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Loss of a prolyl oligopeptidase confers resistance to lithium by elevation of inositol (1,4,5) trisphosphate

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The therapeutic properties of lithium ions (Li^+) are well known; however, the mechanism of their action remains unclear. To investigate this problem, we have isolated Li^+-resistant mutants from *Dictyostelium*. Here, we describe the analysis of one of these mutants. This mutant lacks the *Dictyostelium* prolyl oligopeptidase gene (*dpoA*). We have examined the relationship between *dpoA* and the two major biological targets of lithium: glycogen synthase kinase 3 (GSK-3) and signal transduction via inositol (1,4,5) trisphosphate (IP_3). We find no evidence for an interaction with GSK-3, but instead find that loss of *dpoA* causes an increased concentration of IP_3. The same increase in IP_3 is induced in wild-type cells by a prolyl oligopeptidase (POase) inhibitor. IP_3 concentrations increase via an unconventional mechanism that involves enhanced dephosphorylation of inositol (1,3,4,5,6) pentakisphosphate. Loss of DpoA activity therefore counteracts the reduction in IP_3 concentration caused by Li^+ treatment. Abnormal POase activity is associated with both unipolar and bipolar depression; however, the function of POase in these conditions is unclear. Our results offer a novel mechanism that links POase activity to IP_3 signalling and provides further clues for the action of Li^+ in the treatment of depression.

Keywords: clinical depression/*Dictyostelium discoideum*/inositol triphosphate signalling/lithium sensitivity/prolyl oligopeptidase

Introduction

Lithium is a major treatment for clinical depression and is taken by 1% of the population. It is the standard treatment for bipolar depression and is also effective against recurrent unipolar depression. It can be used in conjunction with antidepressants (Heit and Nemeroff, 1998) or as a temporary mood stabilizer between treatments (Lenox *et al*., 1998). Li^+, however, has a small therapeutic index and a number of hazardous side effects. Overdose can lead to unconsciousness and death (Bowden, 1998). The molecular basis of these disorders and their treatment by Li^+ remains unknown. A strongly supported theory was proposed by Berridge *et al*. (1989). In their inositol depletion theory, Li^+ inhibits inositol monophosphatase (IMPase) and blocks the conversion of IMP to inositol. This causes a reduction of the free pool of inositol and ultimately lowers the cellular concentration of phosphatidyl (4,5) bisphosphate (PIP_2). In addition, Li^+ inhibits the enzyme inositol polyphosphatase (IPP) which is required for the dephosphorylation of inositol (1,4) bisphosphate to IMP (Gee *et al*., 1988; Inhorn and Majerus, 1988). In response to receptor stimulation, PIP_2 is cleaved by phospholipase C (PLC) to form the second messengers inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG). These molecules cause the release of Ca^{2+} from intracellular stores and activation of protein kinase C (PKC), respectively (Nishizuka, 1992). Li^+ depletion of PIP_2 therefore has the potential to cause a significant effect on signal transduction.

Although there is little doubt that Li^+ inhibits IMPase in brain slices (Kennedy *et al*., 1990; Hokin, 1993), it has not been possible to link changes of phosphoinositide signalling directly to depression. Recently, Li^+ has been shown to inhibit glycogen synthase kinase 3 (GSK-3) and this can account for many of its teratogenic properties (Klein and Melton, 1996). Members of the GSK-3 family have been found in all eukaryotic groups where they mediate a wide range of cellular processes, including intermediate metabolism, gene expression and cytoskeletal integrity (Plyte *et al*., 1992). In metazoa, they act within the Wnt-1 signal transduction pathway which is required for pattern formation during development. GSK-3 is abundant in brain tissue (Woodgett, 1990) and causes hyperphosphorylation of tau protein in patients with Alzheimer's disease (Mandelkow *et al*., 1995). GSK-3β is also required for cytoskeletal organization during development of cerebella granule neurons (Lucas *et al*., 1998). Inhibition of GSK-3 therefore presents an alternative mechanism for the treatment of depression and could certainly explain a number of Li^+ side effects.

Dictyostelium development is sensitive to Li^+ treatment. *Dictyostelium* cells grow as unicellular amoebae, but when starved enter a developmental programme. This consists of two phases: aggregation, in which amoebae form a cellular mass called the mound, and multicellular development, where the processes of differentiation and morphogenesis transform the mound to a fruiting body. This structure comprises three elements: the spore head, the stalk which supports the spore head and the basal disc into which the stalk is embedded. In addition to these three cell types, cells of the lower and upper cup form a cradle to suspend the spore head (Williams and Jermyn, 1991). Maeda (1970) showed that treatment with 7 mM LiCl causes the formation of a mis-proportioned fruiting body, in which the spore head is almost completely lost and the base is expanded into a mound of stalk cells. This morphology is likely to arise by inhibition of GSK-3, as it is also seen in a mutant which lacks *gskA*, the *Dictyostelium* GSK-3 homologue (Harwood *et al*., 1995), and arises due

to mis-specification of the pre-spore and pre-stalk B cell types (Williams *et al*., 1989). Treatment of wild-type cells with higher concentrations of LiCl blocks aggregation. This phenotype is not associated with loss of *gskA* and suggests that other targets of Li^+ are important for development (Maeda, 1970).

To investigate the molecular basis of Li^+ action, we have generated Li^+-resistant mutants in *Dictyostelium*. Analysis of these mutants offers a unique opportunity to dissect the effects of Li^+ on GSK-3 and IP_3 signalling. Here we describe one of these mutants, in which the *Dictyostelium* prolyl oligopeptidase (*dpoA*) is disrupted. This mutant has elevated IP_3 concentrations and consequently escapes Li^+ inhibition of IMPase. The interaction revealed in these studies relates directly to observations made on patients with depression, and suggests a mechanism that could explain both cause and treatment of depression.

Results

Generation of Li^+-resistant mutants

Exposure to 10 mM LiCl severely retards aggregation of *Dictyostelium* cells (Figure 1A and B). In most cases, however, aggregation is not totally blocked and will occur after a prolonged period of time. The effect of Li^+ is reversible and cells will form a wild-type fruiting body when Li^+ is removed (Figure 1C). Cells are sensitive to Li^+ throughout development, suggesting that Li^+ inhibits processes which are required at all developmental stages. Li^+ is also effective on cells grown in association with bacteria. Clonally plated cells multiply on a bacterial lawn to form a plaque where the cells in the centre starve and develop. When plated in these conditions in the presence of 10 mM LiCl, wild-type cells grow but aggregate poorly and form plaques with few developed structures. The ability to develop in these conditions was used in a genetic screen to isolate mutants resistant to the effects of Li^+.

From a screen of 30 000 mutagenized cells, 13 mutants were isolated that could aggregate well in 10 mM LiCl. Eleven of these mutants showed morphological defects during later developmental stages in the presence of LiCl, but in its absence all but two could develop fruiting bodies with a wild-type morphology. Here we describe our analysis of one of the genes identified from this screen, denoted as *dpoA*. The *dpoA* mutant aggregates in 10 mM LiCl but forms abnormal fruiting bodies (Figure 1D–G). Treatment with 10 mM NaCl has no effect on the development of either wild-type or the *dpoA* mutant cells (Figure 1A, D and F). Aggregation of the *dpoA* mutant, however, is retarded at higher Li^+ concentrations, suggesting that it has reduced sensitivity rather than complete resistance to Li^+ (Figure 1H and I). Mixing wild-type and *dpoA* mutant cells in a ratio of 1:10 does not restore Li^+ sensitivity to *dpoA* mutant cells nor does it confer Li^+ resistance to wild-type cells (Figure 1J, K and L).

