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The effects of inositol metabolism

On lithium sensitivity and chemotaxis

In *Dictyostelium discoideum*

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PhD Thesis

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SUMMARY OF THESIS:

Dictyostelium discoideum is a eukaryotic amoeba that has proven to be an excellent model system for the study of chemotaxis and development. Upon starvation, individual amoebae are able to aggregate and undergo development to form a fruiting body. Previously it has been demonstrated that this process is disrupted by two drugs commonly used to treat bipolar disorder, lithium and valproic acid (VPA), and that a ablation of the gene encoding prolyl oligopeptidase (PO) is able to confer resistance to this effect. Both drugs also have the common property of depleting intracellular inositol levels, and the observation that PO null cells have increased production inositol 1,4,5 trisphosphate (Ins(1,4,5)P_3) from the dephosphorylation of $InsP_5$ therefore suggests that this may be responsible for lithium resistance.

In this study I have cloned and generated mutants of the gene encoding this activity and shown that it is essential for lithium resistance in PO null cells and that this resistance is due to upregulated transcription of the lithium-sensitive IMPase family of genes. In addition I have shown that cells lacking phospholipase C activity are also lithium resistant, indicating that the recycling of inositol phosphates and lipids plays an important role in maintaining cell movement and lithium action.

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Abstract

Dictyostelium discoideum is a eukaryotic amoeba that has proven to be an excellent model system for the study of chemotaxis and development. Upon starvation, individual amoebae are able to aggregate and undergo development to form a fruiting body. Previously it has been demonstrated that this process is disrupted by two drugs commonly used to treat bipolar disorder, lithium and valproic acid (VPA) and in this study I show that this disruption is due to the inhibition of cell movement. Previous work has shown that ablation of the gene encoding prolyl oligopeptidase (PO) is able to confer resistance this effect.

Both drugs have the common property of depleting intracellular inositol levels, and the observation that PO null cells have increased production of inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$) from the dephosphorylation of $InsP_5$ therefore suggests that this activity may be responsible for lithium resistance. In this study I have cloned, and generated mutants of, the gene encoding this inositol phosphatase activity and shown that it is essential for lithium resistance in PO null cells and that this resistance is due to upregulated transcription of the lithium-sensitive IMPase family of genes. In addition I have shown that cells lacking phospholipase C activity are also lithium resistant, indicating that the recycling of inositol phosphates and lipids plays an important role in maintaining cell movement and lithium action.

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Chapter 1:

Introduction

1.1 *Dictyostelium* and lithium

The effects of lithium on *Dictyostelium* development were originally studied in 1970 by Maeda (Maeda, 1970) and it was observed that when cells are starved and allowed to undergo development in the presence of lithium, two effects are seen. Treatment with 7mM LiCl affects differentiation, causing the fruiting body to suffer from a mis-proportioning of cell types where the spore head virtually disappears and the basal disc is enlarged (Maeda, 1970; Peters et al., 1991). This is likely to be due to inhibition of glycogen synthase kinase a (GskA, the *Dictyostelium* homologue of GSK3) as it is reminiscent of the phenotype seen upon inactivation of this gene (Harwood et al., 1995). In addition, when cells develop at concentrations of lithium chloride above 10mM, aggregation is blocked (an effect also seen in the presence of 1mM valproic acid). As cells lacking any GskA activity are still able to aggregate, albeit with morphological defects, this suggests that other targets of lithium are responsible (see fig 1.1) (Harwood et al., 1995).

Biochemically, lithium treatment of *Dictyostelium* has also been shown to disrupt phosphoinositol metabolism, and is able to decrease both the basal as well as the receptor stimulated levels of Ins(1,4,5)P_3 (Peters et al., 1991) as well as affecting the activation of G-proteins, and the production of cyclic GMP (Peters et al., 1992). Indeed, depletion of inositol is a common property of both lithium and another mood-stabilising drug, valproic acid (VPA) which is also able to deplete intracellular Ins(1,4,5)P_3, and therefore

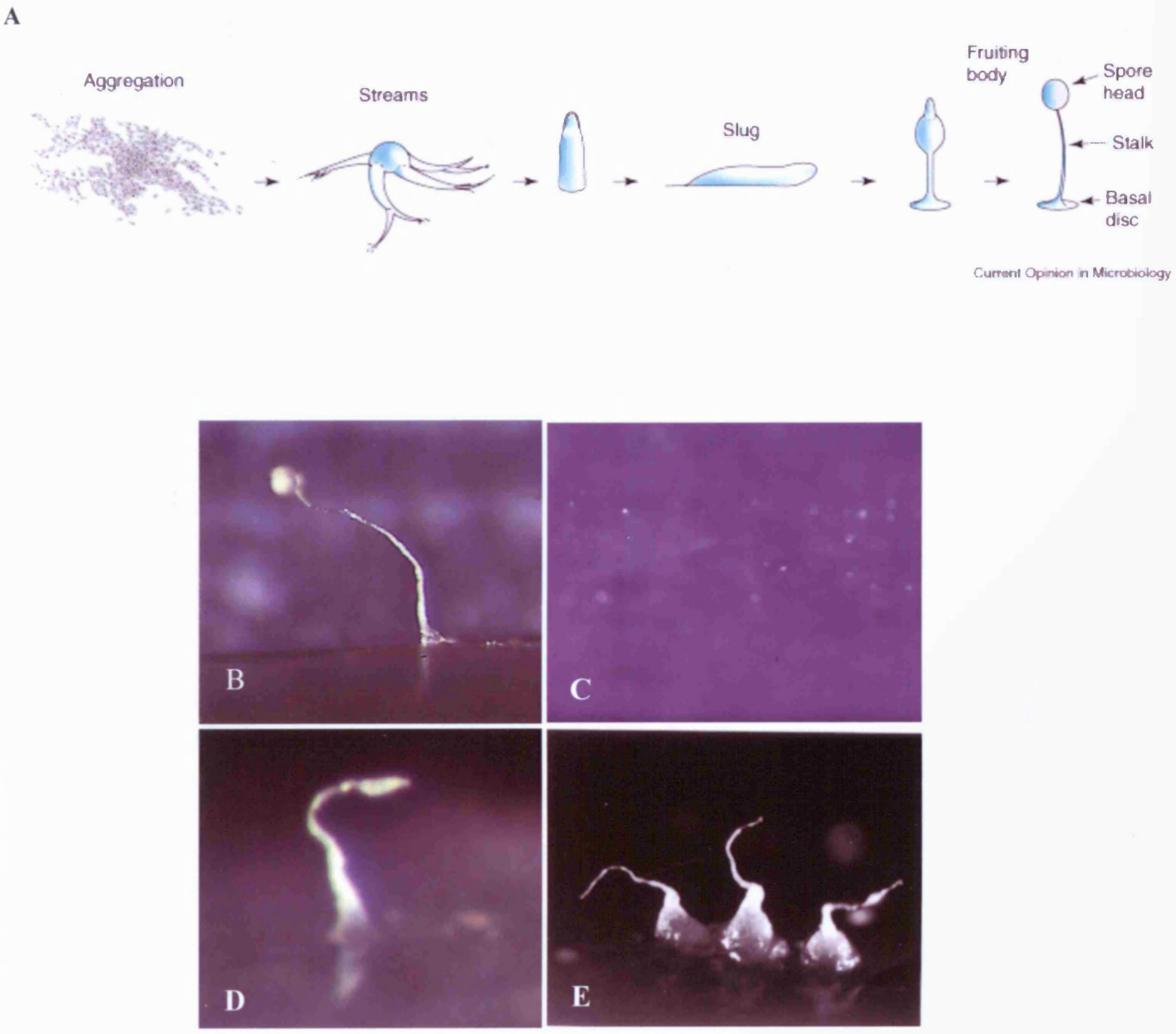


Figure 1.1: The effects of lithium on *Dictyostelium* development. (A) the *Dictyostlelium* developmental cycle, where single cells aggregate together to form multicellular bodies, finally resulting in a fruiting body. (B-D) Wild-type *Dictyostelium* allowed to develop on non-nutrient agar in (B) 10mM NaCl, (C) 10mM LiCl and (D) 7mM LiCl. (E) shows the developmental phenotype of *gskA* null cells, no lithium. Images taken from (Harwood et al., 1995; Williams et al., 1999)

inhibition of inositol signaling may represent a conserved therapeutic activity (Williams et al., 2002). The aim of this study is therefore to determine the mechanism by which lithium is able to block *Dictyostelium* aggregation and understand the role of inositol signaling in both lithium treatment and cell movement in general.

1.2 The *Dictyostelium* model system

Dictyostelium are eukaryotic social amoebae, which modern phylogenic trees place as having diverged from the other metazoa after the animals and plants split (Baldauf and Doolittle, 1997; Eichinger et al., 2005). *Dictyostelium* has a complex life cycle and is able to undergo chemotaxis, development and differentiation and has a large number of functions in common with animal as well as well as plants and fungal cells. For example, *Dictyostelium* is the only lower eukaryote to possess class I phosphatidyl inositol 3'kinases, and outside of the animal kingdom, *Dictyostelium* is the only species which has been shown to have SH2 domain, phosphotyrosine signaling, although no receptor tyrosine kinases could be found (Eichinger et al., 2005). These properties, combined with ease of genetic manipulation and culture make *Dictyostelium* is an excellent and appropriate system for the study of many biological processes.

Dictyostelium cells have a complex life cycle, consisting of single-cell vegetative and multicellular developmental phases. In this system, when bacterial or liquid nutrients are plentiful, the cells grow as independent amoebae and move randomly with amoeboid movement. However, when the food source is in short supply, the cells

initiate a developmental process, and quickly alter the expression of many genes. Two such genes are adenylyl cyclase, which leads to the formation of waves of secreted cyclic adenosine monophosphate (cAMP), as well as its receptor cAR1 (Rathi and Clarke, 1992; Schulkes and Schaap, 1995). In response to the cAMP gradient formed, cells rapidly move towards the source, chemotaxing towards the signal, to form aggregates of up to 200,000 cells. At this stage, the amoebae start to differentiate into different cell types and form a defined multicellular slug, which is able to move along the substratum. Finally a decision is made and the morphology changes again to form a mature fruiting body consisting of a spore head held aloft by a stalk. This fruiting body contains spore cells that germinate when dispersed in the hope of finding more food. This process is summarized in figure 1.1. This lifecycle, which takes approximately 24 hours to complete, represents a robust system for both chemotaxis and development. Along with relatively easy genetic manipulation this makes *Dictyostelium* an excellent model organism for the study of these processes.

The *Dictyostelium* genome is relatively gene-rich, with approximately 12,500 genes encoded in just 34 megabases of DNA. This is split into 6 chromosomes and although it is possible to select and grow diploid cells, cultures are generally haploid. With the recent completion of the genome sequence, this therefore allows for fairly straightforward genetic manipulations such as the generation of null mutants, and the ability to generate large numbers of mutants to carry out genetic screens (Kuspa and Loomis, 1992).

1.3 Chemotaxis in *Dictyostelium*

Upon lithium treatment, *Dictyostelium* aggregation is disrupted. Although the mechanism of this is not understood, it must involve either disruption of developmental or chemotactic signal transduction, or the ability to act upon it and move efficiently towards each other. By studying the chemotactic behaviour of cells treated with lithium as well as the properties of lithium resistant mutants is should be possible to clarify this and understand more about the processes involved in cell migration.

The process of chemotaxis has been extensively studied in *Dictyostelium*. As also seen in neutrophils, cells respond to a gradient of chemoattractant by the induction of actin polymerization at the leading edge of the cell, along with myosin contraction at the rear. Cells are exceptionally sensitive to even very shallow gradients, being able to respond to just a 2% difference in chemoattractant concentration between the front and the back of the cell (Mato et al., 1975). In the absence of a gradient, cells move around randomly producing new pseudopodia in every direction, but when a chemoattractant gradient is introduced, this is biased such that the cell preferentially produces and maintains pseudopodia towards the source, whilst suppressing them elsewhere. This polarizes cell morphology towards the cAMP source, although the cAMP receptors remain uniformly distributed at all times (Funamoto et al., 2002). This polarity can however be transient as, if the chemotactic source is moved the cell is able to overcome this lateral pseudopod repression, and re-orientate itself by the production of a new leading edge, (Chen et al., 2003; Swanson and Taylor, 1982). Although this method of cell turning does happen in the presence of steep gradient of

cAMP, *Dictyostelium* cells will also frequently do a U-turn, retaining the same primary pseudopod. This is the preferred mechanism of turning in neutrophils (Zigmond, 1977), and the degree to which a cell will make a new pseudopod rather than turn may even be actively regulated as *Dictyostelium* cells become more strongly polar as they progress through development.

It is in the formation and maintenance of this polarity that inositol phospholipids have been strongly implicated. In response to activation of the cAMP receptor, the class I PI-3' kinases are rapidly activated, producing a patch of PtIns(3,4,5)P_3 at the plasma membrane (Stephens et al., 1993). This occurs in a spatially regulated manner, with PtIns(3,4,5)P_3 tightly localised at the leading edge of the cell, in the direction of the chemoattractant source. In addition, the 5' phosphatase PTEN forms a reciprocal pattern, repressing PtIns(3,4,5)P_3 accumulation everywhere else (Funamoto et al., 2002). This can be visualised using a specific PtIns(3,4,5)P_3-binding plecstrin homology (PH)-domain probe fused with GFP, and has been used to show PtIns(3,4,5)P_3 production in areas of actin polymerization in neutrophils, *Dictyostelium*, and fibroblasts (Haugh et al., 2000; Parent et al., 1998; Servant et al., 2000).

Using this ability to bind PH-domain containing proteins, PtIns(3,4,5)P_3 is able to recruit proteins, including the cytosolic regulator of adenylyl cyclase (CRAC), Akt/PKB and PhdA specifically to the leading edge of the cell, coinciding with actin polymerization and pseudopod formation (Funamoto et al., 2001; Meili et al., 1999; Parent et al., 1998; Servant et al., 2000). Indeed, *Dictyostelium* cells contain six

potential class I PI3' kinases and although there is some degree of functional redundancy, a PI3K1/2 double mutant has a severe loss of cell polarity, producing multiple pseudopodia along the periphery of the cell (Chung et al., 2001; Funamoto et al., 2001). There are two groups of proteins able to negatively regulate the level of PtIns(3,4,5)P_3; these are the 3' phosphatase PTEN which reverses the action of PI3K, and a family of inositol 5'phosphatases that produce PtIns(3,4)P_2. Although inactivation of the 5'phosphatases does not appear to affect movement (Loovers et al., 2003), disruption of the single PTEN gene results in a reduction in both polarity and chemotaxis efficiency (Funamoto et al., 2002; Iijima and Devreotes, 2002) and this has lead to the proposal that localized PtIns(3,4,5)P_3 production plays a major instructive role in cell migration by biasing the direction of movement towards a chemoattractive source (Funamoto et al., 2002; Funamoto et al., 2001; Insall and Weiner, 2001; Niggli and Keller, 1997; Postma et al., 2004; Wennstrom et al., 1994). Recently however, a further study using a combination of *pi3k* null cells and a PI3K inhibitor, has shown that although the severe inhibition of cAMP-mediated PtIns(3,4,5)P_3 production leads to inhibition of cAMP relay (presumably via CRAC), cell elongation and aggregation, cells are still able to orientate towards the chemoattractant source (Loovers et al., 2006). Therefore it may be that PtIns(3,4,5)P_3 production may simply provide a directed extra motile force, rather than being responsible for determining the orientation.

