determined by OD_{600}, t is the incubation time with the substrate, and 212.6 corrects for dilutions.

Analysis of Cell Growth and Arrest

Cells were fixed in 3.7% formaldehyde/0.15M NaCl solution and turbidity measurements taken 550nm at appropriate times. Cell cycle arrest was determined by scoring cells as budded or unbudded (600 cells per sample).

Results

Prepared *a*-factor has bioactivity

The STE3-LacZ reporter gene was used to test the ability of prepared *a*-factor to increase the transcription from a pheromone responsive promoter. *a*-Factor caused a large increase in the levels of ß-galactosidase activity produced from the STE3-LacZ construct. The relative activity of ß-galactosidase in the treated flask reached a maximum of 7.1 times over control after 90 minutes

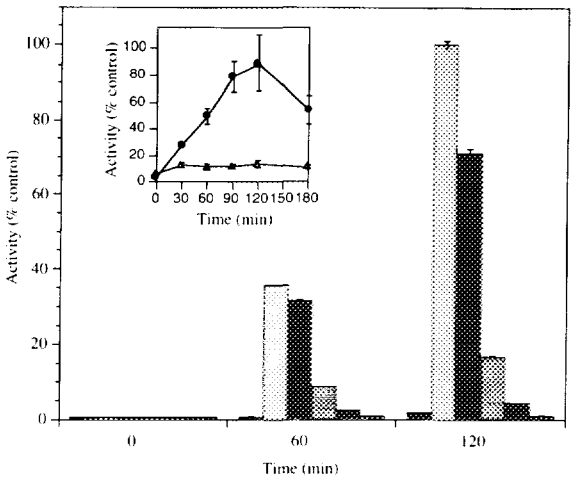

Figure 1. The effect of *a*-factor and LiCl on the production of ß-galactosidase from STE3-LacZ. ß-Galactosidase Assays were performed as described in Materials and Methods. Cells were treated with *a*-factor alone or in combination with LiCl. Errors bars denote the standard of the mean of three replicate samples. YY69 cells (1 x 10^7 cells/ml) were treated with 20µl methanol (▓) (control), 20µl *a*-factor (1 U/ml)(▒), or *a*-factor (1 U/ml) and 15mM LiCl (▓), 30mM LiCl (▒), 45 mM LiCl (▓), or 60 mM LiCl (▒). Data shown are one trial of three separate experiments that gave similar results. (**Inset**) The effect of *a*-factor on the production of ß-galactosidase from STE3-LacZ. YY69 cells were grown to a density of 1 x 10^7 cells/ml and treated with 1 U/ml of *a*-factor (in methanol)(●) or an equal volume of methanol (▲). Shown is one trial of three separate experiments that gave similar results. Error bars represent the standard error between three replicate samples.

(figure 1, inset). The activity in the untreated sample increased approximately two-fold throughout the experiment, a trend present in the majority of experiments (see discussion).

Lithium chloride affects transcription of STE3-LacZ

The addition of LiCl caused a reduction in the levels of ß-galactosidase in a concentration dependent manner (figure 1). At a concentration of 15mM, LiCl caused a 30% reduction in activity after 2 hours (Student's t-test, $p<0.02$), while 30mM LiCl lead to an 83% reduction ($p<0.001$). Very low levels of activity were observed when the concentration of LiCl was 45 or 60mM. Each doubling of the Li^+ concentration caused approximately a 4 fold reduction in the observed levels of ß-galactosidase activity. Treatment with Li^+ without *a*-factor did not affect ß-galactosidase activity (data not shown).

In order to eliminate the possibility of non-specific effects of the Cl^- ion, the experiments were repeated with NaCl. In contrast to LiCl, NaCl did not affect the levels of ß-galactosidase activity. There was no significant difference in the activity between treatments with *a*-factor alone and with both *a*-factor and 20mM NaCl (figure 2). The addition of NaCl without *a*-factor did not have any significant effect on the measured amount of ß-galactosidase activity (figure 2A, $p>0.5$). Two further experiments were designed to further establish the role of lithium as the causal agent of the observed effects on the STE3-LacZ construct. In the first experiment lithium acetate was