To confirm the effects of Li^+ on early development, the expression of the gene for contact site A (csA) was examined (Figure 2A). This gene is expressed during aggregation in response to pulses of extracellular cAMP (Siu *et al*., 1988). CsA protein was expressed in wild-type cells after 6 h of development in shaking culture. Addition of 10 mM LiCl completely inhibits csA expression. Expression is not rescued by addition of cAMP

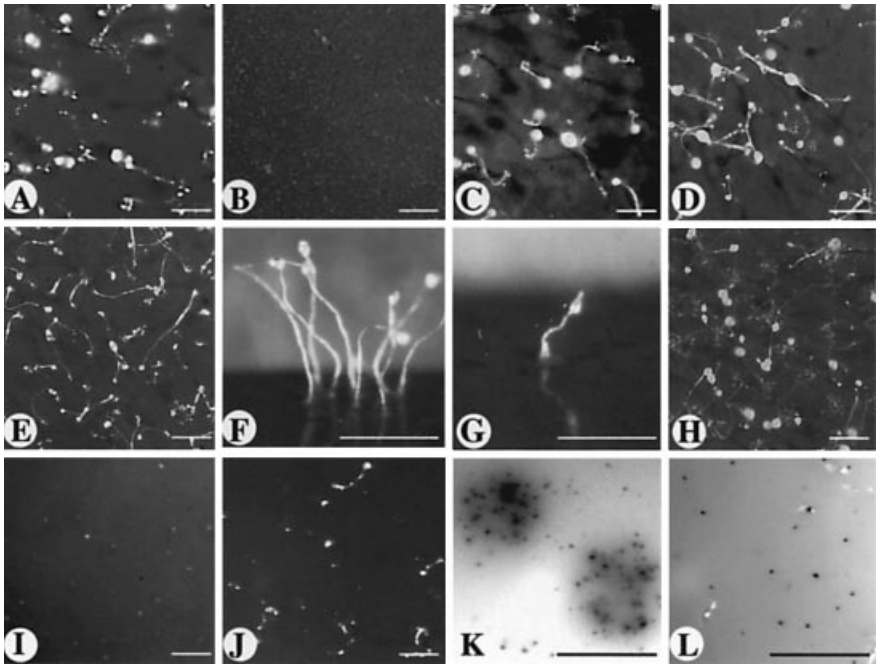


Fig. 1. Comparison of lithium sensitivity of wild-type and *dpoA* mutants. Cells (1×10^7) were plated in KK_2 buffer onto nitrocellulose membranes and developed for 40 h. Aggregation in wild-type *Dictyostelium* is not affected by 10 mM NaCl (**A**), but is impeded by 10 mM LiCl (**B**). Cells treated with 10 mM LiCl for an initial 6 h of development aggregate normally when lithium is removed (**C**). *dpoA* mutant cells aggregate and form fruiting bodies in either 10 mM NaCl (**D** and **F**) or 10 mM LiCl (**E** and **G**). Lithium-treated *dpoA* mutant cells, however, have an enlarged basal disc and reduced spore head as also seen with a *gskA* mutant (G). Aggregation of *dpoA* mutant cells is not affected by 20 mM NaCl (**H**), but is sensitive to 20 mM LiCl (**I**). Mixing wild-type and *dpoA* cells (1:10 respectively) does not induce loss of lithium resistance in the *dpoA* mutant cells (**J**). Mixtures of wild-type cells expressing β-galactosidase (stained) and *dpoA* cells (1:10 respectively) aggregate normally in 10 mM NaCl (**K**), and wild-type cells are not included in fruiting bodies in the presence of 10 mM LiCl (**L**) at 16 h. Bar = 0.5 mm.

pulses (Figure 2B), indicating that Li^+ blocks the cellular response to cAMP. The *dpoA* mutant expresses csA in the presence of 10 mM LiCl. The *dpoA* mutant also expresses csA during growth, suggesting that the mutant may initiate development prematurely. *dpoA* mutant cells grow on bacteria or when plated in medium on tissue culture plates; however, the same cells are unable to sustain prolonged growth in suspension culture (data not shown). The basis of the effect on cell growth is not known.

dpoA encodes a prolyl oligopeptidase

The original *dpoA* mutant was generated by insertional mutagenesis using REMI (Kuspa and Loomis, 1992), and a genomic fragment of the *dpoA* gene was cloned by plasmid rescue. The rescued plasmid was used to disrupt the *dpoA* gene of wild-type cells to confirm that its loss results in Li^+ resistance. The recreated *dpoA* mutant strains had exactly the same phenotype as the original strain and were used in all subsequent experiments. Both the genomic fragment and its corresponding cDNA were sequenced and found to encode a prolyl oligopeptidase enzyme (POase; Figure 3A and B). The name *dpoA* derives from *Dictyostelium* prolyl oligopeptidase. The *dpoA* cDNA contains an open reading frame of 760 amino acids which shows 41% identity to its human homologue (Shirasawa *et al*., 1994; Vanhoof *et al*., 1994). The highest homology occurs within the C-terminal third of the gene, and this region contains the amino acid triad (Ser–Asp–His) that is essential for enzymatic activity (Figure 3C; Goossens *et al*., 1995). The gene contains a single intron of 82 bp. The *dpoA* mutant results from a plasmid insertion into the 5′ region of the coding sequence and is likely to cause a complete loss of DpoA protein (Figure 3B). The human POase enzyme cleaves neuropeptides, some of which may be involved in the pathophysiology of depression (Cunningham and O'Connor, 1997). This function and the recent report that abnormal POase activity is a state marker for both unipolar and bipolar depression (Maes *et al*., 1995) prompted a detailed analysis of the *dpoA* gene.

DpoA activity was examined in wild-type and *dpoA* mutant cell extracts. Z-Gly-Pro-pNA (ZGPpNA) is a specific substrate of POases and is cleaved in wild-type extracts (Figure 4A). The K_m of this reaction compares well with that measured for other POase enzymes (Table I). POase activity is completely absent in the *dpoA* mutant (Figure 4A), suggesting that DpoA is the only POase in *Dictyostelium* during growth and development. POase enzymes are serine hydrolases and consequently are inhibited by chymostatin through a non-competitive mechanism. The same non-competitive inhibition is observed when DpoA is incubated with chymostatin ($K_i = 12.0 \pm 2.3$ μg/ml). BOC-Glu(NHO-Bz)-Pyr is a competitive inhibitor of the proline-specific endopeptidase enzyme group including POase. DpoA is competitively inhibited by BOC-Glu(NHO-Bz)-Pyr with a K_i comparable with that of mammalian POase (Table I). Finally, we tested DpoA against the inhibitors Z-Pro-L-prolinal dimethyacetal and the peptide H-H-L-P-P-P-V-OH, which specifically target POase. Both of these inhibit DpoA activity (Table I). These results indicate that the biochemical properties of DpoA are typical of the POase enzyme family.