Although in most cells PtIns(4,5)P_2 levels are much higher than PtIns(3,4,5)P_3 and therefore do not undergo any change large enough to be a direct signal and specific PtIns(4,5)P_2 binding PH domains fused to GFP indicate a uniform distribution along the plasma membrane, it has been proposed that it may still play an important,

permissive role in cell movement. In order for a cell to move its membrane forwards, it must activate actin polymerization and the formation of actin filaments. This is a highly regulated process and PtIns(4,5)P_2 has been shown to interact with a number of proteins involved in the control of actin dynamics, including, profilin, filament-capping proteins and the Wiscott-Aldrich syndrome protein N-WASP (Higgs and Pollard, 2000; Hilpela et al., 2004; Miki et al., 1996; Ostrander et al., 1995; Palmgren et al., 2001). Therefore, although the concentration and dynamics of PtIns(4,5)P_2 do not appear to be sufficient for direct signaling, as it is tightly localised to the plasma membrane it may play an important, but more instructive role by directing the machinery required for actin polymerization to the cell periphery (Insall and Weiner, 2001).

1.4 Ins(1,4,5)P_3 and Calcium signaling in chemotaxis

In addition to the activation of class I PI3'kinases, chemoattractant stimulation of cells also results in an increase in phospholipase C (PLC) activity, leading to the transient accumulation of Ins(1,4,5)P_3 and diacyglycerol (DAG) due to the hydrolysis of PtIns(4,5)P_2. This increased Ins(1,4,5)P_3 is then capable of binding to its receptor on the surface of intracellular calcium stores and the plasma membrane, leading to both calcium influx and release from intracellular stores (Bumann et al., 1984; Schlatterer et al., 2004; Yumura et al., 1996). This results in a rapid increase in intracellular calcium levels, and the activation of numerous calcium-dependant proteins, some of which have been demonstrated to induce actin polymerization, including protein kinase C (Kishimoto et al., 1980).

However, although this calcium response is common to many cell types, there is evidence to suggest that it is not important for chemotaxis. For example, the *Dictyostelium* genome contains a single PLC (a delta class) which, upon cAMP stimulation is activated by a G-protein dependant pathway, producing a transient rise in Ins(1,4,5)P_3 (Bominaar et al., 1994; Europe-Finner et al., 1989; Okaichi et al., 1992). Upon inactivation of this gene, all PLC activity is lost and yet the cells are still able to grow, chemotax and develop normally (Drayer et al., 1994). In fact so far, the only defect to be found in this mutant is the ability to control spore germination and levels of Ins(1,4,5)P_3 are maintained due to a compensatory up-regulation of InsP_5 hydrolysis (Van Dijken et al., 1997; Van Dijken and Van Haastert, 2001).

Other studies have also questioned the role of this signaling pathway in chemotaxis, as disruption of the single *Dictyostelium* Ins(1,4,5)P_3 receptor-like gene *iplA* results in cells in which calcium influx is abolished, yet again the cells can chemotax with a similar efficiency to wild-type (Traynor et al., 2000). This is in agreement with some previous studies where the addition of calcium chelators did not affect neutrophil chemotaxis (Marasco et al., 1980; Maxfield, 1993), although there is also evidence that *iplA*$^-$ cells can still undergo some calcium influx, through a capacitance-driven entry that is unaffected by this mutation. This, and other studies have also shown that chemotaxis is severely disrupted by the presence of calcium chelators in the cytoplasm (Schlatterer and Malchow, 1993; Unterweger and Schlatterer, 1995; Van Duijn and Van Haastert, 1992), although this may well be due to lowered basal calcium levels disrupting the cell in a more general sense, and not a direct result on Ins(1,4,5)P_3 signaling. If calcium signaling does not prove to be important for chemotaxis it is an interesting question why the cell the cell initiates PLC activation

and produces Ins(1,4,5)P_3 so sharply in response to stimulation. There is some evidence that Ins(1,4,5)P_3 may be able to regulate developmental gene expression and therefore, although why it should be activated in a cyclical nature is unclear, alternative more subtle roles may exist (Ginsburg and Kimmel, 1989; Kimmel and Eisen, 1988).

One of the problems in the study of the pathways described above is the degree of functional redundancy in this system, indicating the fundamental importance of cell movement in survival. From many of these studies, it is clear that in when cells are aggregating efficiently, a number of processes are involved and often the loss of one may be compensated by the up-regulation of others. Both lithium and VPA however, are able to completely block aggregation as well as deplete Ins(1,4,5)P_3 levels (Williams et al., 2002) and therefore by the study of this mechanism we hope to gain further insight into this process.

1.5 Lithium and bipolar disorder

Both lithium and VPA are widely used as mood stabilizers for the treatment of bipolar affective disorder (BAD), previously known as manic depression. Although they have both been used for many years and are effective, the therapeutic mechanism of their action as well as the underlying cause of the condition remain unclear. Bipolar disorder is a mood disorder that affects up to 1% of the population and although the condition is alleviated in most treated patients, both drugs have unpleasant side effects and are teratogenic and some patients fail to respond (Lenox et al., 1998; 2003).

The main difficulty facing the development of new treatments is that neither the condition, nor the action of the drugs is clearly understood. One approach to resolve this is to look for common signaling /metabolic pathways affected by different drugs. To date, three classes of proteins have been shown to be directly inhibited by lithium at therapeutic doses (about 0.5-1.5mM for lithium, and 0.5mM for valproic acid) (Manji et al., 1995). These are the glycogen synthase kinase-3 (GSK3) family of serine/threonine protein kinases, regulating numerous cellular events including glycogen metabolism and wnt signaling (1996); a family of phosphomonoesterases, typified by inositol monophosphatase (IMPase) and 3'(2')-phosphoadenosine (PAP) phosphatase (1995; Murguia et al., 1995; 1994; York et al., 1995); and the enzyme phosphoglucomutase, involved in glucose metabolism (Ray et al., 1978).

Valproic acid has also been shown to affect both wnt signaling (although by an indirect, undetermined mechanism), and inositol synthesis (2002; O'Donnell et al., 2003; Williams et al., 2002; Williams et al., 1999; 2001). In addition, at therapeutic levels (K_i =0.4mM) it is also an inhibitor of histone deacetylase activity and therefore may affect transcriptional regulation and explain its teratogenicity (Phiel et al., 2001).

Specificity is therefore a problem with both drugs, both leading to unpleasant side-effects and complicating the study of their therapeutic action. However hitting multiple targets may also actually be critical for the drugs to work, and therefore in order to try and generate better, alternative treatments, we must endeavour to understand the relative contribution of each. In *Dictyostelium* both lithium and VPA have the common action of blocking aggregation and therefore in understanding both

the relevant targets and the mechanism by which this happens we also learn more about the therapeutic affects of these drugs in the brain.

1.6 The known molecular targets of lithium

1.6.1 Inhibition of glycogen synthase kinase

GSK3 is a multifunctional protein kinase found in all eukaryotes. It was first identified as a kinase that phosphorylates and inactivates glycogen synthase (Frame and Cohen, 2001), a central enzyme in the insulin response but has since been discovered to mediate a number of other pathways (Doble and Woodgett, 2003). The most well known of these is the canonical wnt signaling pathway, whereby phosphorylation of β-catenin by GSK3 leads to its degradation. In response to wnt binding to the Frizzled or LRP5/6 cell surface receptors this phosphorylation is inhibited and β-catenin accumulates in the nucleus, leading to transcriptional activation.

In *Dictyostelium* it has been shown that lithium can directly inhibit GskA (the *Dictyostelium* homologue) with a K_i of 2 mM (2001). As wnt signaling is highly active during development it would seem probable that GskA inhibition can explain the teratogenic effects observed with lithium treatment. Indeed, when *Dictyostelium* cells lacking GskA activity undergo development, cell-type specification goes awry, leading to increased numbers of stalk cells and a decrease in spores as has also been observed at low concentrations of lithium (Harwood et al., 1995; Peters et al., 1989).

Although it is unlikely that GskA inhibition is responsible for inhibition of *Dictyostelium* aggregation, it should not be disregarded merely as a mechanism for the teratogenic side effects of lithium and a number of its substrates have the potential to be involved in neuronal function. Neurogenesis, and therefore neuronal development has recently been reported in the adult brain of several species (Alvarez-Buylla et al., 2001; Gould and Gross, 2002; Gould et al., 1999; Sanai et al., 2004) and GSK-3 has also been implicated in the regulation of neuronal apoptosis (Longo et al., 2002; Takashima et al., 1996; Takashima et al., 1993). In addition, lithium and wnt signaling have been shown to modulate axonal structure, modulating microtubule stabilization and branching (Lucas et al., 1998; Lucas and Salinas, 1997) and other studies have also shown effects of VPA on Wnt signaling, maintaining the possibility that GSK3 could be a therapeutic target (2002; 2001).

1.6.2 Inhibition of IMPase and inositol depletion

An alternative theory for the mood-stabilizing properties of lithium and VPA is by interference with inositol metabolism. The six carbon sugar inositol forms many different phospholipid and soluble polyphosphates within the cell, many of which play important and diverse roles in signaling and movement, some of which have already been described.

As previously mentioned, lithium is a non-competitive inhibitor of the IMPase family, binding the enzyme-substrate complex with a K_i of ~1.0mM dependant on species (Gee et al., 1988). In many cell types this has been shown to reduce the levels of *myo*-inositol and other inositol phosphates leading to the suggestion in 1989 by Berridge

and colleagues that a mechanism of lithium action was to deplete inositol phospholipids and therefore suppress inositol-based signaling in the brain (1989). In addition, VPA has recently been shown to inhibit inositol synthase, the enzyme involved in the *de novo* synthesis of inositol from glucose–6-phosphate, and both drugs have the common ability to reduce the levels inositides, including $Ins(1,4,5)P_3$ in both rat brains and *Dictyostelium* (O'Donnell et al., 2003; Shaltiel et al., 2004; Williams et al., 2002; Williams et al., 1999).

Studies using sensory neuronal growth cones also implicate inositol depletion as a possible therapeutic mechanism. Normal migrating growth cones cycle between phases of rapid extension, with contracted ends, and stationary phases when the cones are spread. In this system, using cells from rat dorsal root ganglia (DRG's), lithium, VPA and carbemazepine (an anti-epileptic drug with mood-stabilizing properties) all inhibit cell migration, as shown by increased spreading - an effect which was reversible by the addition of exogenous *myo*-inositol (Lucas et al., 1998; Williams et al., 2002). In addition, a recent study has also found some evidence for a bipolar-linked polymorphism in the promoter of the IMPase 2 gene (Sjoholt et al., 2000), although a mouse knockout of this gene has no obvious behavioral phenotype (Cryns et al., 2006).

In other model organisms, result have also been confusing, in yeast lithium is inhibitory to growth, but disruption of both IMPase genes gave neither inositol auxotrophy nor any growth problems, indicating that a different mechanism is important for this effect. In addition, the same study also show that although IMPase overexpressing yeast are resistant to lithium, this is due to increased ion-channel

activity and therefore lithium not accumulating within the cell (Lopez et al., 1999). This study was however merely looking at cell growth and used concentrations of lithium far in excess of those used therapeutically (up to 100mM). In the brain, the actions of lithium and VPA are likely to be much more subtle, involving mechanisms that simply do not exist in yeast, such as chemotaxis. Indeed, in *Drosophila melanogaster* mutants lacking all IPP activity, a defect in synaptic transmission is seen. This is due to increased vesicle release at neuromuscular junctions – an effect which is phenocopied by the lithium treatment of wild-type synapses (Acharya et al., 1998).

Although some of this evidence may prove to be misleading, there are nevertheless many lines of evidence that point towards a role for inositides in lithium action. Indeed, it may well be more than coincidence that all three known direct targets of lithium are involved in sugar metabolism and therefore may work co-ordinately along the same pathway to inhibit inositol production within the cell. Were this the case it would certainly make lithium an excellent drug, and may explain the difficulties in discovering more effective treatments. It therefore seems likely that inositol metabolism and signaling plays some role in the treatment, and potentially the cause, of bipolar disorder, and that the inositol-depletion effects of lithium and VPA are responsible for the inhibition of *Dictyostelium* aggregation.

1.6.3 Inhibition of PAP-phosphatase

3'(2')-phosphoadenosine 5'phosphate (PAP) phosphatase is a member of the same family of enzymes that includes IMPase and therefore is also inhibited by lithium. As the name suggests, the enzyme hydrolyses PAP to form adenosine 5'monophosphate (AMP) and is inhibited non-competitively by lithium with a K_i of 0.3mM (Agam and Shaltiel, 2003; Yenush et al., 2000). Although not extensively studied, loss of the enzyme in yeast leads to PAP accumulation along with RNA processing and stabilization defects, and therefore may explain some of the transcriptional changes seen in mice after chronic lithium treatment (Dichtl et al., 1997). However, whether this is a therapeutic target remains unclear and as PAP phosphatase and IMPase have some cross-reactivity to each others substrate (Lopez-Coronado et al., 1999), PAP and inositol metabolism should not be seen as completely independent systems.

1.6.4 Inhibition of phosphoglucomutase

The enzyme phosphoglucomutase (PGM) is important for the metabolism of galactose, and regulates its entry into glycolysis. When galactose is metabolized, it is first converted into glucose-1-phosphate, which is then isomerised by PGM into glucose-6-phosphate before entering the glycolytic pathway. Interestingly, this is also the substrate of inositol synthase, which converts glucose-6-phosphate into inositol-3-monophosphate. The K_i for lithium of human PGM is 1.5mM and therefore is also likely to be inhibited at therapeutic levels and injection of lithium into brain tissue results in changed glucose phosphate levels. Therefore this effect may also play a significant role in lithium action (Csutora et al., 2005a; Csutora et al., 2005b; Nordenberg et al., 1982; Ray et al., 1978).

1.7 Prolyl oligopeptidase and lithium resistance

In order to determine the mechanism and targets of the lithium treatment on aggregation, previous workers in this laboratory isolated a number of mutants resistant to this effect. As a model organism, *Dictyostelium* is fortunate enough to have a haploid genome; therefore it is possible to make a library of random insertional mutants and screen them for sensitivity to lithium (Kuspa and Loomis, 1992). In this screen, a mutant library was generated and grown clonally on a lawn of bacteria in the presence of 10mM lithium. As each clone grows and clears a plaque in the bacterial lawn, the cells in the centre run out of food and initiate development. Mutants were therefore screened for the ability to form fruiting-body structures under these conditions, where wild-type cells cannot.

The first of these mutants (lisA, for lithium suppressor A) was identified as a disruption of the gene encoding prolyl oligopeptidase (PO) and is also cross-resistant to VPA (Williams et al., 1999). In this mutant, cells will aggregate in up to 12mM LiCl, although they still exhibit the patterning defect attributed to GSKa inhibition. This therefore indicates that the two effects of lithium on *Dictyostelium* development are distinct and that the two pathways can be resolved. Interestingly, when inositol levels are examined in this mutant, increased basal levels of Ins(1,4,5)P_3 are observed due to increased breakdown of more phosphorylated species. This observation has also been seen by others in astroglioma cells (Schulz et al., 2002) and therefore would point towards a mechanism whereby an elevated basal levels of inositides can protect against the inositol depleting effects of lithium and VPA.