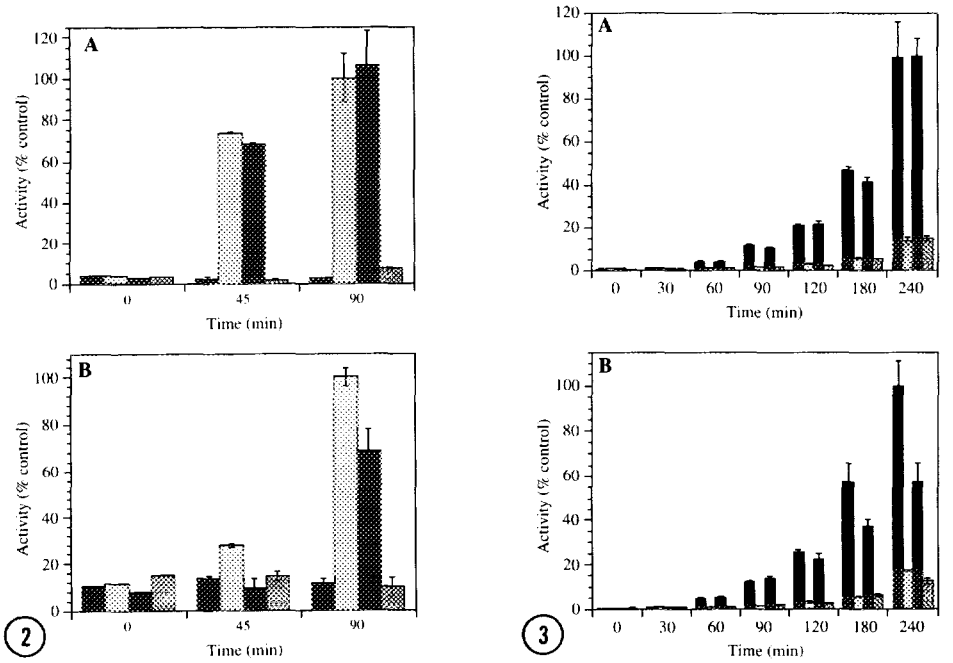

Figure 2. **(A)** The effect of *a*-factor and NaCl on the level of ß-galactosidase activity in YY69. Cells (1 x 10^7 cells/ml) were incubated with methanol as a control (▓), 1 U/ml *a*-factor (▒), 1 U/ml *a*-factor and 20mM NaCl (▓), or 20 mM NaCl alone (▒). ß-Galactosidase assays were performed as described in Materials and Methods and error bars represent the standard error of the mean between three replicate samples. Data shown are one trial of three separate experiments that gave similar results. **(B)** The effect of *a*-factor and lithium acetate on the level of ß-galactosidase activity in YY69. Cells (1 x 10^7 cells/ml) were incubated with methanol (▓), 1 U/ml *a*-factor (▒), 1 U/ml *a*-factor and 20mM lithium acetate (▓), or 20 mM lithium acetate alone (▒). ß-Galactosidase assays were performed as described and error bars represent the standard error between three replicate samples. Data shown are one trial of three separate experiments that gave similar results.

Figure 3. **(A)** Time dependency of the reduction of ß-galactosidase activity in the presence of LiCl and *a*-factor. ß-Galactosidase assays were performed as described except that LiCl was not added until just after the 60-minute sample was removed. Error bars represent the standard error between three replicate samples. YY69 cells (1 x 10^7 cells/ml) were treated with 1 U/ml *a*-factor (▓), 20mM LiCl (▒), 1 U/ml *a*-factor and 20mM LiCl (▓), or methanol (▒). Data shown are one trial of three separate experiments that gave similar results. **(B)** The effect of NaCl 60 minutes after exposure to *a*-factor. ß-Galactosidase assays were performed as described except that NaCl was not added to until just after the 60-minute sample was removed. Error bars represent the standard error of the mean of three replicate samples. YY69 cells (1 x 10^7 cells/ml) were treated with 1 U/ml *a*-factor (▓), 20mM NaCl (▒), 1 U/ml *a*-factor and 20mM NaCl (▓), or methanol (▒). Data shown are one trial of three separate experiments that gave similar results.

used in place of LiCl (see Figure 2B). The affect of lithium acetate was a 30% reduction in activity, an affect similar to that of LiCl. To determine if Li^+ inhibited ß-galactosidase directly, another set of experiments were done. At time=0, *a*-factor was added as usual, however LiCl (figure 3A) or NaCl (figure 3B) were not added to the culture until time=1 hour. The loss of activity was observed with LiCl but the decrease was not significant (35.3%; $p<0.05$) until the 3 hour time point, which is one hour after the affect is usually significant (figure 1). By the fourth hour the difference was 42.5% ($p<0.02$). NaCl had no effect on the ß-galactosidase activity in an identical experiment ($p>0.5$) (figure 3B).