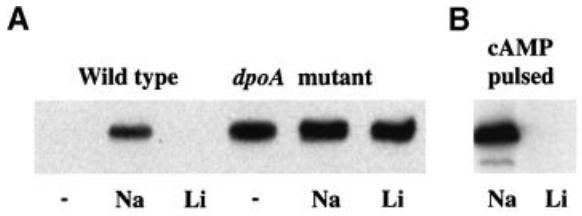


Fig. 2. Lithium inhibits csA expression in wild-type but not the *dpoA* mutant. Western blot with anti-csA monoclonal antibody against *Dictyostelium* cell extracts. (**A**) Wild-type and *dpoA* whole cell extracts from growing cells (–) and cells starved for 6 h with 10 mM NaCl or 10 mM LiCl. (**B**) Wild-type whole-cell extracts from cells developed for 6 h in the presence of cAMP (300 nM pulses, every 6 min) with 10 mM NaCl or 10 mM LiCl.

Localization of DpoA activity

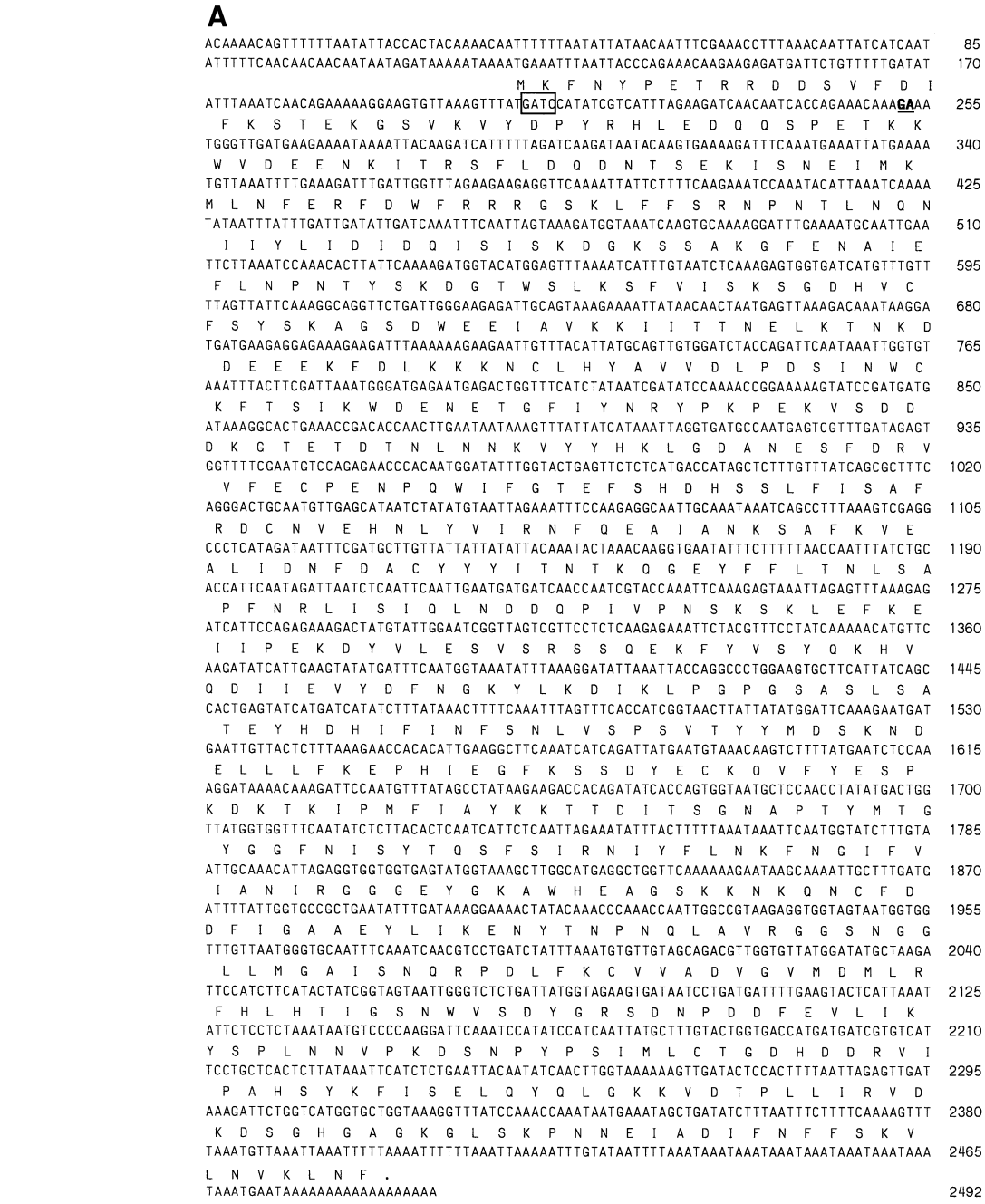
To examine whether DpoA is secreted, we developed cells in culture and assayed both cells and medium for DpoA activity. To assess the degree of enzyme leakage from within the cell, the experiment was carried out using cells transformed with the *Escherichia coli LacZ* gene, expressed from the actin 15 promoter. This allowed a comparison of the cytoplasmically expressed β-galactosidase activity with that of DpoA. The activity of both enzymes in the medium was ~1% of the total (Figure 4B). This strongly suggests that DpoA activity is intracellular and that the small amount of extracellular activity is due to leakage from within cells plated under these conditions. The intracellular localization of DpoA activity is consistent with the cell-autonomous nature of the *dpoA* mutant observed in mixing experiments.

To investigate the subcellular localization of DpoA, cell extracts were made by filter lysis and differential centrifugation. DpoA activity was present in the supernatant following centrifugation at 100 000 *g* (Figure 4C), with only a trace activity (<0.1%) present in the low speed particulate fraction (P10 fraction). Very little cross-contamination was seen between membrane and cytosolic fractions. A similar cytoplasmic localization has been reported for POase enzymes of other species, including the human homologue (Kato *et al*., 1980). Consistent with a cytosolic localization, no signal peptide sequence is present in DpoA or any other POase enzyme.

DpoA is not a Li^+ target

To establish a mechanism for Li^+ resistance in the *dpoA* mutant, we examined the effect of Li^+ on DpoA. Activity was tested in the presence of up to 30 mM LiCl and NaCl, but no effect on enzyme activity was observed in either salt (Figure 5A). This suggests that Li^+ neither directly