Prolyl oligopeptidase is a serine protease with the enzymatic activity of cleaving peptides on the carboxyl side of a proline residue and is not directly inhibited by either lithium or VPA (Williams et al., 1999). Although its cellular function and biological substrates are unclear, the crystal structure predicts an eight-bladed β-propeller fold that would restrict entry into the active site to substrates smaller than 30 amino acids (Hiramatsu et al., 2003; Rasmussen et al., 2003). The mammalian enzyme has been shown to be able to cleave a number of peptide hormones and neuropeptides (Polgar, 2002) although the majority of the enzyme activity appears to be intracellular, and therefore the biological relevance of these as substrates is uncertain. Tantalizingly however, reduced PO activity has also been associated with bipolar patients, as well as the neuronal damage in patients suffering from Alzheimer's disease and cognitive enhancement in rats (Breen et al., 2004; Laitinen et al., 2001; Yoshimoto et al., 1987) and therefore it seems feasible that PO plays some role in brain function. Whether this is due to the regulation of inositol metabolism is unclear, although studies have reported reduced inositol levels in bipolar patients (Davanzo et al., 2001; Shimon et al., 1997). An aim of this project is to investigate the mechanism of lithium resistance in PO null *Dictyostelium* and determine the contribution of inositol metabolism to this.

1.8 Signalling through inositol polyphosphates

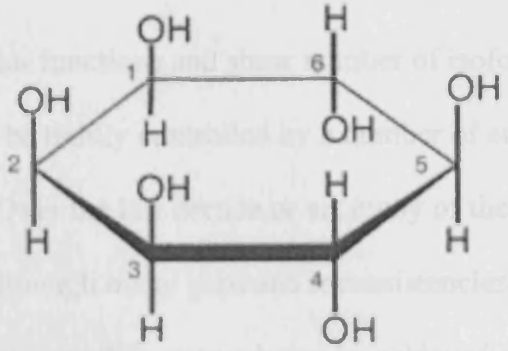
Inositol is a six-carbon sugar molecule which, due to the ability of each carbon to be independantly phosphorylated, can be converted into multiple forms, many of which have been assigned specific biological functions (see Fig. 1.2 for structure and annotation). In addition to single phophorylations, some positions can also be

pyrophosphorylated (PP) so that in cells inositides have been found containing up to 8 phosphate groups (Albert et al., 1997; Laussmann et al., 1996).

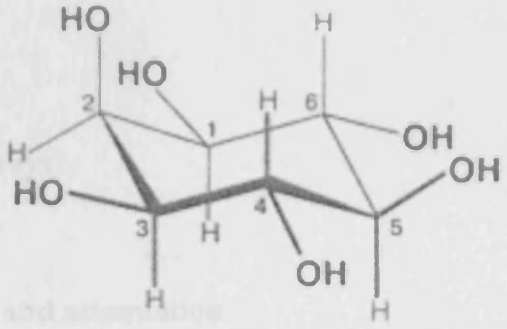
The ability of a single molecule to have so many phosphorylation states as well as multiple isomers within each state provides a powerful mechanism by which many functions can co-ordinately be regulated within the cell. So far these functions range from the stimulation of calcium release ($Ins(1,4,5)P_3$), regulation of chloride ion channels ($Ins(3,4,5,6)P_4$) (Carew et al., 2000; Ho et al., 2002), control of mRNA export from the nucleus ($InsP_6$) (Miller et al., 2004; York et al., 1999), chromatin remodeling (InsP's 4-6) (Shen et al., 2003; Steger et al., 2003) and even direct protein phosphorylation (pyrophosphates) (Saiardi et al., 2004) although there are doubtless others. Indeed, phytate (inositol hexakisphosphate, $InsP_6$) makes up almost two-thirds of the phosphate content of plant seeds, and its synthesis is essential for yolk sac development in mice, therefore is likely to also be used as an energy, or phosphate store (Stevenson-Paulik et al., 2005; Verbsky et al., 2005a).

In addition to the more than 30 known soluble forms, inositol can also be joined to a lipid moiety to form phosphatidyl inositides. These can also be phosphorylated multiple times, and at present 7 forms are known. These again have numerous biological functions such as the regulation of endosomal trafficking, protein kinase B and protein kinase Cε regulation and the recruitment of proteins to specific sections of membrane via pleckstrin homology (PH) domains as will be discussed later (Alessi and Cohen, 1998; Bos, 1995; Comer et al., 2005; Ikonomov et al., 2003; Sbrissa et al., 2002; Stokoe et al., 1997).

A



B



C

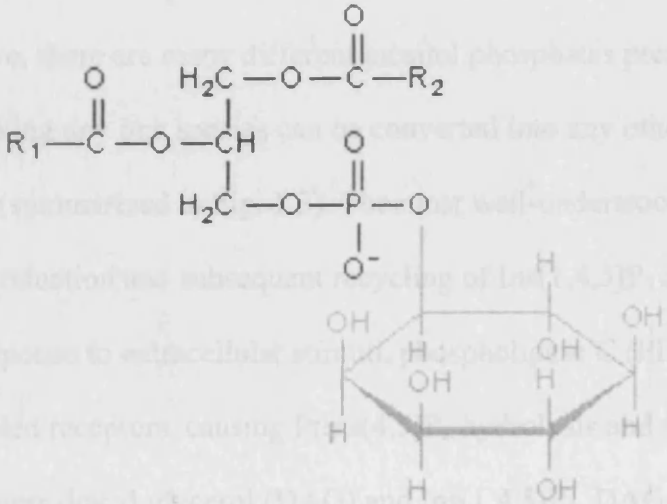


Figure 1.2: The structure of inositol phosphates and phospholipids. The standard numbering system for each carbon is indicated in A and B and the structure of phosphatidyl inositol lipids shown in C.

This astonishing range of cellular functions and shear number of isoforms of inositol polyphosphates must therefore be tightly controlled by a number of enzymes, many of which catalyse multiple steps. Over the last decade or so, many of these have been cloned and characterised and although many gaps and inconsistencies remain, a reasonable understanding of this signalling network can be achieved and is discussed below (Abel et al., 2001).

1.9 Inositol metabolic pathways

1.9.1 Ins(1,4,5)P_3 signalling and attenuation

As mentioned above, there are many different inositol phosphates present in any cell and generally speaking any one species can be converted into any other via various intermediate steps (summarized in Fig. 1.3). The most well-understood pathway is undoubtably the production and subsequent recycling of Ins(1,4,5)P_3 from PtIns(4,5)P_2. In response to extracellular stimuli, phospholipase C (PLC) is activated via G-protein coupled receptors, causing PtIns(4,5)P_2 hydrolysis and the production the second messengers diacyl glycerol (DAG) and Ins(1,4,5)P_3. DAG then activates protein kinase C whereas Ins(1,4,5)P_3 diffuses into the cytosol initiating Ca^{2+} release from intracellular stores, leading to a whole range of cellular responses (see later).

In order to terminate the signal, two different mechanisms are employed; first, Ins(1,4,5)P_3 can be hydrolysed by 5' and 1' phosphatases to form Ins(4)P which is then subsequently converted into *myo*-inositol by IMPase (inhibited by lithium), and

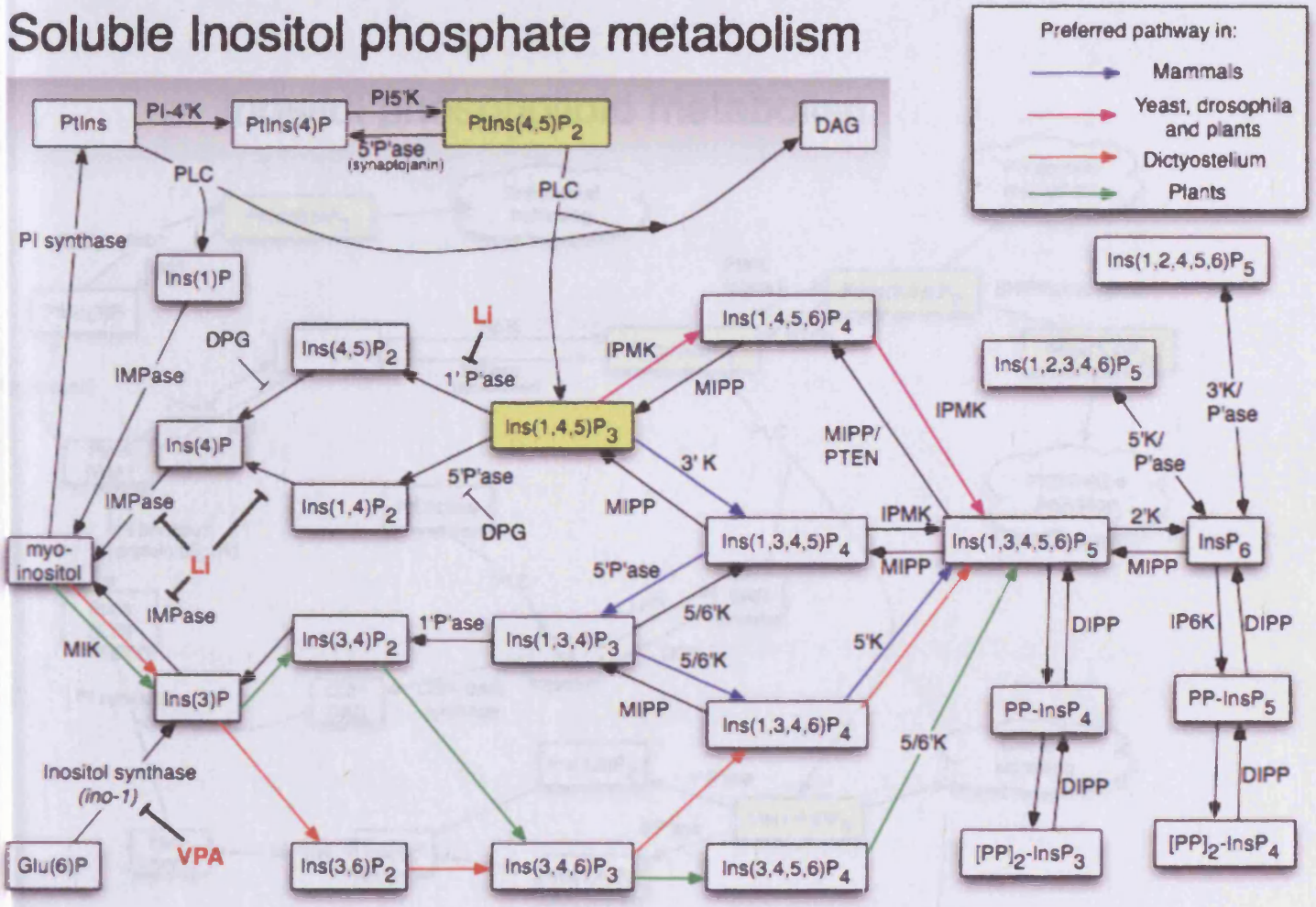


Figure 1.3: The metabolism of inositol polyphosphates. As far as is possible, inter species variation has been indicated by the coloured arrows. These show the preferred routes in these organisms. Abbrevations used: Ins – inositol; P'ase – phosphatase; MIK – *myo*-inositol kinase; VPA – valproic acid; DAG – diacylglycerol; DPG – 2'3'- diphosphoglycerate; DIPP – diphospho inositol polyphosphate phosphatase.

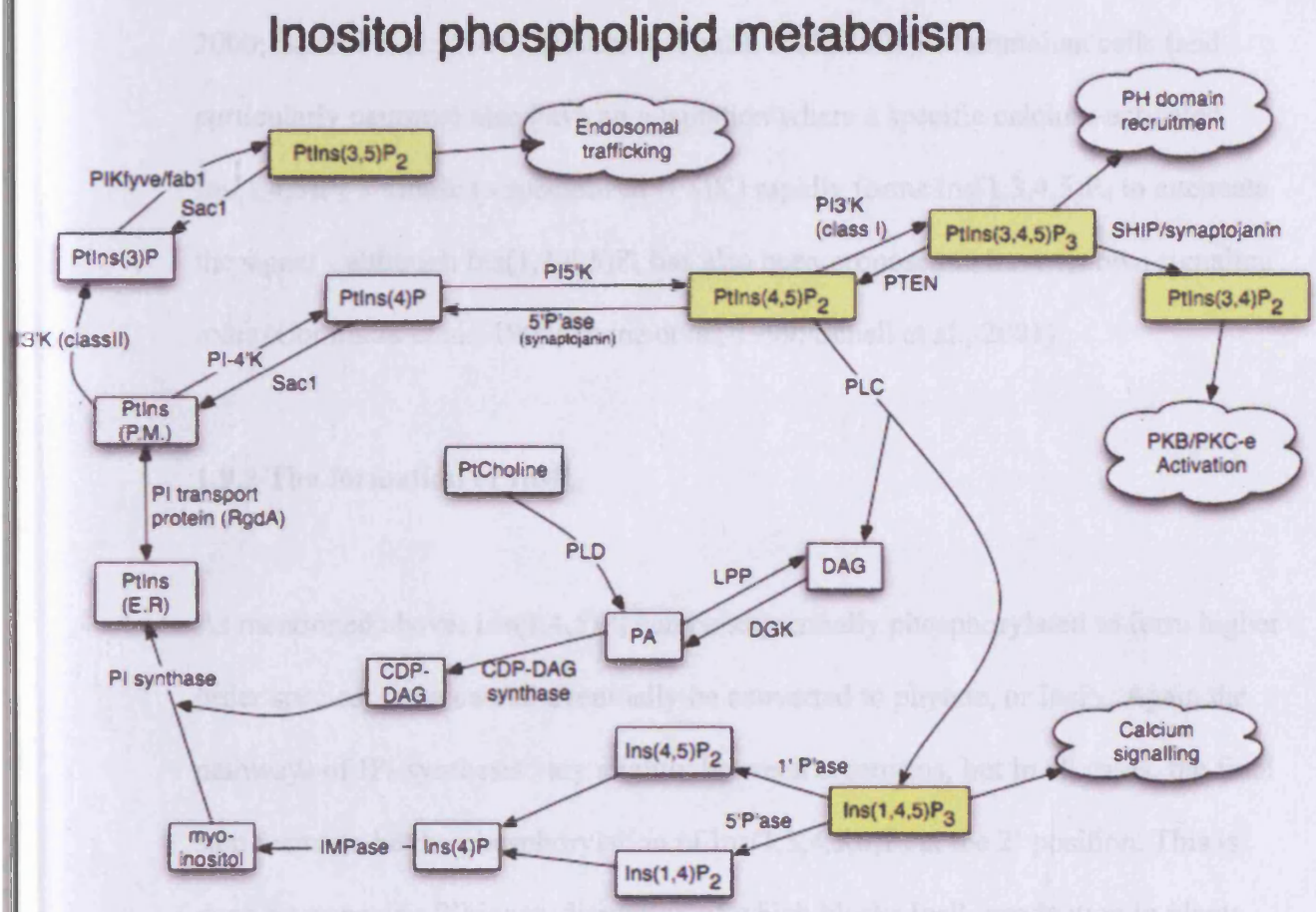


Figure 1.4 : Inositol phospholipid metabolism. Abbreviations used: PtIns, phosphatidyl inositol; P.M. – plasma membrane; E.R. – endoplasmic reticulum; DAG – diacyl glycerol; PA – phosphatidic acid; CDP - DAG -cytidine diphosphodiacyl glycerol.

recycled back to phosphatidyl inositol (PtIns) (Abel et al., 2001; Van der Kaay et al., 1995; van Haastert and van Dijken, 1997). Second, in *Dictyostelium,* yeast, *Drosophila* and plants the Ins(1,4,5)P_3 signal can be attenuated by phosphorylation where a promiscuous inositol polyphosphate multikinase (IPMK) phosphorylates Ins(1,4,5)P_3 on the 6' and/or 3' positions to produce Ins(1,3,4,5,6)P_5 (Odom et al., 2000; Saiardi et al., 1999; Stevenson-Paulik et al., 2002). Mammalian cells (and particularly neurons) also have an adaptation where a specific calcium-activated Ins(1,4,5)P_3 3'kinase (a specialized IPMK) rapidly forms Ins(1,3,4,5)P_4 to attenuate the signal - although Ins(1,3,4,5)P_4 has also been proposed to have its own signaling roles (Communi et al., 1995; Irvine et al., 1999; Schell et al., 2001).