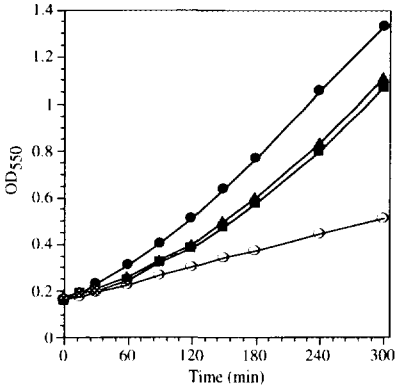

Figure 4. Representative experiment on the effects of salts on the growth of DC6. Turbidity measurements were taken at 550nm. The cultures contained sddH_2O as a control (●), 20mM LiCl (○), 20mM NaCl (▲), 20mM KCl (■). Data shown are one trial of three separate experiments that gave similar results.

Lithium and growth of *S. cerevisiae*

Growth curves were generated for DC6 in the presence of 20mM NaCl, KCl, LiCl, or no salts (figure 4). The addition of NaCl or KCl to the media had little effect on the growth over a five hour period. The average growth rates were also similar to the control (NaCl=0.372, KCl=0.382, control=0.348 odu.hr^{-1}). The salt that caused a large difference in the growth pattern was LiCl. As shown in figure 4, LiCl caused DC6 to grow 35% slower than the control (LiCl=0.229 odu.hr^{-1}).

Lithium and the cell cycle

To determine if Li^+ could affect the cell cycle of yeast, control cells and cells treated with 50mM LiCl were scored as budded or unbudded (table 1). The proportion of unbudded cells in the

Table 1: Cell cycle arrest by lithium chloride. Logarithmically growing DC6 cells at a density of 1 x 10^7 cells/ml were treated with 50mM LiCl. 1 ml samples were periodically removed, fixed, and scored as budded or unbudded. The data shown are the percentage of unbudded cells.

Time (hours)	No Treatment	50 mM LiCl	Increase over Control
0	37%	36%	-
1	23%	49%	2.3 X
2	39%	80%	2.0 X
3	38%	86%	2.3 X
4	54%	76%	1.4 X

untreated population averaged 37%. Throughout the 4 hours of observation this proportion remained relatively stable. Cells incubated with 50mM LiCl showed an increase in the proportion of unbudded cells. After 1 hour with 50mM LiCl the fraction of unbudded cells was 49%, which was 2.1 times higher than in the control. This proportion rose to 80% by 2 hours and 86% by three hours, 2.0 and 2.3 times more than the control values respectively. The fourth hour showed 76% unbudded cells, a slight decrease compared to previous time points, but still 1.4 times over that of the control.

Discussion

a-Factor was partially purified and tested for bioactivity using a STE3-LacZ reporter construct. It was shown that prepared *a*-factor increased the amount of ß-galactosidase activity to a maximum of 7.1 times over control after 120 minutes, levels similar to those reported by Proteau *et al.* (15). A low basal level of ß-galactosidase activity was detected in all assays, due to the "leaky" nature of the STE3 promoter (3). Thus *a*-Factor caused an increase in the level of ß-galactosidase in cells carrying the STE3-LacZ reporter gene presumably through the normal induction of the mating response. In control experiments, the timing of the induction of ß-galactosidase activity was essentially identical to that of Hagen and Sprague (16).

Lithium is an inhibitor of inositol 1-phosphatase, which catalyses the conversion of inositol 1-phosphate to inositol (17). All the pathways of recycling IP_3 (and other inositol metabolites) converge at inositol 1-phosphate, making this enzyme an extremely important for the replenishment of PIP_2 in the cell membrane. When inositol 1-phosphatase is inhibited by lithium, then the amount of PIP_2 available for signalling should decrease. This is the basis of the "inositol depletion" hypothesis currently thought to explain the observed effects of Li^+ in humans (18). If lithium affects an early response to pheromone in yeast (transcription of STE3-LacZ), then it may be that inositol metabolism is involved in the control or completion of that event.

When LiCl or lithium acetate was added to cultures of cells carrying the STE3-LacZ reporter, the levels of measured ß-galactosidase activity decreased significantly. In order to determine the effects, if any, of the negative ion on ß-galactosidase activity, the experiments were repeated with NaCl, which showed no effect. The possibility that Li^+ inhibited ß-galactosidase directly was eliminated by experiments in which the addition of Li^+ was delayed until some ß-galactosidase activity was already present. There was no immediate affect on activity, revealing that Li^+ is not an inhibitor of ß-galactosidase.