Fig. 3. *dpoA* encodes a prolyl oligopeptidase gene (EC 3.4.21.26). (**A**) Sequence of *dpoA* gene cDNA and the predicted protein product. The corresponding genomic sequence is interrupted by a single, 82 bp intron (site underlined). The *dpoA* mutant arises through a plasmid insertion into a site (boxed) at the N-terminus of the predicted product. (**B**) Schematic diagram of the *dpoA* gene showing the two exons (boxed) and the REMI insertion site (arrow). The restriction enzyme sites *Dpn*II (●) and *Cla*I are shown. (**C**) Alignment of the conserved peptidase domain of the *Dictyostelium* (top, amino acids 454–760) and human POase (bottom, amino acids 400–710), showing identical residues (boxed), and the conserved amino acids of the catalytic triad (shaded).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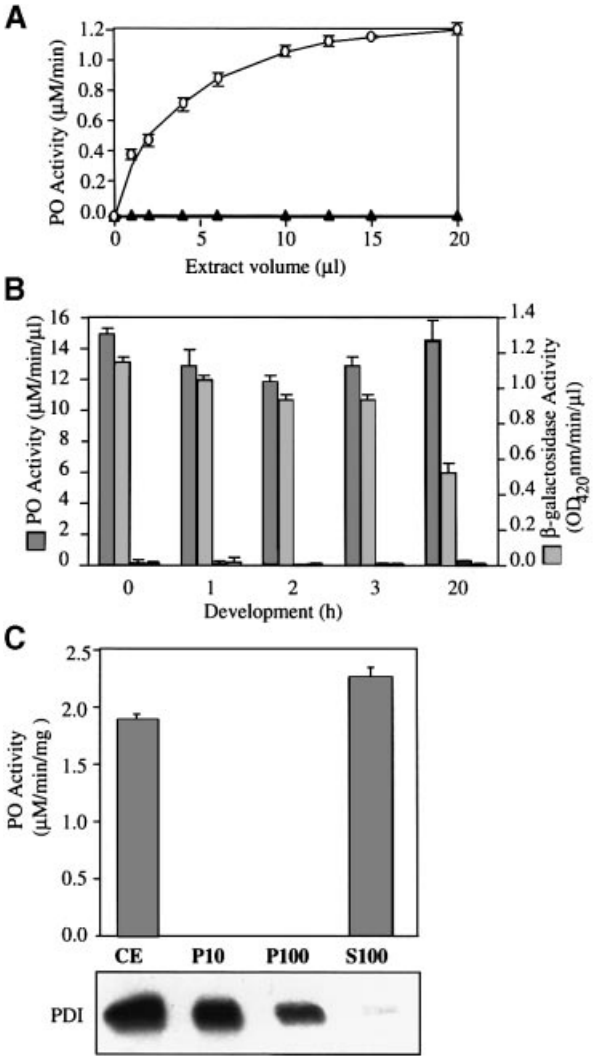

Fig. 4. POase activity is absent in *dpoA* mutant, and present in the cytosol of wild-type cells. (**A**) Extracts from growing cells of wild-type (○) or *dpoA* mutant (▲) cells assayed for POase activity with increasing cell equivalents. (**B**) *Actin15:LacZ*-transformed wild-type cells were incubated in KK_2 medium. Medium and cells were separated and assayed for DpoA activity and β-galactosidase activity. (**C**) Wild-type cells were lysed and fractionated to enrich for vacuole (P10), cell structural components (P100) and cytoplasmic proteins (S100). The total cell extract (CE) and each fraction was assayed for DpoA activity. To estimate the degree of separation, each sample was examined by Western blotting for cross-contamination with the endoplasmic reticulum-specific protein, protein disulfide isomerase (PDI; Monnat *et al*., 1997).

activates nor inhibits DpoA. The effect of Li^+ treatment on intact cells was also examined. Cells in shaking culture were treated with Li^+ for 6 h and then extracts were tested for DpoA activity. No difference was seen in the level of activity in treated or non-treated samples (Figure 5B). As expected, no DpoA activity was seen in the *dpoA* mutant. We also observe no change of DpoA activity in wild-type cells after 6 h of development. These conditions are sufficient to induce csA expression, and we conclude, therefore, that DpoA activity is not regulated during early development. Our observations demonstrate that DpoA is not a direct target of Li^+ and exclude the possibility that Li^+ causes an increase of DpoA activity which then blocks development.

dpoA does not interact with gskA

GskA is inhibited by Li^+ (Ryves *et al*., 1998); however, the biological effects of Li^+ treatment on development are more severe than can be explained by a simple loss of GskA activity (Figure 6A). In particular, *gskA* mutant cells can aggregate in the absence of Li^+ (Harwood *et al*., 1995) but, as seen with wild-type cells, are severely retarded in its presence (Figure 6B). As GskA protein is totally absent from the *gskA* mutant in this case, it cannot be the Li^+ target. Introduction of the *dpoA* mutation into the *gskA* background confers Li^+ resistance on the double mutant (Figure 6C and D). The fruiting body of the *dpoA,gskA* double mutant, however, retains the characteristic morphology of a small spore head and enlarged basal disc seen in a *gskA* mutant. This suggests that the *dpoA* mutation does not suppress the *gskA* mutant phenotype. Similarly, when the *dpoA* mutant is developed in the presence of 10 mM LiCl, it also has a smaller spore head and enlarged basal disc, suggesting that *gskA* is still a Li^+ target in these cells (Figure 1G).

To verify these conclusions, we examined stalk cell differentiation in low density monolayer culture. Stalk cell differentiation is repressed by the presence of extracellular cAMP through a signal transduction pathway which requires *gskA* (Harwood *et al*., 1995). The number of stalk cells formed in the presence of cAMP is therefore a sensitive indication of the activity of GskA and its associated signal transduction pathway. We find that cAMP strongly represses stalk cell differentiation of both wild-type and *dpoA* mutant cells (Figure 6E). No cAMP repression is seen with either *gskA* or double *dpoA,gskA* mutant cells. Addition of low concentrations (2.5 mM) of LiCl reverses the effects of cAMP on both wild-type and *dpoA* mutant cells. As we see no effect of 2.5 mM LiCl on the *gskA* mutant, we conclude that this effect is due to inhibition of GskA activity. The effect of LiCl on wild-type and *dpoA* mutant cells further demonstrates that Li^+ ions enter the cell and target GskA. We can therefore find no evidence for an epistatic interaction between the *dpoA* and *gskA* genes, and all of our observations suggest that they are on different signal transduction pathways.

Loss of dpoA elevates IP_3 concentrations by dephosphorylation of IP_5

We investigated the possibility of an interaction between DpoA and phosphoinositide signalling. Treatment of *Dictyostelium* cells with 10 mM LiCl reduces the IP_3 concentration by 20% (Peters *et al*., 1989). We compared the IP_3 concentration of wild-type and *dpoA* mutant cells during growth and development in shaking culture. The basal IP_3 concentration in *dpoA* mutant is 3-fold higher than in wild-type cells during the growth phase and continues to be higher during development (Figure 7A). The IP_3 concentration of *dpoA* mutant cells is sensitive to Li^+, but is still higher than in the wild-type after Li^+ treatment. To confirm that the reduction of IP_3 concentration was due to inhibition of IMPase activity, wild-type cells were treated with Li^+ in the presence of 10 mM myo-inositol. This restored cellular IP_3 concentrations to untreated levels (Figure 7B). These results suggest that loss of DpoA activity leads to an elevated concentration of IP_3. To test this conclusion, wild-type cells were developed in the presence of the POase-specific inhibitor

Table I. The profile of DpoA activity and inhibition is characteristic of a prolyl oligopeptidase

Substrate	Organism	K_m	Reference
Z-Gly-Pro-pNA	*Dictyostelium* (DpoA)	115 ± 13 μM	
	porcine (brain)	53 μM	Rennex *et al*. (1991)
	human (lung)	360 μM	Zolfaghari *et al*. (1986)
	Treponema denticola	83 μM	Makinen *et al*. (1994)
	ovine (kidney)	100 μM	Yoshimoto *et al*. (1978)
Inhibitor		K_i	
Z-Pro-L-prolinal dimethylacetal	*Dictyostelium* (DpoA)	1.15 ± 0.19 μM	
	human	130 nM (IC_{50})	Goossens *et al*. (1997)
H-H-L-P-P-P-V-OH	*Dictyostelium* (DpoA)	338 ± 91 μM	
	F.meningiosepticum	80 μM	Maruyama *et al*. (1992)
BOC-Glu(NHO-Bz)-Pyr	*Dictyostelium* (DpoA)	99 ± 9.8 nM	
	human	30 nM	Demuth *et al*. (1993)
	porcine	100 nM	Demuth *et al*. (1993)
	bacterial	11.7 μM	Demuth *et al*. (1993)
Chymostatin	*Dictyostelium* (DpoA)	12.0 ± 2.3 μg/ml	