1.9.2 The formation of $InsP_6$

As mentioned above, Ins(1,4,5)P_3 can be sequentially phosphorylated to form higher order species; these can all eventually be converted to phytate, or $InsP_6$. Again the pathways of IP_6 synthesis vary slightly between organisms, but in all cases, the final step seems to be the phosphorylation of Ins(1,3,4,5,6)P_5 at the 2' position. This is done by a specific 2'kinase, disruption of which blocks $InsP_6$ production in plants, yeast, and mice (Miller et al., 2004; Stevenson-Paulik et al., 2005; Verbsky et al., 2005a; Verbsky et al., 2005b; Verbsky et al., 2002). Once made, $InsP_6$ can then be pyrophosphorylated to form $InsP_7$ and $InsP_8$, or in *Dictyostelium* it is involved in futile cycles of interconversion to Ins(1,2,3,4,6)P_5 and Ins(1,2,4,5,6)P_5 with no apparent signaling purpose (Huang et al., 1998; Saiardi et al., 2001; Stephens and Irvine, 1990).

In mammals, yeast and *Drosophila*, $InsP_6$ is only formed from $Ins(1,4,5)P_3$ and this activity is predominantly nuclear. In mammals, this is done via a dephosphorylation of $Ins(1,3,4,5)P_3$ to give $Ins(1,3,4)P_3$ which is then sequentially phosphorylated at the 6' and 5' positions by an $Ins(1,3,4)P_3$ 5/6'kinase to give $Ins(1,3,4,5,6)P_5$ (Verbsky et al., 2005b). In lower eukaryotes and plants, although this pathway exists, $InsP_5$ is generally produced by the phosphorylation of $Ins(1,4,5)P_3$ at the 6' and 3' positions by IPMK to give the same product (Estevez et al., 1994; Ongusaha et al., 1998; Ongusaha et al., 1997).

In addition to these pathways, it is interesting that there is a further pathway for the production of $InsP_6$ so far only identified in plants and *Dictyostelium*. In the cytoplasm of these organisms, $InsP_6$ is rapidly made by the sequential phosphorylation of *myo*-inositol. Although the path is slightly different, both require an initial 3'phosphorylation and involve the production of an $Ins(3,4,6)P_3$ intermediate (Brearley and Hanke, 1996; Stephens and Irvine, 1990). Therefore in these organisms $InsP_6$ can be synthesized by a route independent of $Ins(1,4,5)P_3$ production and calcium signaling.

Why do these organisms have such a unique pathway? It is certainly no coincidence that both plants and *Dictyostelium* contain large amounts of $InsP_6$ – approximately 1% of the dry seed weight in plants and at concentrations of almost 1mM in *Dictyostelium* (Martin et al., 1987; Raboy, 2001). One explanation is their use as an energy or phosphate store; both organisms have a dormant phase of their lifecycle either as a seed or a spore, which would be obsolete in other organisms such as yeast and animals. On the other hand it is highly possible that this pathway does exist at a low

rate in other organisms – only metabolic emphasis has shifted for survival in their respective evolutionary niches.

1.9.3 Inositol phosphatases

In addition to the kinases mentioned above, there are a number of inositol phosphatases responsible for the converse reactions. The most versatile of these is the multiple inositol polyphosphate phosphatase (MIPP) family. These enzymes are part of the histidine acid phosphatase family and are the only known means by which Ins(1,3,4,5,6)P_5 and $InsP_6$ can be broken down in the cell (Craxton et al., 1995; Nogimori et al., 1991). Indeed this activity was originally identified in *Dictyostelium* as a means of Ins(1,4,5)P_3 synthesis in cells lacking phospholipase C activity and it appears to be the same activity that is upregulated in cell lacking PO and may be responsible for lithium resistance (Van Dijken et al., 1995a; Williams et al., 1999). *In vitro* this enzyme has been shown to act on a number of inositol phosphates, although greatly favouring substrates with 4 or more phosphates, and will generally attack the 2' and then the 3' and 6' positions (Caffrey et al., 1999; Craxton et al., 1995; Yu et al., 2003). To date however, the *in vivo* role of this enzyme remains unclear, mainly due to the fact that it appears to be localized within the endoplasmic reticulum (ER), where so far none of its substrates have been reported. Also, although a mouse knockout has been made, no phenotype was found – possibly due to compensatory upregulation of a second gene (Ali et al., 1993; Chi et al., 2000).

In addition to MIPP, another family of enzymes have been isolated which remove the β-phosphate from a pyrophosphorylated inositide (Caffrey et al., 2000; Safrany et al.,

1998). Although the regulation and cellular function of these also remains unclear, it seems that these enzymes along with MIPP account for all the production of $InsP_3$ from the higher inositol phosphates, although some 5' phosphatase enzymes may be able to produce Ins(1,3,4)P_3 from Ins(1,3,4,5)P_4. The phosphates remaining after these activities are then subsequently removed by 1', 4' and 5' phosphatases. The most intensively studied are the 5'phosphatases that have been shown to be active on Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and the lipids PtIns(4,5,)P_2 and PtIns(3,4,5)P_3. These can be subdivided into three related classes, each with varying substrate specificity (reviewed in (Majerus et al., 1999)).

In addition to these there is a distinct 1'phosphatase, encoded by a single gene in mammals and able to dephosphorylate Ins(1,4)P_2, Ins(1,4,5)P_3 and Ins (1,3,4)P_3 (Van Dijken et al., 1996b; Van Dijken et al., 1995b; van Haastert and van Dijken, 1997). This is a member of the lithium inhibited gene family that includes IMPase, although structural homology seems to be restricted to the active site (York and Majerus, 1990; York et al., 1994; York et al., 1995). Finally, the dephosphorylation of inositol is completed by the activity of IMPase. In cells, only Ins(1)P, Ins(3)P and Ins(4)P have been found and all of these can be used as substrate for a single enzyme (Gee et al., 1988; Shears, 1989). However, as with many of these enzymes, some organisms have multiple genes, each with different substrate preferences (Van Dijken et al., 1996b).

1.10 Inositol phospholipid metabolism

In addition to the soluble inositide pathways above, inositol phospholipid metabolism is also intimately interconnected (summarized in fig. 1.4). All lipid inositides can only

be produced via *myo*-inositol, and therefore the recycling of soluble forms may prove to be critical for lipid signaling. PtIns synthesis occurs in the endoplasmic reticulum by the fusion of cytidine diphosphodiacyglycerol (CDP-DAG) and *myo*-inositol by PI synthase (Gaigg et al., 1995; Gardocki et al., 2005; Zinser et al., 1991) and is then transported to the plasma membrane where the various phosphatases and kinases act.

At the plasma membrane, PtIns is then phosphorylated at either the 3' or the 4' positions by class II PI3'kinases or PI4'kinases respectively. These two pathways are then extended by further phosphorylation, either by PIKfyve/fab1 5'kinases to form PtIns(3,5)P_2 and mediate endosomal trafficking, or PI-5-kinase to form PtIns(4,5)P_2 (Desrivieres et al., 1998; Homma et al., 1998; Ikonomov et al., 2003; Odorizzi et al., 1998; Odorizzi et al., 2000; Sbrissa and Shisheva, 2005). As described previously, PtIns(4,5)P_3 is a central molecule in a number signaling pathways - as a substrate for PLC to invoke calcium signaling, for the regulation of actin dynamics and as a precursor for PtIns(3,4,5)P_3 production (Nebl et al., 2000; Ostrander et al., 1995; Sciorra et al., 1999). In higher eukaryotes (yeast lack the enzyme), extracellular stimuli are then able to activate the class I PI3'kinases through G-protein coupled receptors, producing PtIns(3,4,5)P_3 and initiating a whole range of cellular signaling events (Leevers et al., 1999; Shepherd et al., 1998). In order to restrict and/or terminate the signal, PtIns(3,4,5)P_3 is then broken down by the actions of either the tumour suppressor PTEN (phosphatase and tensin homologue) , or SHIP (SH2-containing inositol phosphatase) - removing the 3' or 5' phosphates respectively (Maehama and Dixon, 1999; Pesesse et al., 1998).

Although some of these molecules, such as PtIns(4,5)P_2 are precursors for the production of second messengers, the most important role for many of these lipids is the recruitment of proteins to specific parts of specific membranes. This is done through conserved lipid-binding domains, most notably pleckstrin homology (PH) domains and are involved in a number of processes including, chemotaxis, endocytosis, phagocytosis and cytokinesis. PH domains are structurally conserved modules of approximately 100 amino acids that bind to the head groups of inositol lipids, and it has recently been argued, soluble inositol phosphates (Kavran et al., 1998; Lemmon et al., 1996; Razzini et al., 2000). Many PH-domain containing proteins have been identified and although a common structure is conserved, subtle differences lead to different binding preference, and therefore localization.

1.11 Complexity in inositol metabolism and signalling

One of the more distinctive features of inositol phosphate metabolism is its complexity and the variety of signaling pathways that can be regulated. This is further compounded by the fact that many of the enzymes can use multiple substrates and produce multiple products. For example, the Ins(1,3,4)P_3 5/6 kinase family of enzymes have been reported to also be able to use Ins(1,4,5)P_3 and Ins(3,4,5,6)P_4 as substrates, adding phosphates at the 3' and 1' positions respectively (Field et al., 2000; Yang and Shears, 2000). In addition, some of the enzymes involved in inositol metabolism have dual functionality, such as IPMK which was first identified as *Arg82* - involved in the transcriptional regulation of arginine metabolism in *S. cerevisiae*, an activity which is independent of inositol kinase activity (Dubois et al., 2000). In addition, it has been suggested that Ins(1,3,4)P_3 5/6 kinase can also act as a protein

kinase, although the data for this is conflicting (Caffrey et al., 2001; Resnick et al., 2005; Wilson et al., 2001).

Inositol phosphate metabolism is further complicated by the fact that many proteins may not distinguish between soluble and lipid inositides. *In vitro* studies have shown that MIPP, IPMK and PTEN are all able to use both as substrates and there are also indications that the pyrophosphates may be able to compete for the lipid binding of PH-domains (Caffrey et al., 2001; Luo et al., 2003). Whether this proves to be significant *in vivo* may be debatable, particularly as many of the enzymes have a highly regulated localization as well as spatial and temporal activation, but it does suggest the possibility that the relative levels of soluble and lipid inositides may be able to affect the signaling of the other and it may well be naïve to think of each in isolation. In addition, inositol signaling is an extremely robust system, and often there is a significant degree redundancy between enzymes, or alternative pathways such that when the system is perturbed, homeostasis and signaling can be maintained.

1.12 Regulation of phospholipid synthesis

Inositol signaling is important for a number of cellular process and therefore the total amount of inositol within the cell is carefully maintained. In addition to the recycling pathway of inositol lipids, *myo*-inositol is made *de novo* from glucose-6-phosphate by the action of inositol synthase, producing Ins(3)P which is subsequently dephosphorylated by IMPase. As IMPase is inhibited by lithium and inositol synthase is indirectly inhibited by VPA, this pathway is a common target for both drugs and therefore is a good candidate for the action of these drugs on aggregation.

Inositol synthase is the protein product of the *ino-1* gene - disruption of which leads to inositol auxotrophy in both yeast and *Dictyostelium (Fischbach et al., 2006; Hirsch and Henry, 1986)*. The expression of *ino-1* is tightly regulated by a number of factors involved in sensing the inositol and lipid status of the cell. Upon addition of inositol to the medium of yeast, expression of *ino-1* is repressed 12-fold, an effect which is amplified to 33-fold if choline is included as well (Hirsch and Henry, 1986). In the absence of inositol, transcription is derepressed by the binding of the transcriptional activators Ino2p and Ino4p as a heterodimer to the UAS_{INO} sequence, found upstream of *ino-1* and other inositol-sensitive genes, including *Ino2* (Ambroziak and Henry, 1994). Upon addition of inositol, transcription is blocked by the translocation of a further element, Opi1p, from the ER to the nucleus where it is able to mediate repression though a direct interaction with Ino2p (Gardenour et al., 2004; Heyken et al., 2005). Opi1p is used as a sensor of the lipid status of the cell and is regulated by interaction with ER-protein including the proteins vesicle-associated-membrane protein associated protein (VAP), Scs2p and phosphatidic acid (Loewen et al., 2004; Loewen et al., 2003). In addition, it has also been shown that Opi1p can be phosphorylated by protein kinases A and C and casein kinase II, altering its ability to repress transcription (Chang and Carman, 2006; Sreenivas and Carman, 2003; Sreenivas et al., 2001).

As this process is designed to up-regulate inositol production when intracellular levels of inositol are limiting, it is perhaps no surprise that treatment with lithium and VPA also increases the expression of *ino-1* and *ino-2*, although not sufficiently to stop inositol depletion (Vaden et al., 2001). However, upon disruption of the yeast *opi1*

gene, *ino-1* becomes constitutively expressed, which leads to increased resistance to growth in lithium and therefore a similar mechanism may also be responsible for lithium resistance in *Dictyostelium*.

1.13 Inositol phosphates and the nucleus

Recently it has emerged that in addition to the roles of inositol lipids and phosphates in the cytosol and at the plasma membrane, there is a distinct nuclear pool of phosphoinositides, independent from the rest of the cell, with its own quite separate metabolism (reviewed in (Cocco et al., 2004; Hammond et al., 2004; York, 2006)). This nuclear pool has been shown to be important for a number of nuclear functions; in yeast loss of either PLC, IPMK or $InsP_5$ 2' kinase results in defective mRNA export (Odom et al., 2000; York et al., 1999) and the pyrophosphates appear to be involved in both telomere maintenance and DNA repair (Luo et al., 2002; Saiardi et al., 2005; York et al., 2005) although the mechanisms of these are unclear.

In addition, both the higher order inositol polyphosphates and the enzymes involved in their metabolism are involved in transcriptional regulation. For example, IPMK was also identified as a transcription factor involved in the regulation of arginine-responsive genes, known as Arg82 and associates with the ArgR-Mcm1 complex required for an arginine-specific transcriptional response (Yoon et al., 2004). Interestingly, the recruitment of this complex to arginine responsive DNA elements and transcriptional regulation requires IPMK/Arg82 protein but not kinase activity and therefore it appears that IPMK/Arg82 has two independent functions (Dubois et al., 2000; El Alami et al., 2003).

IPMK/Arg82 and changes in the higher order inositol polyphosphates have also been implicated in transcriptional regulation via chromatin remodeling complexes. In eukaryotic cells the DNA is tightly wrapped around nucleosomes and thus packaged into chromatin. In the nucleus this is generally repressive to transcription by limiting the access of the RNA polymerase machinery to the DNA. Therefore there are many complexes of proteins whose function it is to move these nucleosomes along the DNA. Using the energy from ATP hydrolysis, these complexes move the nucleosomes out of the way, unblocking the promoter sites and leaving the DNA accessible for transcription (Fyodorov and Kadonaga, 2001; Olave et al., 2002).