At a point of the G1 phase of the cell cycle called "START", the cell may proceed along one of two routes (19). If culture conditions are poor the cell may arrest at this point. Alternatively, the cell may commit to another completion of the cell cycle, including DNA replication and mitosis, resulting in the formation of a bud (reviewed in 20). PIP_2 may play an important role in this "decision" at START (8).

If PIP_2 is required for the progression of the cell cycle and Li^+ can reduce the amount of PIP_2 available for signalling, then Li^+ should cause two things to occur. First, the average growth rate should decrease in the presence of Li^+, and second, the cells should arrest in G_1. Lithium was

shown to cause a 35% reduction in the average growth rate of DC6 cells. The ratio of budded to unbudded cells in a population is a good indicator of the proportion of cells in G1, as cells in this stage do not posses a bud (20). The proportion of unbudded DC6 cells increased from approximately 40% to 85% in the presence of 50mM Li+, indicating G1 arrest. Therefore, PIP_2 may be required for the normal progression through the cell cycle. Lithium can be used for DNA transformations of yeast and it is therefore presumed to have an effect on the permeability of yeast plasma membranes (21). However, these data suggest that, in this case, Li+ has a specific affect on inositol metabolism rather than a general permeability effect.

In this work we have demonstrated the importance of inositol phosphates in both the mating response and the cell cycle of yeast. Recent experiments demonstrated that overexpression of phosphatidylinositol 4-kinase, an enzyme involved in inositol metabolism, led to an enhanced sensitivity to mating pheromone (14). Other experiments revealed that a phosphorylation cascade is activated by the mating pheromones (reviewed in 22). The complete response may involve both phosphorylation and inositol signalling pathways to accomplish cell cycle arrest and mating-specific gene expression.

References

1. Nakayama N., Miyajima A., Arai K. (1985) *EMBO. J.* **4**, 2643-2648.
2. Burkholder, A.C. and Hartwell, L.H. (1985) *Nuc. Acid Res.* **13**, 8463-8474.
3. Hagen D.C., McCaffrey G., and Sprauge Jr. G.F. (1986) *Proc. Nat. Acad. Sci. USA* **83**, 1418-1422.
4. Dohlman H.G., Thorner J., Caron M.G., and Lefkowitz R.J. (1991) *Ann. Rev. Biochem.* **60**, 653-688.
5. Deckmyn H., Whiteley B.J., and Majerus P.W. (1990) *G Proteins* (R.C. Bruch, ed.), Academic Press, New York, 429-452.
6. Berridge M.J., and Irvine R.F. (1984) *Nature* **312**, 315-321.
7. Dudani A.K., Trivedi A., and Prasad R. (1983) *FEBS Letts.* **153**, 34-36.
8. Uno I., Fukami K., Kato H., Takenawa T., and Ishikawa T. (1988) *Nature* **333**, 188-190.
9. Uno I., and Ishikawa T. (1990) *Calcium as an intracellular messenger in eucaryotic microbes* (D.H. O'Day, ed.), American Society for Microbiology, Washington, 52-64.
10. Homann M.J., and Carman G.M. (1983) *Anal. Biochem.* **135**, 447-452.
11. Nikawa J.I., Kodaki T., and Yamashita S. (1987) *J. Biol. Chem.* **262**, 4876-4881.
12. Dean-Johnson M., and Henry S.A. (1989) *J. Biol. Chem.* **264**, 1274-1283.
13. Auger K.R., Carpenter C.L., Cantley L.C., and Varticovski L. (1989) *J. Biol. Chem.* **264**, 20181-20184.
14. Flanagan C.A., Schnieders E.A., Emerick A.W., Kunisawa R., Admon A., and Thorner J. (1993) *Science* **262**, 1444-1448.
15. Proteau G., Gelaznikas R., and Merante F. (1990) *Biochem. Biophys. Res. Comm.* **170**, 182-186.
16. Hagen D.C., Sprague G.F. (1984) *J. Mol. Bio.* **178**, 835-852.
17. Gee N.S., Ragan C.I., Watling K.J., Aspley S., Jackson R.G., Reid G.G., Gani D., and Shute J.K. (1988) *Biochem. J.* **249**, 883-889.
18. Berridge M.J., Downes C.P., and Hanley M.R. (1989) *Cell* **59**, 411-419.
19. Hartwell L.H. (1980) *J. Cell Biol.* **85**, 811-822.
20. Herskowitz I. (1988) *Microbiol. Rev.* **52**, 536-53.
21. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153**, 163-168.
22. Errede, B., and Levin D.E. (1993) *Curr. Opin. Cell Biol.* **5**, 254-260.