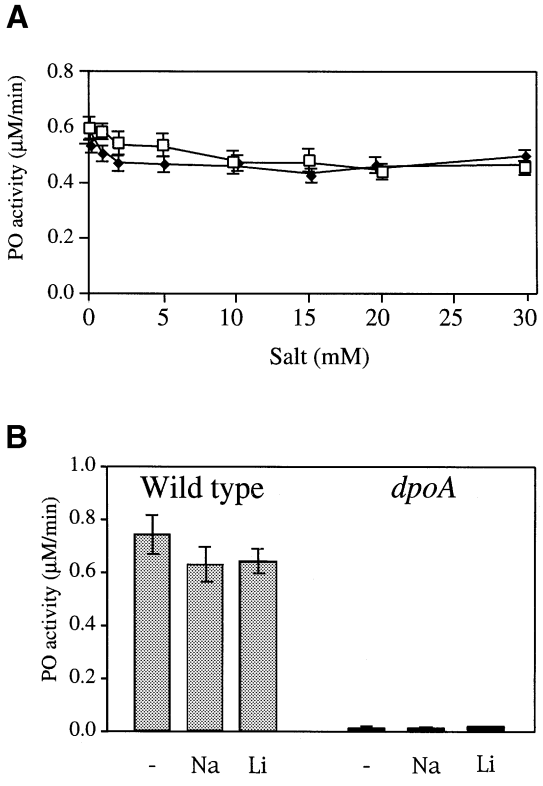

Fig. 5. DpoA is not a direct target of lithium. (**A**) Wild-type cell extract (equivalent to 2.5×10^5 cells per point) was assayed with increasing concentrations of LiCl (□) or NaCl (◆). (**B**) POase activity for wild-type (solid) or *dpoA* mutant (shaded) cell extracts from growing cells and after 6 h development in the presence of 10 mM LiCl or NaCl (equivalent to 5×10^6 cells per point).

Z-Pro-L-prolinal dimethylacetal. This overcame the effect of Li^+ and elevated IP_3 to a concentration higher than ever seen in untreated wild-type cells and matches that seen in the *dpoA* mutant (Figure 7B). We therefore conclude that the cellular IP_3 concentration is linked to DpoA activity and that the *dpoA* mutant is less sensitive to Li^+ treatment as a consequence of its elevated IP_3 concentration. Treatment of wild-type cells with POase inhibitor had no effect on fruiting body formation even at concentrations 1000-fold higher than the K_i (data not shown). This is expected as loss of *dpoA* has no obvious effect on morphogenesis in the absence of LiCl, and indicates that the effects of the POase inhibitor are likely to be specific to DpoA.

To determine the cause of the increased IP_3 concentration in the *dpoA* mutant, we examined synthesis and degradation of IP_3. The breakdown of PIP_2 to IP_3 was examined in membrane fractions prepared from wild-type and *dpoA* mutant cells by the method described by Bominaar and Van Haastert (1994). This method assays the breakdown of endogenous PIP_2 by PLC and hence would be sensitive to changes in both PLC activity and PIP_2 concentration. We could detect no substantial differences in IP_3 synthesis between wild-type and *dpoA* mutant cells (Figure 8A). As PIP_2 hydrolysis is unaffected by the *dpoA* mutation, we examined whether a reduced rate of IP_3 degradation could account for the elevation in cellular IP_3 concentration. Extracts containing IP_3 phosphatase activity were prepared from both wild-type and *dpoA* mutant cells and the rate of breakdown of exogenous IP_3 was measured (Bominaar *et al*., 1991). Rather than having reduced breakdown of IP_3, degradation was increased in the *dpoA* mutant, giving a rate of 1.9 pmol/min/mg of total protein for the *dpoA* mutant compared with 0.7 pmol/min/mg (Figure 8B). Whatever the cause of this increased activity, it cannot account for the elevated IP_3 levels present in the *dpoA* mutant. An alternative source of IP_3 has been reported in both mammalian cells and *Dictyostelium*. In this case, IP_3 can be formed by dephosphorylation of inositol (1,3,4,5,6) pentakisphosphate (IP_5) via inositol (1,4,5,6) and (1,3,4,5) tetrakisphosphate intermediates (Van Dijken *et al*., 1995a,b). This enzyme activity was prepared from wild-type and *dpoA* mutant cells using the method described by Van Dijken and coworkers (1997), and the dephosphorylation of exogenous IP_5 was examined. In a 5 min time period, the *dpoA* mutant was found to synthesize 63% more IP_3 from IP_5 than wild-type cells, with a rate of ~17 pmol/min/mg compared with 10 pmol/min/mg (Figure 8C). We therefore conclude that the elevated concentrations of IP_3 seen in *dpoA* mutant cells are due to increased dephosphorylation of IP_5.

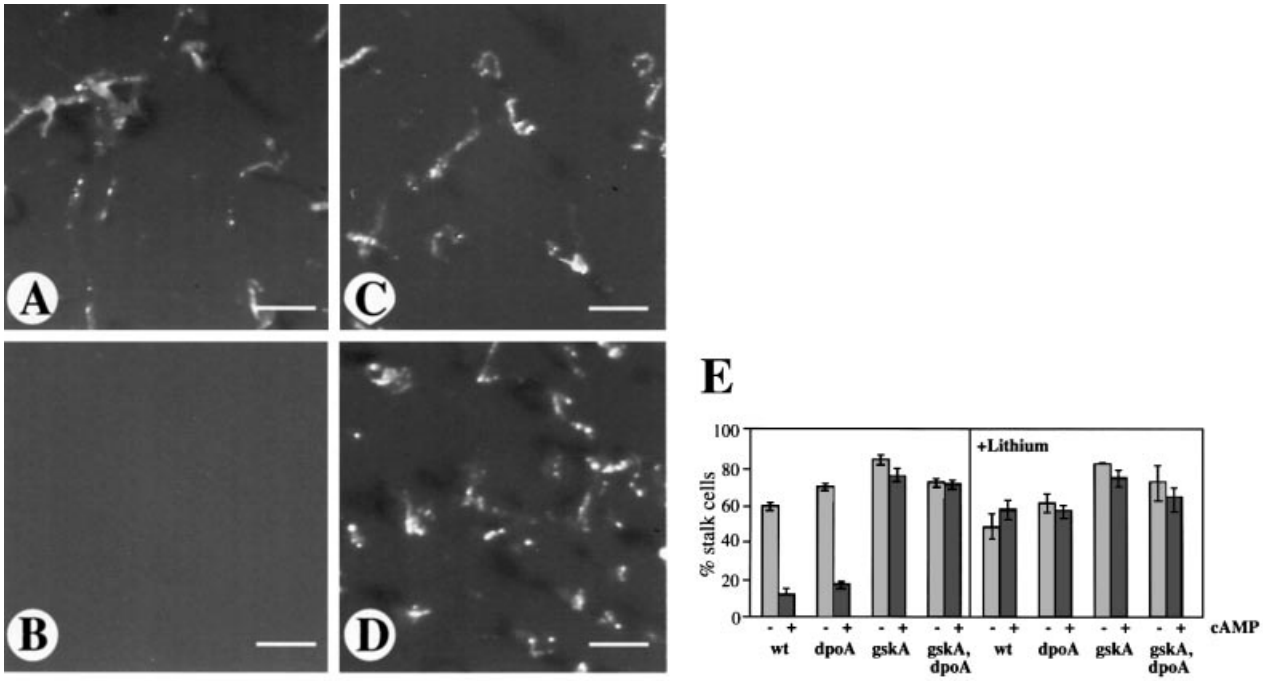

Fig. 6. DpoA does not interact with the GSK-3 signalling pathway. Aggregation of a *gskA* mutant is not affected by 10 mM NaCl (**A**), but is impeded by 10 mM LiCl (**B**). The *dpoA*,*gskA* double mutant aggregates normally in the presence of 10 mM NaCl (**C**) and 10 mM LiCl (**D**). Bar = 0.5 mm. (**E**) Monolayer analysis of wild-type, *dpoA*, *gskA* and *dpoA*,*gskA* cells (Harwood *et al*., 1995). Cells were treated with cAMP or 2.5 mM LiCl as indicated.