In a study by Shen *et al.* it was demonstrated that *in vitro*, different higher order inositol polyphosphates can directly interact with and either activate, or inhibit chromatin remodeling complex activity at the *Ino-1* promoter. In this study it was seen that the ATPase and nucleosome mobility activities of the NURF, ISW2 and INO80 complexes is directly inhibited by $InsP_6$, but no other inositides. Conversely they also demonstrate that the remodeling activity of the Swi/Snf complex was stimulated by both Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 although this time independently of ATPase activity (Shen et al., 2003). Others have shown that *in vivo* the induction of the yeast *pho-5* gene requires the inositol kinase activity of IPMK/Arg82, as well as PLC (Auesukaree et al., 2005), and Steger *et al.* went on to demonstrate that null mutations of these genes reduce the recruitment of INO80 and Swi/Snf complexes to this promoter. This therefore again points towards a role for $InsP_4$ and/or $InsP_5$ production in chromatin remodeling, although this time they found no requirement for $InsP_6$ production (Steger et al., 2003). These experiments would

therefore predict that under conditions with high nuclear $InsP_4$ and $InsP_5$ and low $InsP_6$, expression is increased. In addition to this, other studies have also found that in lymphocytes, nuclear $PtIns(4,5,)P_2$ can also regulate the binding of the Brahma associated factor (BAF) complex (of the Swi/Snf family) to chromatin (Zhao et al., 1998). And therefore changes in the nuclear inositol metabolism may be responsible for transcriptional regulation on a genome-wide scale co-ordinately regulating the metabolism of inositol, phosphate and arginine.

If these processes prove to be significant *in vivo*, they indicate mechanisms by which inositol metabolism may affect gene expression, potentially across the whole genome and potentially explains some of the transcriptional changes observed upon the chronic lithium treatment of both mice and *Dictyostelium* (Bosetti et al., 2002; Keim-Reder, 2006). Therefore, in addition to a direct effect on cell signalling, changes in inositol metabolism may also induce transcriptional changes and alter cell behaviour - potentially providing an alternative mechanism for how alterations in higher order inositol polyphosphate metabolism, as seen in the PO mutant, may confer drug resistance.

1.14 Aims of this study

In *Dictyostelium* the loss of prolyl oligopeptidase activity confers resistance to the effects of lithium and VPA on aggregation. These cells also have elevated $Ins(1,3,4,5,6)P_5$ phosphatase activity resulting in increased $Ins(1,4,5)P_3$ production and therefore it is likely that this is may be able to compensate for the inositol-depleting effects of both drugs. The aim of this study is to clone the MIPP gene

responsible for this activity, and by generating null and overexpressing mutants, determine the effect altered MIPP activity has on lithium sensitivity. If MIPP activity proves to be important for lithium resistance, the role of the higher order inositol phosphates and the mechanism of resistance will be investigated by generating further mutants in other genes involved in their regulation such as the inositol polykinases.

In addition, the ability of lithium and VPA to inhibit aggregation indicates that the recycling of inositol and the synthesis of new phospholipids may have an important role in cell migration. PLC represents the major link between inositol lipid signaling and the soluble inositides and loss of this gene should result in reduced inositol recycling. Therefore by studying the effect of this mutation on lithium resistance, the roles of both PLC and phospholipid recycling on cell movement will hopefully be better understood.

Chapter 2:

Materials and methods

2.1 Bioinformatics

Indentification of *Dictyostelium* homologues was done using the Blast portal on the Dictybase website (www.Dictybase.org) (Altschul et al., 1997). Where known, the conserved or catalytic domains of proteins in other species were used to search the predicted protein database. If such details were unavailable, whole protein sequences were used and the "expect value" decreased until some potential proteins found. In this case, *Dictyostelium* homologues were verified by comparison with the same protein across evolution. Dictybase reference codes are given for all *Dictyostelium* genes. Alignments were constructed using the Clustal algorithm as part of the MacVector 7.2 software suite (Accelerys).

2.2 *Dictyostelium* cell culture

Dictyostelium cells were grown axenically in either HL-5 medium (14 g/L proteose peptone, 7 g/L yeast extract, 13.5 g/L glucose, 0.5 g/L Na_2HPO_4, 0.5 g/L KH_2PO_4,pH 6.4.) for normal culture and antibiotic selections, or in FM minimal medium (Formedium) which contains no inositol, prior to inositol phosphate determination. Cells were also grown and allowed to develop on SM agar plates (10 g/L proteose peptone, 1 g/L yeast extract, 10 g/L glucose, 1.9 g/L KH_2PO_4, 1.3 g/L $K_2HPO_4.3H_2O$, 0.49 g/L $MgSO_4$, 17 g/L agar) on a lawn of *Klebsiella aerogenes* by standard methods.

In all cases, except where otherwise stated, the AX2 strain was used as wild-type and is the parent of all mutants. Cells were both starved and washed in KK2 buffer (16.5

mM KH_2PO_4, 3.8 mM K_2HPO_4 pH 6.2). When required, cells were pelleted by centrifugation at 700 x g for 2 minutes.

2.3 RNA extraction and cDNA synthesis

Prior to extraction, 5 x 10^6 cells were seeded in a 10cm Petri dish and left to grow overnight. The following day, cells were pelleted and washed once in KK2 buffer before total RNA extraction using a Roche kit. RNA quality was then checked by running 1µl on a 1% agarose/ethidium bromide gel. Any samples lacking two clear ribosomal bands were discarded. RNA was then quantified by measuring absorbance at 260 and 280nm in a spectrophotometer. Reverse transcription was done using 1µg total RNA using first strand synthesis kit (Roche) as per the manufacuters instructions. In all cases random primers were used.

2.4 PCR analysis of expression

To check for expression of each gene, cDNA was analysed by PCR. For each gene the following primer pairs were used to generate a 400-600bp specific product for each gene: *mppA* GTGTACCCTATCCAAGTGATA / TACTTAGAAGTGAACACCAA TG; *mppB* TATTGGTCGTCATGGTTCAAGA / CATTGTCATTAATTGATTGAT CTCG; *ipkA1* TAGTCCACCATCATCCAATGA / GAATGTGGTTTCATATGAG ATGG; *ipkA2* AATGGGTGTAAGACAGCACG / GAGTAAAAGTGAACCACCA TAG; *ipkA3* GTGGTGCAAAACTTGTGCATCC / GAGTTAATGGATCTACATG AGCG; *ipkB* CTAACTGATGAGTTAGGTGAC / GCGTTATGATTAA TGAACTCTTG.

PCR conditions used were 30 cycles of 92° x 1 min., 52° x 1 min. and 68° x 1 min. Biotaq DNA polymerase (Bioline) was used in the supplied buffer supplemented with 2 mM MgCl, 0.1mM dNTP's and 0.4mM each primer. 10ng cDNA was used as template in all reactions. PCR products were then examined using ethidium bromide in a 1.5% agarose gel.

2.5 Extraction of genomic DNA

For use as either a PCR template or for Southern blotting, 10^7 cells were pelleted and washed once in KK2 before being dissolved in 1ml DNAzol reagent (Invitrogen). Cell debris was then removed by centrifugation at 15,000 x g, and DNA precipitated from the supernatant by the addition of 0.5 ml 100% ethanol. This was left for 5 minutes at room temperature before centrifugation at 15,000 x g for 5 minutes. The DNA pellet was then washed once in 70% ethanol and finally dissolved in 100µl 8mM NaOH. The pH was then adjusted to 7.0 using 3.2µl 1M HEPES buffer.

2.6 General cloning

Throughout this project, all plasmids constructed were primarily screened by restriction digest. All enzymes were supplied by New England Biolabs and used in the appropriate buffer. For screening of plasmid clones, approximately 0.5 µg of DNA from each was digested with enzyme and analysed by agarose gel electrophoresis. Clones to be used for protein expression were then subsequently sequenced. Ligations were carried out at room temperature for approximately 3-4

hours using T4 DNA ligase (New England Biolabs). Generally a 3:1 molar ratio of vector : insert was used.

2.7 Cloning of *mppA* and generation of knockout and overexpression constructs

A full-length clone of *mppA* was made by PCR from genomic DNA using the primers CACA AACCAACTATGATGGTAAAAAT / TTATTGGATCAATGGAGATCT - TGA. This was then cloned into the TOPO blunt II vector (Invitrogen) as per the manufacturers instructions, sequenced and used as the starting clone for all other constructs. To facilitate PCR screening of potential disrupted clones, the full-length clone was first truncated at its 3' end by digestion with BglII and BamHI restriction enzymes and subsequent re-ligation. The loxP flanked blasticidin selection cassette from pBLPBSR (Faix et al., 2004) was then excised by SmaI digest and inserted into the middle of the middle of this truncated *mppA* clone at the BstBI site, which was blunted using Klenow. The Blasticidin cassette was found to be inserted in the same orientation as the *mppA* gene, and this construct was named pJSK116. The entire disruption construct was then excised from the vector backbone by NotI digest prior to *Dictyostelium* transformation.

To generate a completely untagged, full-length *mppA* expression construct the RHI8 vector was used (a gift from R. Insall, unpublished). This is a G418 selectable, Ddp1-based episomal plasmid using an actin15 promoter to drive expression of a gene of interest.

Wild-type genomic DNA was used as template to generate a full-length clone of *mppA* with additional 5'BamHI and 3' NotI restriction sites. PCR was done as normal, using the acuzume proofreading polymerase (Bioline) and the following primers: GGATCCCACAAACCAACTATGATGGTAAAAAT / GCGGCC GCTTATTGGATCAATGGAGATCTTGAATC. The PCR product was then gel-purified and cloned into TOPO blunt II (Invitrogen) before validation by sequencing. This was then cut with BamHI and NotI and ligated into RHI8 cut with the same enzymes to generate the final construct, named pJSK 166.

2.8 Construction of Inositol kinase overexpression constructs

For expression in *Dictyostelium*, the pTX-FLAG episomal vectors were used (Levi et al., 2000). A full-length genomic clone of *ipkB* was obtained from wild-type cells using the primers: ATGGGTCGAAATAATACATTAC / GTTTTTATACC GATGAT TTTCTT, and subsequently clones in the TOPO blunt II vector (pJSK 102). A 5'BamHI restriction site in frame with the initiating ATG was then added by using this as a template for PCR using the GGATCCATGGGTCGAAATAATACA TTAC and the initial reverse primer and again cloned into TOPO blunt II (pJSK114). The whole gene was then cut out with BamHI and Xho I (from the TOPO multiple cloning site) and ligated into pTX-FLAG cut with the same enzymes (pJSK123).

The *ipkA1* expression vector was cloned in an identical fashion except that the original clone used was the cDNA obtained from the Japanese cDNA sequencing constortium (CHB152). The intermediate clone, made using the primers GGATCCATGAATGACCAAAATAAATTTCAAG / CTCGAGTTTTTGTGGT

TGTTGAGGTTGTTG, to add 5' BamHI and 3' Xho I restriction sites was named pJSK130, and the final N-terminal FLAG fusion plasmid pJSK134.

2.9 Construction of a PLC knockout construct

The *plc* knockout vector was made with a few refinements for ease of cloning. Two regions of the *plc* gene, at the 5' and 3'ends were amplified from wild-type genomic DNA using the following primers: 5' fragment – GTCGATTTATCATTAG AATCATTAGC / AGATCTTACGTCGACCCATCCCAAACGTCCAATTCAACG C; 3' fragment – GTCGACGTAAGATCTCTTCACCATATCCTGTCATACTCAG / GGAATTGCATTCATTTTCTTTGTATC. These two segments do not cover the whole gene such that a double crossover event into the genomic locus will result in a deletion of 100bp of sequence and the 5' fragment was designed to start just after the 5' end of the gene so that further primer could be used for screening.

The PCR primers were also designed such that SalI and BglII sites were added to the 3'end of the 5' fragment and the 5'end of the 3' fragment. This overlapping sequence means that the two fragments could be combined by PCR, by mixing the two fragments together and using the two end primers. This product was then cloned into the TOPO blunt II vector (Invitrogen). Finally, this plasmid was cut with SalI/BglII and the loxP – flanked blasticidin cassette from pBLPBSR was excised by SalI/BamHI digest, gel purified and ligated in. This final construct (pJSK163) was then cut with EcoRI to release the knockout cassette before transformation.

2.10 *Dictyostelium* transformation

Cells were transformed by electroporation as previously described (2002). Briefly, 10^7 cells, growing in log-phase were washed once in ice-cold electroporation buffer (50 mM sucrose in KK2) before being resuspended in a final volume of 800µl in an electroporation cuvette. For integrating plasmids, 20µg and for episomal plasmids 10µg DNA was then added and incubated on ice for 10 minutes. Cells were then electroporated at 1kV before a further 10 minute incubation on ice. The cells were then transferred into a 10cm petri dish and 8µl of salt solution (0.1 M $MgCl_2$, 0.1 M $CaCl_2$) added. After 20 more minutes the cells were then finally resuspended in 10 ml HL-5 medium.

After 24 hours, transformations of knockout vectors were cloned into 6 x 96 well plates in the presence of 10 µg/ml blasticidin S. After approximately 2 weeks, multiple clones were seen and transferred into individual wells of a 24 well plate for screening. Transformations with episomal expression vectors were simply expanded in 10cm dishes and selection started 24 hours post-transformation with 20 µg/ml G418. Due to the extrachromasomal nature of these vectors, all clones should have the same copy number and have no positional effects. Therefore, sub cloning is unnecessary and all colonies produced were pooled.

2.11 Screening of *mppA* and *plc* knockout clones

After the isolation of independent clones, each was grown in 1ml culture in a 24 well plate. When each wells became confluent, they were resuspended and the cells

harvested into microfuge tubes. Medium was replaced in each well so that the residual cells would repopulate. The harvested cells were then pelleted and solubilized using 250µl DNAzol reagent (Invitrogen). Genomic DNA was then precipitated by addition of 125 µl 100% ethanol and incubater at 22°C for 10 minutes. DNA was pelleted and washed once in 250 µl 70% ethanol before dissolving in 20µl 8mM NaOH (freshly prepared).

To differentiate between integration at the desired locus or at a random position, the configuration of the genomic locus was analysed by PCR. As the *mppA* knockout construct was truncated at its 3'end, the original primers used to obtain the initial genomic clone could be used as only the endogenous locus will give a product. The *plc* knockout construct is also slightly truncated at its 5'end and therefore the following primers could be used : ATGGATACTTTAACAAATTCCCAAG / GAT GACCAATTCTATTGTGACCAAC, again as the 5'primer will not bind the original construct, only the genomic locus will be amiplified. In both cases, homologous recombination and integration of the blasticidin cassette, will lead to a 1.3Kb increase in the size of the PCR product.

Therefore 1µl of genomic DNA from each sample was used as template for PCR using Biotaq polymerase (Bioline). The conditions used were: 30 cycles of 92° x 1 min., 49° x 1 min. and 68° x 1.5 min. The supplied NH4 buffer was again supplemented with 2 mM MgCl, 0.1mM dNTP's and 0.4mM of each primer.

2.12 Southern blotting

Positive *mppA* knockout clones were verified by Southern blot. Genomic DNA from 1 x 10^7 cells was cut overnight at 37° with ClaI and SpeI restriction enzymes, run on an 0.6% agarose gel and blotted onto nitrocellulose membrane by standard methods (Southern, 1975). A probe was then generated using the 500bp PCR product generated by the primers: GTGTACCCTATCCAAGTGATA / TACTTAGAAGTG AACCCAATG with the knockout construct as template. PCR was done using Biotaq as described above. The PCR product was then purified using a G-50 column (Amersham Biosciences), and the megaprime kit (Amersham Biosciences) used to label it with ^{32}P as per the manufacturers instructions, using random primers. This was again purified with a G-50 column and boiled for 5 minutes before addition to Church hybridization buffer (0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA) (Church and Gilbert, 1984).

The blot was then incubated with the probe at 65°C overnight, before washing twice with 2 x SSC, 0.1% SDS and twice with 0.5 x SSC, 0.1% SDS (20x SSC stock: 3 M NaCl, 0.3 M sodium citrate, pH 7.0). The membrane was then examined using a phosphoimager screen.