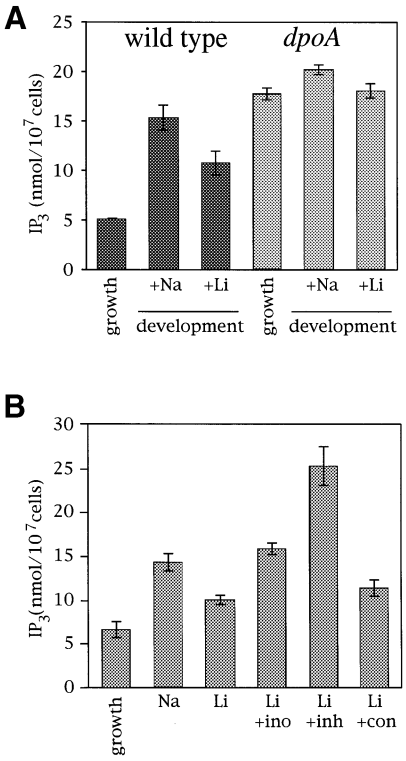

Fig. 7. Loss of DpoA activity elevates the IP_3 concentration during growth and development. (**A**) IP_3 concentration of wild-type (solid) and *dpoA* (shaded) cells during growth and after 6 h development in the presence of 10 mM NaCl or 10 mM LiCl. (**B**) IP_3 concentration of wild-type cells during growth and after 6 h development in the presence of 10 mM NaCl or 10 mM LiCl, or with LiCl and 10 mM inositol (ino), 650 μM Z-Pro-L-prolinal dimethyacetal in methanol (inh) or methanol (con).

Discussion

Li^+ has multiple effects on Dictyostelium development

Many of the effects of Li^+ on *Dictyostelium* are consistent with its inhibition of GSK-3 activity. Li^+ produces a patterning defect in which a disproportionate number of stalk cells are formed at the expense of spore cells (Maeda, 1970). Li^+ treatment can elevate expression of a pre-stalk enriched gene, *cysteine proteinase-2*, and reduce that of a pre-spore gene, *PsA* (Peters *et al*., 1989). In this report, we show that Li^+ treatment inhibits the repressive effects of cAMP on stalk cell differentiation in low density monolayer culture. These are all features consistent with a loss of GskA activity and this similarity is one of the factors which led Klein and Melton (1996) to consider Li^+ as a GSK-3 inhibitor. It has been shown further that Li^+ treatment can affect the Wnt-1 signalling pathway in a manner consistent with a reduction of GSK-3β activity (Stambolic *et al*., 1996; Hedgepeth *et al*., 1997). In *Dictyostelium*, Li^+ reverses the effects of loss of the cAMP receptor cAR4, an effect consistent with it acting through GskA (Ginsburg and Kimmel, 1997).

Although inhibition of GSK-3 can explain the teratogenic activity of Li^+, it does not explain all of the biological effects of Li^+ treatment on *Dictyostelium* development. Maeda (1970) describes a differential effect, where high Li^+ concentrations block aggregation. Furthermore, the effects on gene expression observed by Peters and coworkers (1989) only occur at low Li^+ concentrations, and at high concentrations all developmental gene expression was lost. We also observe an early developmental effect of Li^+, which in our conditions shows a severe retardation of aggregation and loss of expression of the aggregation-specific marker csA. Aggregation of the *gskA* mutant is

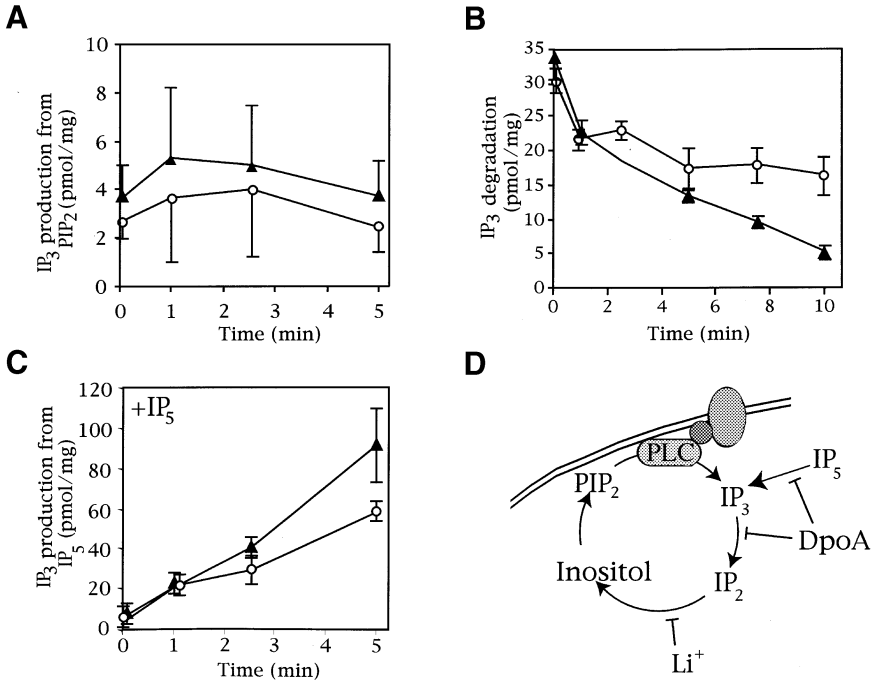

Fig. 8. Loss of DpoA activity causes increased higher order phosphoinositol breakdown. (**A**) PLC activity in cell pellet fractions from the wild-type (○) and *dpoA* (▲) mutant (±SEM). (**B**) IP_3 degradation from the wild-type (○) and *dpoA* (▲) mutant supernatant fractions (S100) in the presence of 200 μM IP_3 (±SEM). (**C**) IP_3 generation from pellet fractions of the wild-type (○) and *dpoA* (▲) mutant in the presence of 100 μM IP_5 (±SEM). (**D**) Model showing the interaction of DpoA and phosphoinositide metabolism.

also inhibited, indicating that in this case GskA is not the Li^+ target. To identify the biologically relevant targets of Li^+, we isolated Li^+-resistant mutants. These fall into two groups: ones which affect stalk cell differentiation and hence could act through *gskA* (data not shown), and a second group in which cells overcome the effect of Li^+ on aggregation. The *dpoA* mutant falls into this second group. We can find no genetic evidence for an epistatic interaction between *dpoA* and gskA and conclude that they lie on separate pathways. These results suggest that there are at least two separate targets of Li^+ during *Dictyostelium* development, and the effect of Li^+ described in earlier reports arises from the simultaneous inhibition of both.

As in previous reports, we find that Li^+ treatment decreases the cellular IP_3 concentration, but this decrease is relatively small. This may be due to two distinct and Li^+-resistant IMPase activities that are also present in *Dictyostelium* cells (Van Dijken *et al*., 1996). Accordingly, it has been suggested that IP_3 signalling is not a significant Li^+ target in *Dictyostelium*. The failure to find a developmental phenotype associated with loss of the *Dictyostelium* PLC gene further strengthened this view (Drayer *et al*., 1994) although, in this case, IP_3 is generated by dephosphorylation of IP_5 (Van Dijken *et al*., 1997). The discovery that elevated IP_3 confers Li^+ resistance demonstrates that IP_3 signalling is a biologically significant target of Li^+ and is required during early *Dictyostelium* development. Interestingly, cells appear largely unaffected by possession of a high cellular concentration of IP_3, although they do show premature expression of the *csA* gene and are incapable of sustained growth in shaking culture.

A Dictyostelium prolyl oligopeptidase

The unique conformation of the prolyl bond protects peptides that contain proline residues from enzyme degradation, and many hormone and neuropeptides are enriched in proline residues. Prolyl oligopeptidases [EC 3.4.21.26] are endopeptidases that cleave Pro–X amino acid bonds, but are only active on polypeptides <3 kDa. They are required to degrade bioactive peptides (Cunningham and O'Connor, 1997) and in some cases to generate new signals, such as seen with the conversion of angiotensin I to angiotensin (1-7) and during host invasion by *Trypanosoma cruzi* (Burleigh *et al*., 1997). POase enzymes are found in metazoa, protozoa and bacteria, but none have been found among the fungi and actinomycetes. The mammalian POase is found in all tissues, but is highly active within the brain, particularly in the frontal cortex, nucleus caudatus and amygdala (Kato *et al*., 1980; Goossens *et al*., 1997). POase enzymes have been associated with neural processing, and POase inhibitors affect learning and memory (Toide *et al*., 1997). It is interesting to note that these processes are attenuated by Li^+ treatment (Barber *et al*., 1998; Cook and Persinger, 1998). We have identified the first and probably only *Dictyostelium* member of this family and we have shown that DpoA has biochemical properties similar to those of other members of the group. The targets of DpoA are not known, although one candidate could be CMF, a protein factor which is processed into peptides and acts via PLC (Brazill *et al*., 1998). CMF sensitizes cAMP receptors on the surface of developing *Dictyostelium* cells (Van Haastert *et al*., 1996) and is required for aggregation.

The known substrates of mammalian POase enzymes are extracellular signal peptides; it is therefore unexpected

that POases are almost exclusively intracellular and are predominantly cytosolic (Kato *et al*., 1980). Some activity has been found associated with synaptosomal membranes, although it is not clear whether this is the same POase present in the cytosol (O'Leary and O'Connor, 1995). Significantly, only 0.1–1% of activity is found in serum (Kato *et al*., 1980). We find DpoA is almost totally cytosolic, with <0.1% of activity present in the 10 000 *g* particulate fraction. We find only 1% of DpoA activity in the extracellular medium. This level of extracellular activity, however, is also seen for β-galactosidase, an enzyme that is definitively cytosolic, and is likely to arise from leakage of damaged cells. This creates a sensitivity limit for measurement of extracellular POase activity; however, our mixing experiments enable us to exclude the action of even undetectable levels of extracellular POase activity as mixing with small quantities of wild-type cells does not restore Li^+ sensitivity to the *dpoA* mutant. As wild-type cells are not induced to aggregate in the presence of 10 mM LiCl when mixed with the *dpoA* mutant, we can also eliminate the possibility that the mutant overproduces an extracellular signal and escapes the inhibition by high levels of autocrine stimulation. There are two possibile mechanisms for the action of POases in the cytosol: they may either act on a peptide signal generated within the cell or they degrade extracellular peptides which function after import into the cell. Such a mechanism has been observed in the bacterium *Bacillus subtilis* where peptide signals are imported into the cell during regulation of sporulation (Lazazzera *et al*., 1997).

An association between prolyl oligopeptidase activity and IP_3 concentration

We have demonstrated an inverse relationship between POase activity and IP_3 concentration. This is seen either in a mutant which lacks all POase activity or in wild-type cells that are treated with a POase inhibitor. In both cases, basal concentrations of IP_3 increase to 3–4 times that seen in untreated wild-type cells. This relationship is unexpected and, to our knowledge, novel. To investigate further, we have examined the enzyme activities that generate or dephosphorylate IP_3. The conventional mechanism of IP_3 production is via activation of PLC through activation of cell surface receptors by extracellular signals. We can exclude chronic stimulation of PLC in the *dpoA* mutant cells on two counts. First, the cytosolic localization of DpoA prevents it from acting on signal peptides outside the cell. Secondly, the rate of hydrolysis of PIP_2 in the *dpoA* mutant is the same as that seen in wild-type cells. We can also exclude the possibility that the higher cellular IP_3 concentration is due to decreased dephosphorylation, and in fact we find higher rates of IP_3 dephosphorylation in the *dpoA* mutant. This suggests a higher rate of phosphoinositide turnover in the absence of DpoA activity. IP_3 can also be generated by breakdown from the higher order phosphoinositides. This has been observed both in mammalian and *Dictyostelium* cells and involves enzyme activities which can dephosphorylate IP_5, $Ins(1,3,4,5)P_4$ and $Ins(1,4,5,6)P_4$ to form IP_3 (Van Dijken *et al*., 1995b). This could involve a single enzyme, multiple inositol polyphosphatase (MIPP), which removes phosphate from both the 6- and 3-positions (Shears, 1998). This enzyme activity has been reported in AR4-2J pancreatoma cells, erthryocytes and liver cells (Estrada-Garcia *et al*., 1991; Nogimori *et al*., 1991; Oliver *et al*., 1992). Curiously, enzyme purified from this last source appears to be compartmentalized to the lumen of the endoplasmic reticulum (ER) which would isolate it from its substrate (Ali *et al*, 1993). MIPP has been cloned from these cells and contains a putative ER retention site, SDEL (Craxton *et al*., 1997; Caffrey *et al*., 1999). In *Dicytostelium*, this enzyme activity is regulated by extracellular cAMP and can account for IP_3 production in the absence of PLC (Drayer *et al*., 1994). We find that in the *dpoA* mutant the conversion of IP_5 to IP_3 is increased. We conclude that loss of DpoA activity causes enhanced turnover of phosphoinositides which has the effect of increasing the cellular concentration of IP_3 (Figure 8D). This would occur if the breakdown of IP_5 exceeds that of IP_3, as reported in mammalian systems (Hughes *et al*., 1989). Our results therefore demonstrate a novel mechanism to control the cellular IP_3 concentration.