2.13 MIPP activity assays

MIPP activity assays were essentially done as in (Van Dijken et al., 1995a) with a few refinements. Protein samples were prepared by resuspending cells at 2 x 10^8 cells /ml in TEE (20mM triethanolamine pH6.5, 5.9mM EGTA, 0.5mM EDTA) and lysing them through nucleopore filters (5μm pore size). Samples were then centrifuged at 100,000 x g for 30 minutes, and the superatant removed. Pellets were then

resuspended in the original volume of TEE and equal protein levels between samples verified by Bradford assay.

Unless activity was to be analysed by HPLC, MIPP activity was determined by the rate of Ins(1,4,5)P_3 production, as measured using the isotope dilution assay. Reaction conditions were as follows: cell extracts were mixed with an equal volume of TEE supplemented with 20mM CaCl, 50mM LiCl and 200μM $InsP_6$ (final substrate concentration 100μM). 50μl samples were removed at the start and after 30 minutes, and were immediately quenched by the addition of 10μl ice-cold 100% (w/v) trichloroactetic acid (TCA). Quenched samples were then mixed, and left on ice for 10 minutes before the addition of 440μl water. Precipitated protein was removed by centrifugation and TCA removed by solvent extraction using 1ml of 3:1 1,1,2-trichloro-1,2,2 trifluoro ethane : trioctylamine. Ins(1,4,5)P_3 levels were then determined using the isotope dilution assay (Perkin Elmer) as per the manufacturers instructions.

For HPLC analysis, the reactions were set up as above with the exception that in order to prevent $InsP_6$ precipitation, the final $CaCl_2$ concentration was reduced to 1mM $CaCl_2$ (although reaction efficiency is maintained) and the reaction samples were quenched in an equal volume of 20% (w/v) TCA + 2mM EDTA. Precipitated protein was removed by centrifucation and TCA was then largely removed by four sequential extractions in two volumes of diethyl ether (retaining the lower phase), and pH returned to neutrality using 0.5M triethamolamine pH 8.0. Samples were then 0.2 μm filtered before HPLC analysis as described below.

2.14 Prolyl oligopeptidase activity assays

For PO assays, 2 x 10^7 cells were harvested, washed once in KK2 and snap frozen on dry ice. Cells were then resuspended in 50 mM Tris pH7.4, 1mM EDTA supplemented with protease inhibitors (1mM dithiothreitol, 5 μg/ml aprotinin, 1mM benzamidine, 10 μg/ml leupeptin and 0.1mM AEBSF) and lysed by sonication (3 x 10 seconds on ice). Particulate debris was then removed by centrifugation at 15,000 x g for 35 minutes at 4 °C and protein levels in the supernatant quantified by Bradford assay (using BioRad reagent).

PO activity was then measured by cleavage of Z-gly-Pro-pNA (Bachem) to form the coloured product p-nitroaniline (Makinen et al., 1994). 2.5 μg of protein was incubated with 141 μM Z-gly-Pro-pNA (dissolved in methanol) in 50 mM HEPES pH 7.8, 1mM EDTA and 1mM DTT, 0.25% dimethylsulphoxide in a 60μl reaction for 90 minutes at 37°C. Reactions were then quenched with 200 μl 1.5M acetic acid and the absorbance at 490 nm measured. Duplicate reactions were also set up and quenched immediately and these were subtracted to give an absolute reading. Activity was subsequently calculated as a percentage of that in wild-type cell extracts.

2.15 Extraction of Inositol phosphates for HPLC analysis

For the quantitation of intracellular inositol phosphates, cells were harvested from bacterial clearing plates, thoroughly washed free of bacteria and pellets of 1 x 10^9 cells snap—frozen on dry ice. Upon defrosting, cell pellets were resuspended in 1ml ice-cold 20% (w/v) trichloroacetic acid, 5mM NaF and incubated in ice for 15

minutes. At this stage samples were routinely spiked with an internal standard of 7nmol Ins(1,4,5,6)P_4 as none was detectable in any of the mutants used. Samples were then spun at 15,000 x g for 10 minutes at 4°C, the supernatant transferred into a new tube and the extraction repeated again. The two supernatants were then combined and the TCA largely removed as before by four sequential extractions in two volumes of diethyl ether. Sample pH was then returned to 6.0 using 0.5M triethanolamine pH 8.0 and the extracts were freeze-dried.

Nucleotides were then removed by charcoal extraction. Lyphilised samples were resuspended in 400µl pellet wash (0.1M NaCl, 15mM NaF, 0.5mM EDTA pH 6.0), and treated three times with 150µl charcoal suspension (0.1mM NaCl, 50mM sodium acetate, 20% w/v acid-washed Norit A, pH4.0). Charcoal was removed by centrifugation at 13,000 rpm for 5 minutes at 4°C in a microfuge. Pellets were retained and sequentially resuspended in 100µl pellet wash which was then combined with the cell extract. All samples were then filtered to 0.2µm before injection. Quantification of the internal control indicated that consistently over 70% of the sample remained after extraction. After quantification, most of the variability appeared to come from the batch of SM nutrient agar on which the cells were originally grown, and therefore in each case control cells were grown and extracted in parallel.

2.16 HPLC-MDD analysis

Quantification of inositol phosphates by metal-dye detection was based on that described in (Casals et al., 2002; Guse et al., 1995; Mayr, 1988) using Tricorn Mini Q

4.6/50 PE columns (Amersham/GE healthcare). Chromatography solutions used were A: (21μM YCl_3) and B (0.8M HCl, 25μM YCl_3) and the post-column detection reagent was C (2.13M triethanolamine, 500μM 4-(2'-Pyridylazo)-Resorcinol (PAR), pH 9.75). Elution of inositol phosphates were detected by a drop in absorbance at 520nm, although for ease of analysis, the connection to the computer was reversed such that this becomes a peak. All reagents were of analytical grade and supplied by Merck unless otherwise stated. Flow rate was maintained at 1ml/min (A/B) and 0.4 ml/min C and the gradient used was as follows (time(min):%B): 0'-1%, 4'-1%, 6'-2.5%, 7'-2.5%, 8' – 4%, 10' – 5%, 11' – 5.5%, 12' - 6.2%, 13' – 8.5%, 15' – 10%, 19' – 11.2%, 20' – 13.7%, 25' – 14%, 35' – 21.2%, 41' – 35%, 45' – 36%, 50' – 50%, 60' – 90%, 65' – 100%. This gradient was sufficient to separate all phosphorylation states, including the pyrophosphates and many of the isomers of each phosphorylation state. Where possible, peaks were identified using authentic standards, if this was not possible, as the order of elution remains constant between systems, peaks were identified by the order in which they eluted compared to a partial hydrolysis of $InsP_6$ reported in (Hull et al., 1999) and other studies.

For quantification, in the middle of each sample set a four-point calibration was run using 0.5, 1, 2 and 4 nmoles each of Ins(1,4,5)P_3, Ins(1,5,6)P_3, Ins(1,3,4,6)P_4, Ins(1,2,5,6)P_4, Ins(1,4,5,6)P_4, Ins(1,2,3,4,6)P_5, Ins(1,3,4,5,6)P_5 and $InsP_6$ (Cell Signals Inc.). The area of each peak on each chromatogram was then calculated using the Star software suite (Varian), and used to verify a linear response and generate a calibration curve for each phospho inositide. To quantify inositol phosphates other than those commercially available or included in the calibration, a calibration curve was estimated from similar isomers. In the case of the $InsP_7$ and $InsP_8$ as no isomers

are commercially available, calibration was estimated from the $InsP_6$ values corrected for phosphate content. For quantification of cell extracts, a 500µl injection was used (the extract from approximately 7 x 10^8 cells), both of neat sample and a 1:10 dilution and samples were normalised for extraction efficiency using the internal control. For analysis of enzyme assays, all samples were also diluted for 500µl injections.

2.17 Determination of intracellular and secreted Ins(1,4,5)P_3 levels

For the specific measurement of Ins(1,4,5)P_3 the isotope dilution assay was used. For the determination of intracellular Ins(1,4,5)P_3 levels, exponentially growing cells were pelleted, washed once in FM minimal medium, and resuspended at 5 x 10^6 cells/ml in KK2 buffer. Cells were then placed in shaking flasks for 10 minutes to allow any effect of cell stress to be neutralized. 1ml samples were then taken and immediately extracted by addition of 200µl 100% trichloroacetic acid (TCA). TCA was then largely removed by solvent extraction in twice the equivalent volume of 3:1 1,1,2 trichloro 1,2,2 trifluoro ethane : trioctylamine.

For the measurement of secreted Ins(1,4,5)P_3, log-phase cells were washed and resuspended at 3 x 10^6 cells/ml in 10 ml FM minimal medium. Cells were then placed in shaking flasks and 1ml samples removed at the time intervals indicated. Cells were quickly removed from samples by centrifugation at 10,000 x g for 30 seconds in a microcentrifuge and the supernatant removed to a fresh tube. Inositol phosphates in the supernatant were then extracted in 100% TCA and neutralized as above. Samples

were then quantified using the isotope dilution assay (Perkin-Elmer) as per the manufacturers instructions.

2.18 Western blotting

For western blotting, cells were lysed in Laemelli buffer (50 mM Tris-HCL pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 minutes to denature proteins before running on a 10% polyacrylamide gel. The protein from 2 x 10^5 cells was loaded per lane. Western blotting was then carried out using standard methods, and probed with either a rabbit α-PLC antibody (Gift from P.J.M Van Haastert (Drayer et al., 1994)) followed by a secondary horse raddish peroxidase (HRP)-conjugated α-rabbit antibody (Sigma), both used at 1:10,000; or HRP conjugated α-FLAG antibody (Sigma) used at 1:1,000 dilution. In all cases Pierce ECL reagent was used as HRP substrate.

2.19 Analysis of cell movement

For analysis of random cell movement, 24 hours prior to analysis, cells were seeded at 3 x 10^5 /ml in FM minimal medium. On the day of the experiment, the cells were resuspended and 40 μl transferred to a glass-bottomed chamber slide (Lab-Tek) containing 2 ml medium. Cells were then left for 30 minutes to attach and recover before the medium was aspirated and replaced with KK2 containing either sodium or lithium chloride. They were then left for 1 hour before analysis.

For chemotaxis assays, growing cells were washed once and resuspended at 5 x 10^6 cells /ml in KK2. Cells were then shaken for 5 hours and subjected to 100 nM cAMP pulses at 6 minute intervals. LiCl treatment was included for the final hour only. After 5 hours a small number of cells was then allowed to adhere to a glass slide before being exposed to a cAMP gradient using a Zigmond chamber (Zigmond, 1988).The chamber was set up with 1μM cAMP (in KK2) on one site and KK2 on the other and left for 15 minutes before analysis.

In all experiments DIC images were taken using an Olympus IX71 inverted microscope with a 20x objective (NA 0.45). As our microscope is equipped with a motorized stage, 8 fields of view were taken simultaneously for each sample. Images were captured every 10 seconds for 10 minutes. DIAS 3.4.1 software (Soll Technologies Inc.) was then used to analysis the images. In this analysis, for each frame of the movie each cell was automatically outlined and cell paths determined. At this stage any cell that contacts another was disregarded. The average speed, persistence, acceleration, direction change, roundness and chemotactic index over the 10 minute period was calculated for each cell and in every case a minimum of 50 cells were measured and averaged. All experiments were repeated at least 4 times and, normalized to a NaCl control value as indicated. In all cases statistical analysis was done using an unpaired 2-tailed T-test.

2.20 Quantitative real-time PCR analysis

Total RNA was isolated from *Dictyostelium* as follows: 5 x 10^6 cells were seeded in 10cm petri dishes and allowed to grow axenically overnight in the presence or

absence of the appropriate inhibitors. 16 hours later cells were harvested by centrifugation and washed once in KK2. RNA was then extracted and cDNA synthesized as described in section 2.3.

Q-RT PCR was then performed using 10ng cDNA as a template and a SYBR green mastermix kit (Abgene) on an Opticon 2 instrument (MJ Research). All reactions with PCR products between 100-200 bp were run for 35 cycles using the following program: 95° x 15 sec, 57°x 15 sec, 70° x 20 sec. Reactions giving products of 300-500 bp were all used the same program but with extension at 70°C for 30 seconds. Each reaction was then subjected to melting curve analysis, to verify product quality.

All samples were run in triplicate, and in at least 3 independent experiments. Fold changes were calculated using the $2^{-\Delta\Delta cT}$ method (Livak and Schmittgen, 2001) using the mean c(T) value from each run and normalised to Ig7 as a loading control. Note that to maintain comparable product sizes for all genes, when *ipma1*, *ippA/B* and *ino1* primer sets were used, the long *ig7* long product primer set was used and for all other genes, the short product primer set was used. For all primer details and validation see appendix.

2.21 Immunofluorescence

For immunofluorescence imaging, cells were seeded onto poly-L-lysine coated coverslips, and allowed to adhere for 30 minutes. Coverslips were then washed twice in PBS and fixed in 3% paraformaldehyde (in PBS) for 20 minutes. They were then washed three times more and permeabilised by two incubations of 15 minutes in 1%

gelatin/ 0.2% saponin. Cells were then stained with M2-mouse α-FLAG primary antibody (1:1,000 dilution Sigma) for one hour, washed three times, and then a FITC-conjugated α-mouse IgG secondary antibody (1:1,000, Sigma) mixed with 1μg/ml 4`,6-Diamidino-2-phenylindole dihydrochloride (DAPI). Coverslips were then washed and mounted on slides using Vectorshield mounting medium (Vector Labs). Images were then taken using a 40x oil immersion objective on a Leica TCS SP2 confocal microscope.

Chapter 3:

Multiple Inositol Polyphosphate phosphatase activity is required for lithium resistance in PO null cells

3.1 Introduction

In *Dictyostelium*, a genetic screen for cells resistant to the effects of lithium on aggregation identified the enzyme prolyl oligopeptidase (PO). In this system, cells lacking all PO activity were able to aggregate at concentrations of lithium where wild-type cells cannot – albeit with an altered morphology, probably due to inhibition of GskA (Williams et al., 1999).

In these cells, it was also noted that disruption of the PO gene leads to increased Ins(1,4,5)P_3 levels due to increased Ins(1,3,4,5,6)P_5 dephosphorylation, and this inositol phosphatase activity is also inhibited in PO overexpressing cells ((Williams et al., 1999) and R. Williams, unpublished data). In astroglioma cells, a similar effect has also been observed, were treatment with a PO specific inhibitor led to increased Ins(1,4,5)P_3 levels, although in this study the mechanism was not investigated (Schulz et al., 2002).

This phosphatase activity was previously identified in *Dictyostelium* as an alternative route of Ins(1,4,5)P_3 production in cells lacking phospholipase C activity (Van Dijken et al., 1997), and when purified from mammalian sources it was shown that the multiple dephosphorylations are catalysed by a single enzyme, Multiple Inositol Polyphosphate Phosphatase (MIPP) (Craxton et al., 1995; Nogimori et al., 1991; Van Dijken et al., 1995a). Therefore, in *Dictyostelium* because of the ability to directly phosphorylate inositol to form $InsP_6$, Ins(1,4,5)P_3 levels can be maintained and regulated completely independently of PLC.

MIPP is therefore a likely candidate as the target of PO which gives lithium resistance (implicating an inositol depletion mechanism) and therefore I have identified, cloned and disrupted the *Dictyostelium* homologue of MIPP in order to test this hypothesis.