POase activity and depression

Our observations link Li^+ sensitivity, POase activity and the cellular IP_3 concentration to a single signal transduction pathway and have interesting implications for the Li^+ treatment of depression. Recently, POase activity has been associated with the mental disorders of unipolar and bipolar depression. POase activity is lowered during depression, but raised during mania (Maes *et al*., 1994, 1995), and antidepressant and antimanic drugs restore POase activity to normal levels (Maes *et al*., 1995). Furthermore, POase is known to degrade Substance P, arginine, vasopressin, β-endorphin, thyroliberin and luliberin, all implicated in the pathophysiology of depression (Cunningham and O'Connor, 1997). If the pathway we observe in *Dictyostelium* is conserved in the brain, it could explain the therapeutic effect of Li^+. Depression could result from a reduction in POase activity that leads to an elevated IP_3 concentration, which in turn is reversed by Li^+ inhibition of IMPase and IPP. If this is the case, it provides support for the inositol depletion theory and suggests that IP_3-, rather than GSK-3-mediated signal transduction is the appropriate target for the treatment of depression. Further insight into the nature of this signal transduction pathway will be obtained by examination of other Li^+-resistant mutants in this pathway.

Materials and methods

Materials

BOC-Glu(NHO-Bz)-Pyr was supplied by Calbiochem, Z-Pro-L-prolinal dimethylacetal and Z-Gly-Pro-pNA by Bachem (UK) Ltd, and myo-inositol was supplied by Sigma-Aldrich Co. Ltd.

Cell culture and gene isolation

Cells were grown at 22°C in shaking culture (HL5 medium) up to 3×10^6 cells/ml or in association with *Klebsiella aerogenes*. REMI mutagenesis was carried out using wild-type cells (Ax2) as previously described (Kuspa and Loomis, 1992), and mutants were screened for the ability to aggregate and develop when grown on *K.aerogenes* in the presence of 10 mM LiCl. Li^+-resistant mutants were designated HAD170–HAD183. The *dpoA* mutant (HAD172) was recapitulated using 30 μg of rescued plasmid, cleaved with *Cla*I and reinserted into wild-type cells (Ax2) to give strains HAD200–HAD204, or into *gskA* mutant cells (HM1435) to give strain HAD205. The encoding cDNA was isolated from a slug stage cDNA library in λZAP (kindly provided by R.Firtel). Sequence analysis was performed by standard protocols using dRhodam-

ine terminator cycle sequencing Ready Reactions (Applied Biosystems). The DDBJ/EMBL/GenBank accession Nos of the *Dictyostelium dpoA* gene and rescued fragment are AJ238018 and AJ238019, respectively. Developmental phenotypes were analysed by plating cells (density = 1×10^7/filter) on 47 mm nitroclellulose filters (Millipore) soaked in KK_2 (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4 pH 6.2) containing either 10 mM LiCl, 10 mM NaCl or 1.3 mM Z-Pro-L-prolinal dimethyacetal. Development was observed after 20 h unless otherwise stated. Staining for β-galactosidase was carried out as described in Dingermann *et al*. (1989).

Immunological techniques

Extracts from 5×10^5 cells were separated by SDS–PAGE and transferred to nitrocellulose (Hybond-C Extra, Amersham Pharmacia Biotech.) following standard procedures (Harlow and Lane, 1988). csA and protein disulfide isomerase (PDI) proteins were detected by monoclonal antibody, mAb 33459 and mAb 221-135-1, respectively (kindly provided by J.Faix and M.Maniak) and visualized by chemiluminescence (Supersignal™, Pierce & Warriner Ltd).

DpoA assays

Wild-type (Ax2) and *dpoA* cells were harvested and washed in KK_2, and resuspended to give 2×10^8 cells/ml in homogenization buffer [50 mM Tris pH 7.4, 1 mM EDTA, 1 mM dithiothreitol (DTT), 5 μg/μl aprotinin, 1 mM benzamidine, 10 μg/μl leupeptin, 0.1 mM TLCK]. Cells were lysed by sonication, and soluble fractions were prepared by centrifugation (100 000 *g*, 35 min, 4°C). Assays were measured in triplicate by combining 3 μl of enzyme extract with 300 μl of assay buffer [50 mM HEPES pH 7.8, 1 mM EDTA, 1 mM DTT, 0.25% dimethylsulfoxide (DMSO), 141 μM Z-Gly-Pro-pNA] and incubating at 37°C for 2 h. Units of enzyme activity were calculated from the increase in absorbance at 405 nm in 2 h (ε_{405} = 8800/M/cm; Makinen *et al.*, 1994), measured in triplicate using a microtitre plate reader. Standard Michaelis–Menten analysis of kinetic data was used to define enzymatic constants.

Cell secretion assay

Actin15:LacZ-transformed wild-type (10^7 cells) were incubated in 10 ml of KK_2 in tissue culture plates. Cells were separated from medium by centrifugation. Filtered medium and the S100 cell fraction were assayed for POase activity and β-galactosidase activity according to Dingermann *et al.* (1989).

DpoA localization

Wild-type cells (1.7×10^8) were resuspended in 1.7 ml of KK_2, diluted with 2 vols of homogenization buffer [30 mM Tris–HCl pH 7.4, 2 mM DTT, 4 mM EGTA, 2 mM EDTA, 5 mM benzamidine, 30% (w/v) sucrose] and lysed through a 5 μM nucleopore filter (Corning). Intact cells were removed by centrifugation at 750 *g* and the crude supernatant was centrifuged at 10 000 *g* to concentrate the particulate fraction (P10), and at 100 000 *g* to concentrate cell membrane and small vacuoles (P100). All pellets were washed in homogenization buffer and assayed for POase activity or for Western analysis with anti-DPI mAb (Monnat *et al.*, 1997).

Stalk cell induction assay

Cells were plated at a density of 10^5 cells/ml into stalk medium (10 mM MES, 2 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 200 μg/ml streptomycin, 20 μg/ml tetracycline; pH 6.2) supplemented with 5 mM cAMP. After 20 h, the medium was removed, the cells washed three times and replaced with fresh medium supplemented with 100 nM DIF (Berks and Kay, 1988); 5 mM cAMP and 2.5 mM LiCl were added as indicated. Stalk cells formed after a further 24 h.

IP_3 assays

Mutant and wild-type cells were grown in shaking culture overnight before assay. Cells were developed as shaking cultures (5×10^7 cells) in 1 ml of KK_2 at 22°C, 150 r.p.m. for 6 h. Aliquots of 200 μl were assayed for IP_3 concentration by an isotope dilution method (Amersham Pharmacia Biotech.), using final reaction volumes of 100 μl.

PLC hydrolysis, and IP_3 and IP_5 dephosphorylation assays

Cell pellet and supernatant fractions were prepared from 2×10^8 cells/ml in buffer A [20 mM HEPES/NaOH pH 7, 0.5 mM EDTA, 200 mM sucrose (Van Dijken *et al.*, 1996)], lysed by passing through a 5 μm nucleopore membrane and separated by centrifugation at 100 000 *g* for 30 min. Pellets were washed and resuspended in equal volumes of buffer A. Bradford assays confirmed equal protein levels in extracts. PLC and IP_5 dephosphorylation were measured by the production of IP_3 from pellet fractions at 22°C in the presence of 10 mM $CaCl_2$ and inhibitors of IP_3 breakdown, 25 mM LiCl and 250 μM diphosphoglycerate (Bominaar and Van Haastert, 1994; Van Dijken *et al*., 1997). IP_5 (100 μM) (Sigma Chemical Co.) was added for the measurement of the conversion of IP_5 to IP_3. IP_3 dephosphorylation was measured by the loss of IP_3 in supernatant fractions in the presence of 5 mM $MgCl_2$ (Bominaar *et al*., 1991). In this assay, exogenous IP_3 was used to adjust the a final concentration to 200 μM. Figures represent average results from at least two independent extracts assayed twice in triplicate.

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