3.2 Identification of *Dictyostelium* MIPP

The first MIPP to be cloned was from rat liver and was found to contain the catalytic motif of the histidine acid phosphatase family (RHGXRXP), distinct from all other known inositol phosphatases (Craxton et al., 1997). When this was cloned the only protein in the animal kingdom found to share homology throughout its length was a partly characterized protein from chick chondrocytes, up regulated when these cells become hypertrophic, HiPER, sharing 64% homology (Reynolds et al., 1996) an effect later observed with rat MIPP (Caffrey et al., 1999). This motif is closely conserved throughout evolution, including the fungal $InsP_6$ phosphatase phytase, although in these enzymes the homology is restricted to this region.

The motif conserved in MIPP and phytase was used to screen the complete *Dictyostelium* genome using a BLAST search in order to identify potential homologues. The search gave only two good candidates - predicted proteins DDB 0186447 and DDB 0218876 hereafter named MippA and MippB respectively (encoded by the genes *mppA and mppB)*. Both genes are located on chromosome 4 (although in distinct regions, co-ordinates 3103340-3105247 and 5169198-5170721 respectively) and consist of a single exon, but neither has any EST data indicating expression (from www.dictybase.org). The two predicted protein products are closely

related to each other, sharing 70% sequence identity although MippA has an extended C-terminus and therefore they may serve different biological functions (see Fig. 3.1).

When compared to other species, both *Dictyostelium* genes share conserved residues with other eukaryotic MIPPs along their length whilst differing significantly from the fungal phytase gene except in the active site (Fig. 3.2).

3.3 The expression of MIPP in *Dictyostelium*

To examine expression, specific primers were designed for *mppA* and *mppB* and expression tested by RT-PCR on a cDNA library constructed from vegetative cells. In this assay, only the *mppA* primers gave a product, although as a control both products could be amplified from a genomic DNA template (Fig. 3.3). This indicates that *mppB* is not expressed in vegetative cells.

To test whether either gene was developmentally regulated, expression at different times post-starvation was analysed by quantitative real time PCR (Q-RT PCR). At all time points, and over two independent experiments, no *mppB* expression could be detected. In contrast *mppA* expression remained fairly constant, although it may drop slightly after 4 hours (Fig. 3.3). In addition the copy number of *mppA* mRNA in vegetative cells was measured (by Q-RT PCR) and it was found to be present at only 3 copies per cell (see appendix), indicating that the protein may have a slow turnover, and possibly explaining why no ESTs have been found.

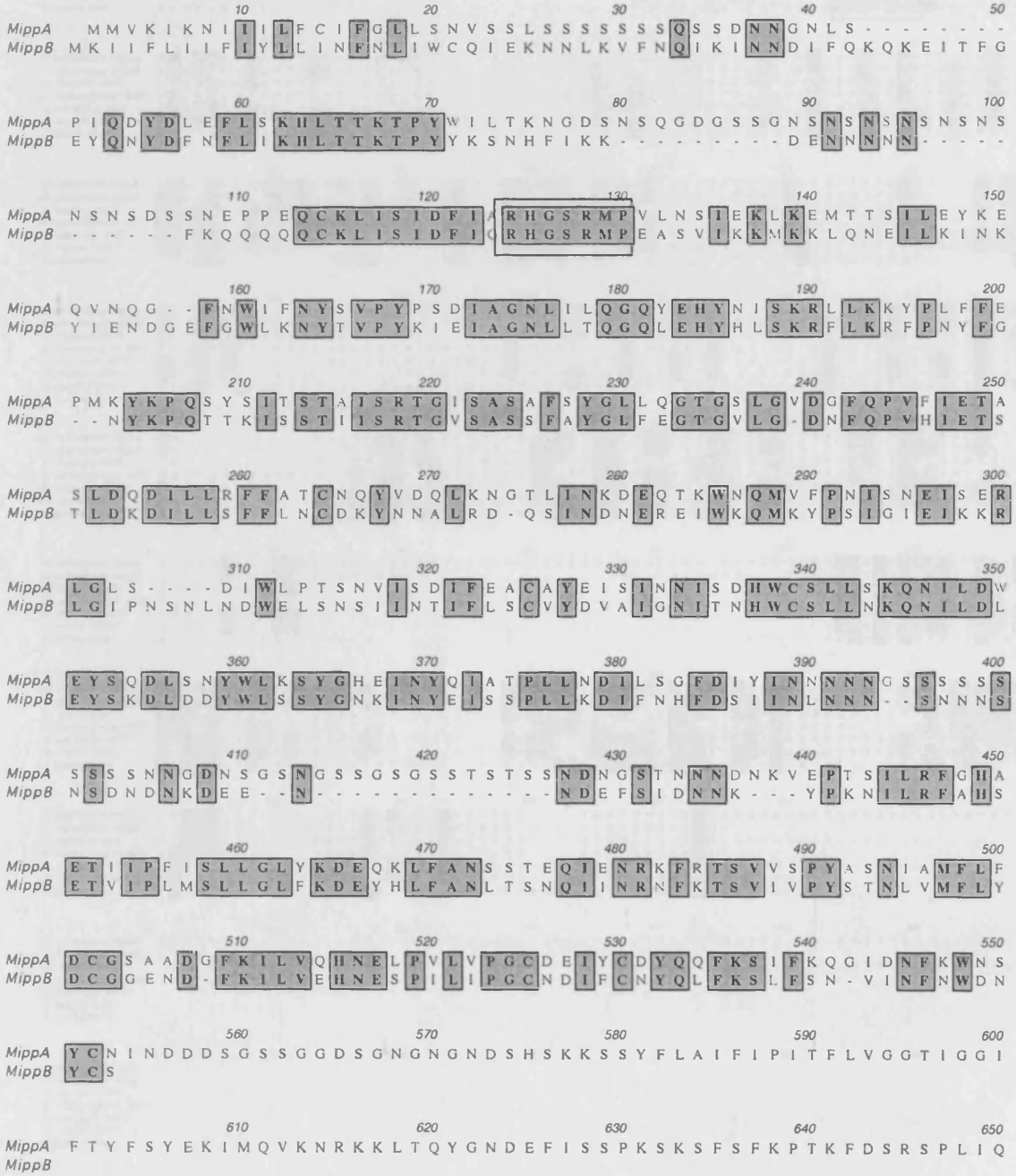


Figure. 3.1: Sequence and alignment of *Dictyostelium* MIPP homologues. The boxed region represents the conserved catalytic motif.

Figure 3.2: Alignment of MIPP genes across evolution. The boxed region represents the conserved catalytic motif.

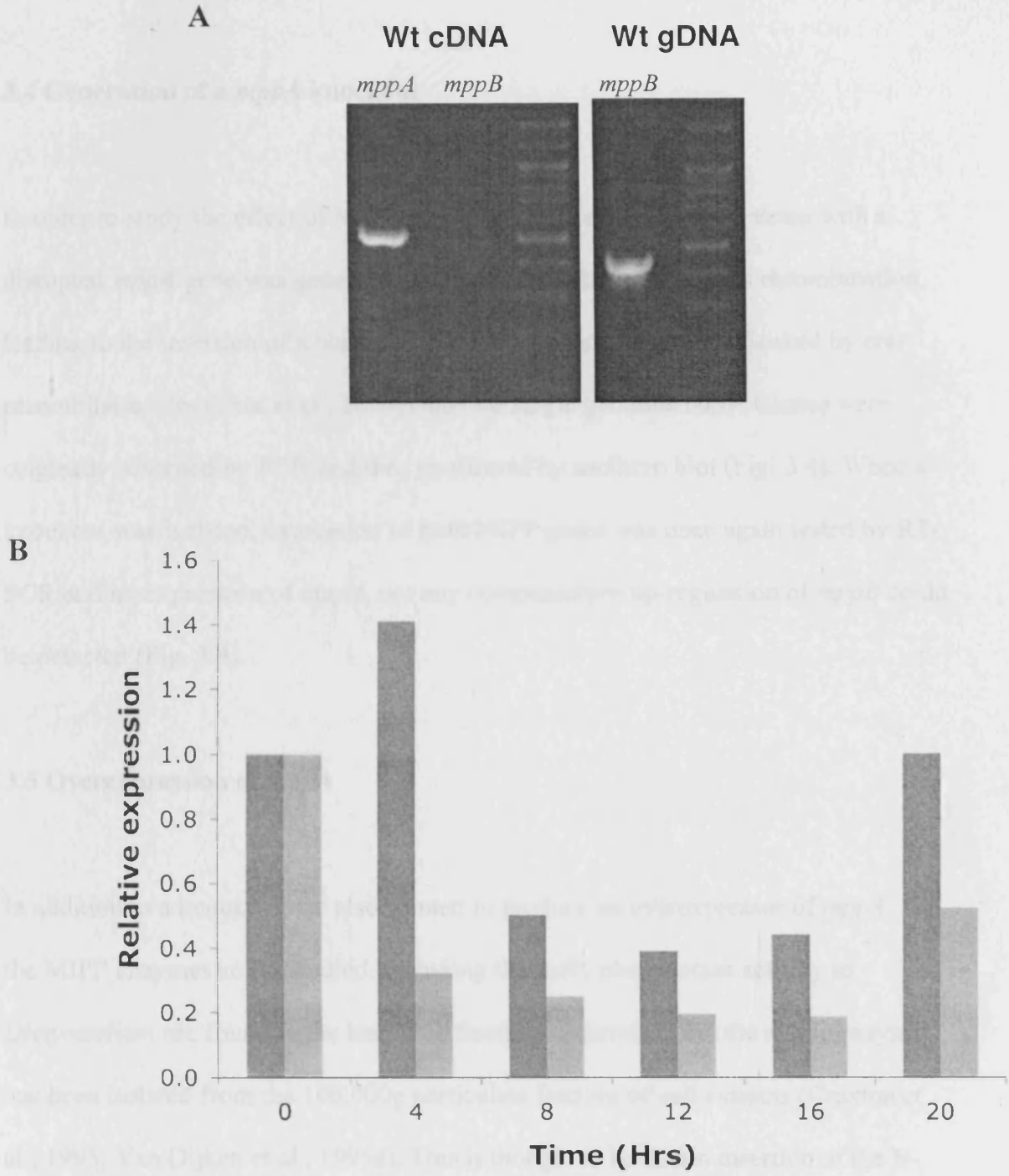


Figure 3.3. The expression of *Dictyostelium* MIPP homologues. (A) RT-PCR analysis of *mppA* and *mppB* in vegetative cells. Second panel indicates a control reaction using a genomic DNA template with the *mppB* primer set. (B) Developmental expression of *mippA* relative to vegetative (0hrs) cells. Samples were measured using quantitative real time PCR, two independent experiments are plotted.

3.4 Generation of a *mppA* knockout

In order to study the effect of MIPP activity on lithium resistance a strain with a disrupted *mppA* gene was generated. This was done by homologous recombination, leading to the insertion of a blasticidin selectable marker cassette (flanked by cre-recombinase sites (Faix et al., 2004)) into the single genomic copy. Clones were originally screened by PCR and then confirmed by southern blot (Fig. 3.4). When a knockout was isolated, expression of both MIPP genes was once again tested by RT-PCR and no expression of *mppA*, nor any compensatory up-regulation of *mppB* could be detected (Fig. 3.4).

3.5 Overexpression of *mppA*

In addition to a knockout, we also wanted to produce an overexpressor of *mppA*. All the MIPP enzymes so far studied, including the $InsP_5$ phosphatase activity in *Dictyostelium* are found in the insoluble fraction of extracts, and the native enzyme has been isolated from the 100,000g particulate fraction of cell extracts (Craxton et al., 1995; Van Dijken et al., 1995a). This is thought to be due to insertion of the N-terminus into a membrane, a truncation of which results in a soluble form of the rat enzyme which is then mis-localised to the cytoplasm and can result in elevated basal calcium levels (Craxton et al., 1997; Yu et al., 2003). In addition, the C-terminus of the human, rat and chick proteins contain putative XDEL motifs which is a signature for proteins found within the ER. Therefore addition of a tag to either end is

problematic, and indeed when I made constructs adding an epitope tag to either end, or even to an N-terminal truncated version of MippA they were not expressed in *Dictyostelium* and were either not expressed, or completely insoluble in *E. coli* (data not shown).

A construct expressing an untagged, full-length MippA under the control of the constitutively active actin 15 promoter was made. When this was transformed into cells, MIPP activity (as measured by Ins(1,4,5)P_3 production from $InsP_6$ in the particulate fraction) was increased by approximately 100 fold. As previously reported, this activity is also moderately increased in PO null cells but is completely absent in both the soluble, or particulate fractions of the *mppA* null mutants described above (Fig. 3.5). We therefore conclude that *mppA* encodes a functional MIPP enzyme and is indeed the only functional enzyme that we can detect in growing, or developing cells.

3.6 The biochemistry of *Dictyostelium* MIPP

To further understand the role of MippA within the cell, its biochemistry was analysed using the MDD-HPLC system, which is able to directly detect and resolve many of the inositol polyphosphate isomers. In crude particulate extracts from MippA overexpressing cells there appear to be two inositol phosphatase activites present: firstly MIPP activity, degrading $InsP_6$ and producing a peak of $InsP_3$, which is abolished in the knockout; and secondly, a MippA-independent 5' phosphatase activity which degrades Ins(1,4,5)P_3 to a single peak co-eluting with an authentic Ins(1,4)P_2 standard (Fig. 3.6 B and C).

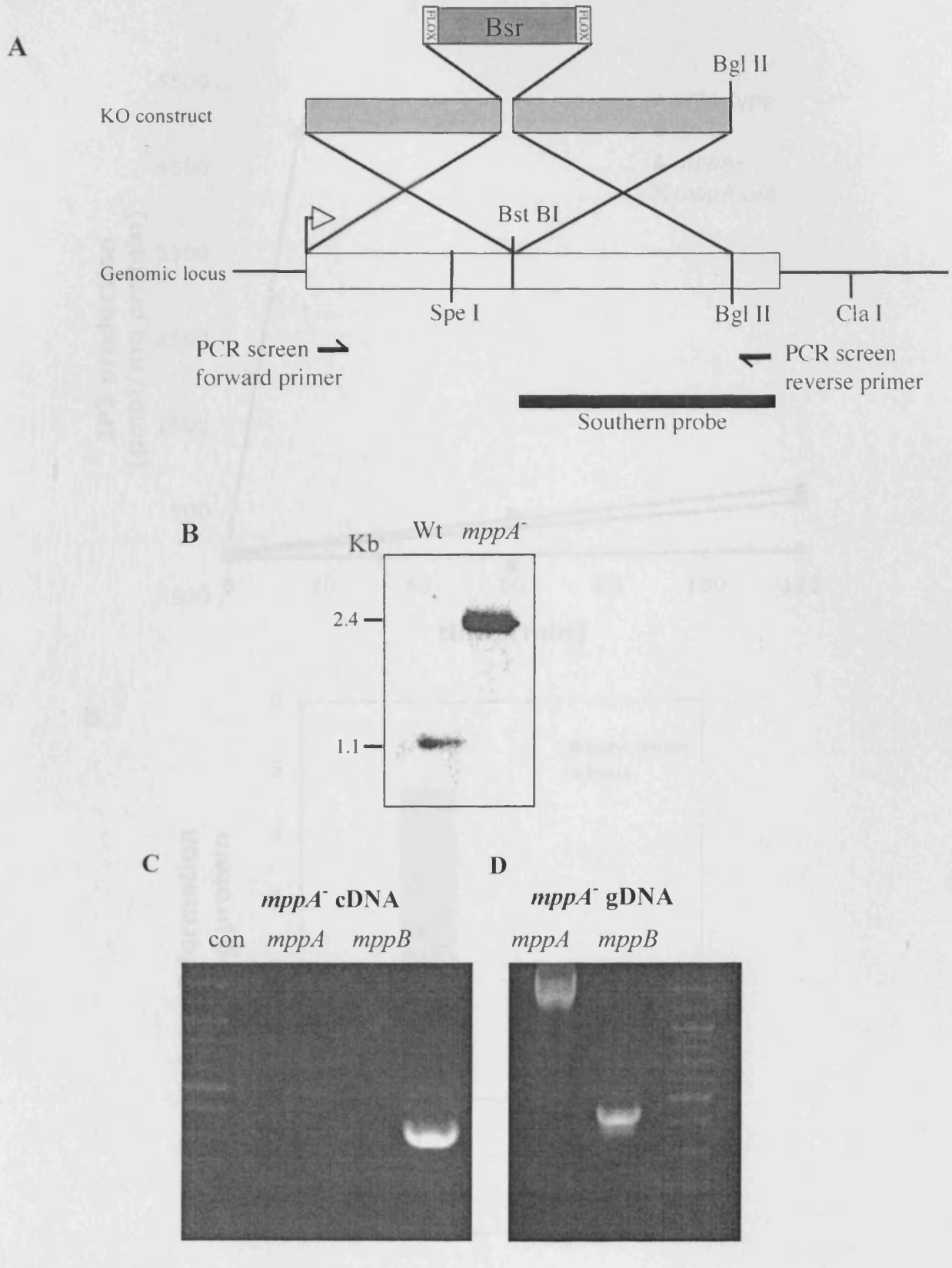
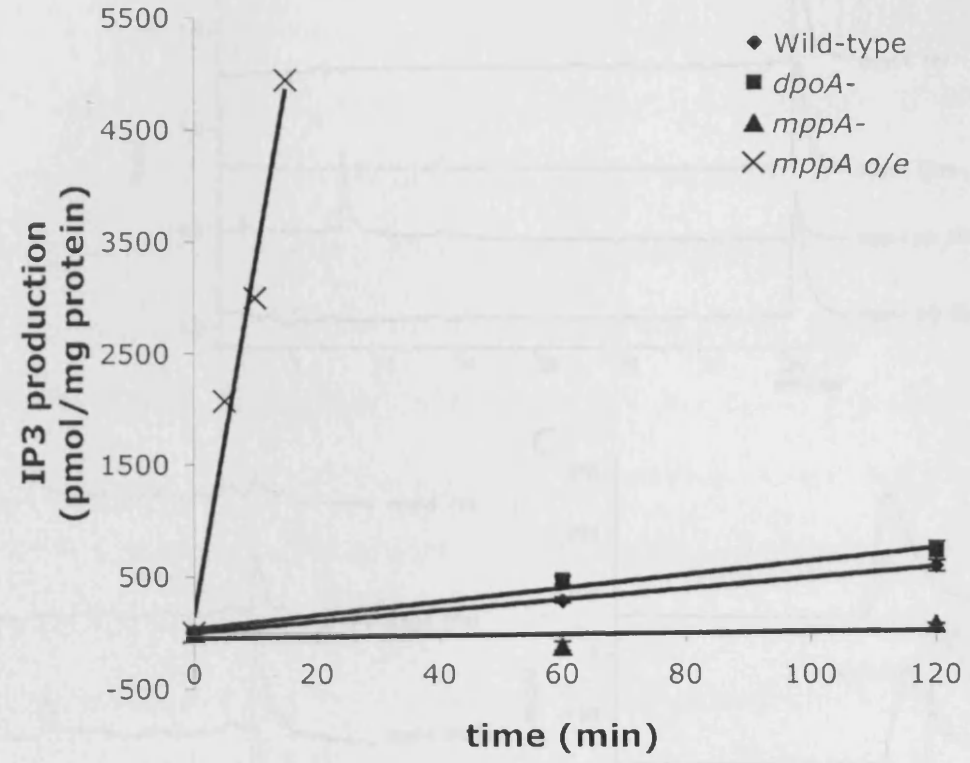


Figure 3.4: Generation of a *mippA* null strain. (A) Schematic of the knockout construct transformed into cells. (B) Southern blot of a wild-type and *mippA*$^-$ cells after digestion with SpeI/ClaI. (C) RT-PCR analysis of *mppA/B* expression in vegetative, *mppA*$^-$ cells. (D) Control PCR using the same primer sets with a *mppA*$^-$ genomic DNA template.



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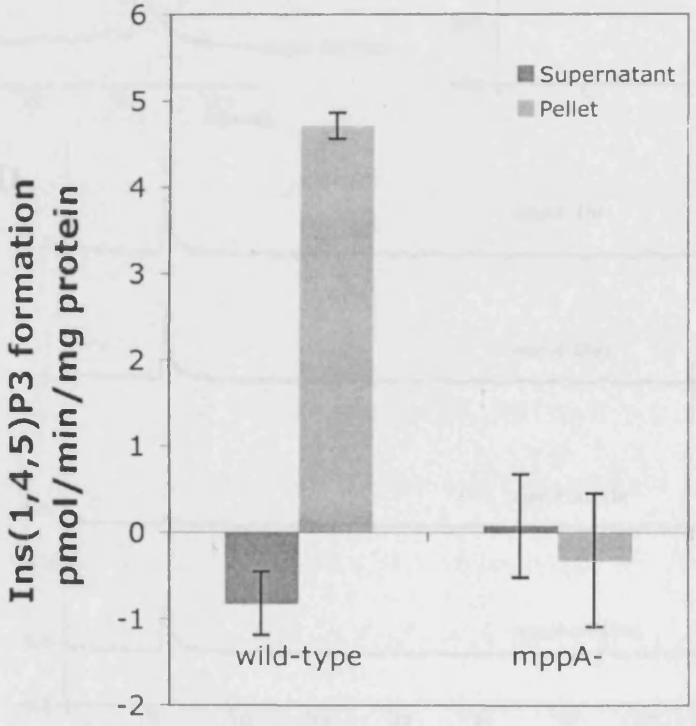


Figure 3.5: MIPP activity in mutant cells. (A) The 100,000g particulate fraction from wild-type, *dpoA*$^-$, *mppA*$^-$ and *mppA* overexpressing cell extracts were incubated with 0.1mM $InsP_6$ and $Ins(1,4,5)P_3$ formation measured at the times indicated. Values are normalised to protein content and are from a typical experiment. (B) Cell extracts from wild-type and *mppA*$^-$ cells were spun at 100,000g and both pellet and supernatant fractions incubated for 30mins before $InsP_3$ determinations. Activity is measured by accumulation of $Ins(1,4,5)P_3$, subtracting the starting concentration. Negative activity is due to both noise within the assay as well as the degradation of $Ins(1,4,5)P_3$ by contaminating 5' phosphatases.

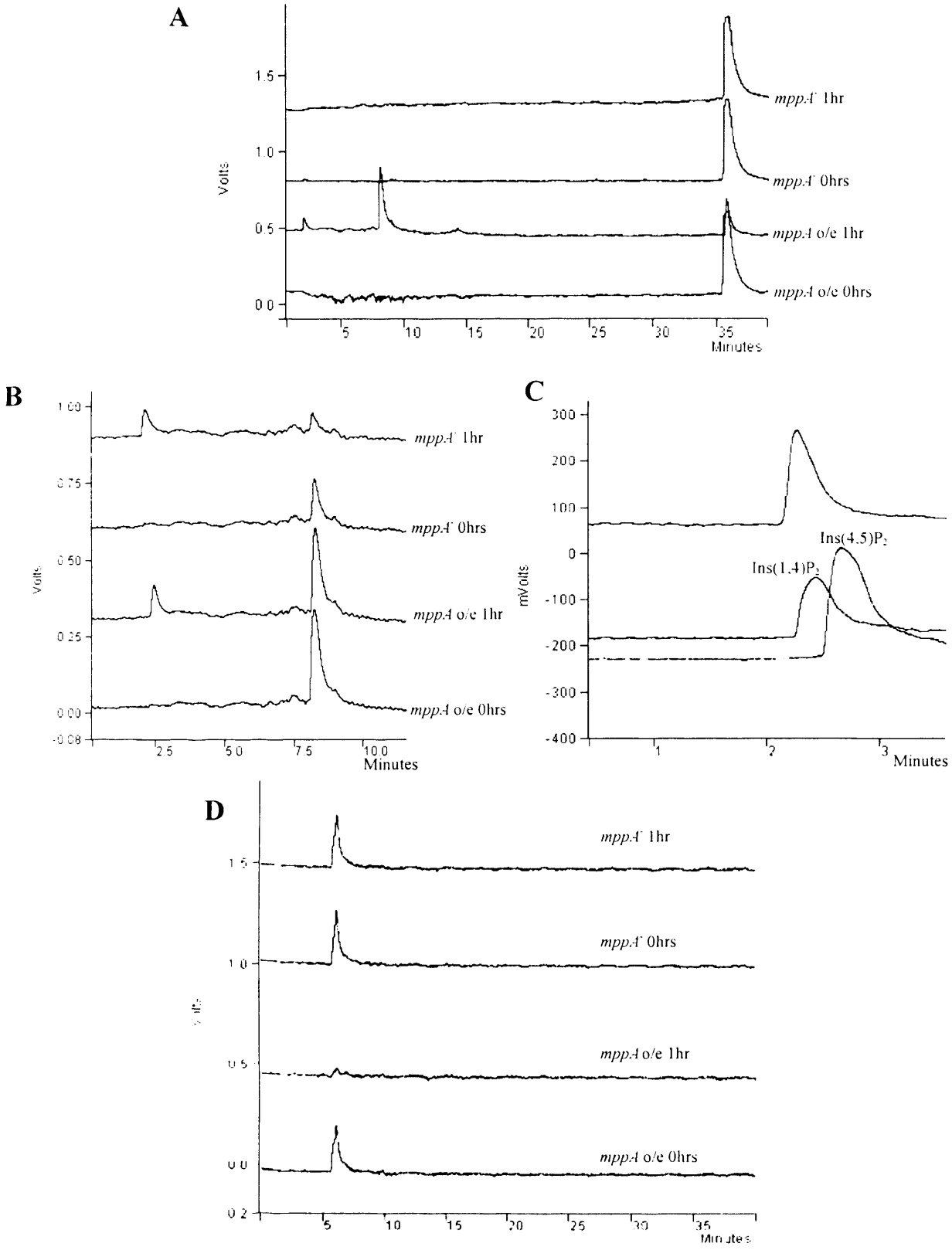


Figure 3.6: Analysis of MIPP substrate specificity. The insoluble fractions from either *mppA* null or overexpressing cells were incubated with either (A) 10 nmoles $InsP_6$ (B) 10 nmoles $Ins(1,4,5)P_3$ or (D) 10 nmoles 2,3-diphosphoglycerate. Samples were removed at the start and after 1 hour as indicated. (C) Shows the product of $Ins(1,4,5)P_3$ dephosphorylation (top trace) compared with authentic $Ins(1,4)P_2$ and $Ins(4,5)P_2$ standards. In all traces the y-axis refers to the bottom trace and the other traces have been offset for clarity.

In addition to inositol polyphosphates, it has also been suggested that MIPP may also be able to use 2,3 diphosphoglycerate (DPG), a glycolytic intermediate as a substrate (S. Shears, personal communication). When incubated with *Dictyostelium* extracts from *mppA* overexpressing cells, DPG was rapidly converted to an undetectable species presumably by dephosphorylation (note that in our system, detection is based on phosphate content). However in *mppA*$^-$ extracts DPG was stable, indicating that this is a MippA specific activity (Fig. 3.6). This is interesting because DPG is also a potent inhibitor of inositol 5'phosphatase activity (Downes et al., 1982), and therefore in conditions where MIPP activity is elevated such as in *dpoA*$^-$ mutants, this 5'phosphatase inhibition may be reduced, allowing the coordinate regulation of both enzymes involved in the conversion of $InsP_6$ to $InsP_2$.

In our assay, when extracts from *mppA* overexpressing cells are incubated with 0.1mM $InsP_6$, the $InsP_6$ is rapidly hydrolysed and only a single peak of $InsP_3$ production is detected (presumably Ins(1,4,5)P_3 which is then in turn hydrolysed by the 5'phosphatase). To our surprise we also observed that when a higher concentration of $InsP_6$ was used, a dramatically different chromatogram was obtained. If MIPP is incubated with 1mM $InsP_6$, it is hydrolysed at either the 3', 5' or 6' positions, to give $InsP_5$ and $InsP_4$ isomers which were not previously seen and do not co-elute with the Ins(1,4,5,6)P_4 or Ins(1,3,4,5)P_4 intermediates which would be expected by the normal reaction. These are then further resolved into a single $InsP_3$ peak. This is not further degraded to Ins(1,4)P_2 indicating that it is a poor substrate for the 5'phosphatase (Fig. 3.7).

This observation indicates that, at high concentrations of $InsP_6$ the enzyme does not attack the 2' position, instead removing alternative phosphate groups. If these phosphoinositides were poor substrates for sequential dephosphorylation they would accumulate slightly. Indeed if this were the case and MIPP only removes the 3', 5' and 6' phosphates (as is indicated by the $InsP_5$ isomers formed) this would result in the formation of Ins(1,2,4)P_3 which would not be a substrate for a 5' phosphatase.

To date no 2' phosphorylated inositides have been found to be biologically active and do not appear to be present in most biological systems. In addition, the reaction observed at lower $InsP_6$ concentrations is much more efficient with no intermediates at all accumulating between $InsP_6$ and $InsP_3$. I suggest that this is more likely to be the more physiological reaction.

3.7 The effect of MIPP mutants on inositol metabolism

To assess what role MippA plays *in vivo* the intracellular inositol phosphates in both null and overexpressor cell lines were quantified by HPLC (Figs. 3.8, 3.9 and table 3.1). I detected no significant difference in the relative abundance of any phosphoinositide species in the *mppA* null cells, compared to wild-type. The overexpressors however show an increase in all the inositol phosphates generated in the reaction described above at high $InsP_6$ concentrations, although many of the other species (including $InsP_6$) remain constant.

This is perhaps not surprising as the intracellular concentration of $InsP_6$ is estimated as ~1mM in *Dictyostelium* (Martin et al., 1987) and therefore indicates that in MippA

overexpressing cells at least, it is present in a compartment with high $InsP_6$ levels. This however may well be misleading as these cells have a ~100 fold increase in activity compared to wild-type and therefore some enzyme may be mis-localised, or leaked into the wrong compartment. It is also of note though, that in these cells there is no detectable Ins(1,3,4,5,6)P_5 (normally present at 44 pmol/10^7 cells), the normal precursor of Ins(1,4,5)P_3 production by MIPP and therefore it appears that both reactions are occurring simultaneously although perhaps not in the same place.

When intracellular Ins(1,4,5)P_3 levels are measured by the isotope dilution method no significant difference could be detected between either *mppA* null or overexpressing cells and wild-type (Fig. 3.8). This assay uses an Ins(1,4,5)P_3-binding protein for detection and therefore should be specific for this isomer. In HPLC analysis of *mppA* overexpressing cells however, a large accumulation of $InsP_3$ is seen and therefore this must consist of an alternative isomer, most likely Ins(1,2,4)P_3.

3.8 Generation of *dpoA/mppA* double mutants

In order to test whether the lithium resistance phenotype see in PO null cells is due to increased MippA activity, a double mutant was generated and lithium sensitivity examined. In isolating the original *mppA* knockout, a cre-lox flanked blasticidin gene was used, therefore these cells were transformed with a cre-recombinase expression construct to allow a sequential knockout to be generated using the same selectable marker (Faix et al., 2004).

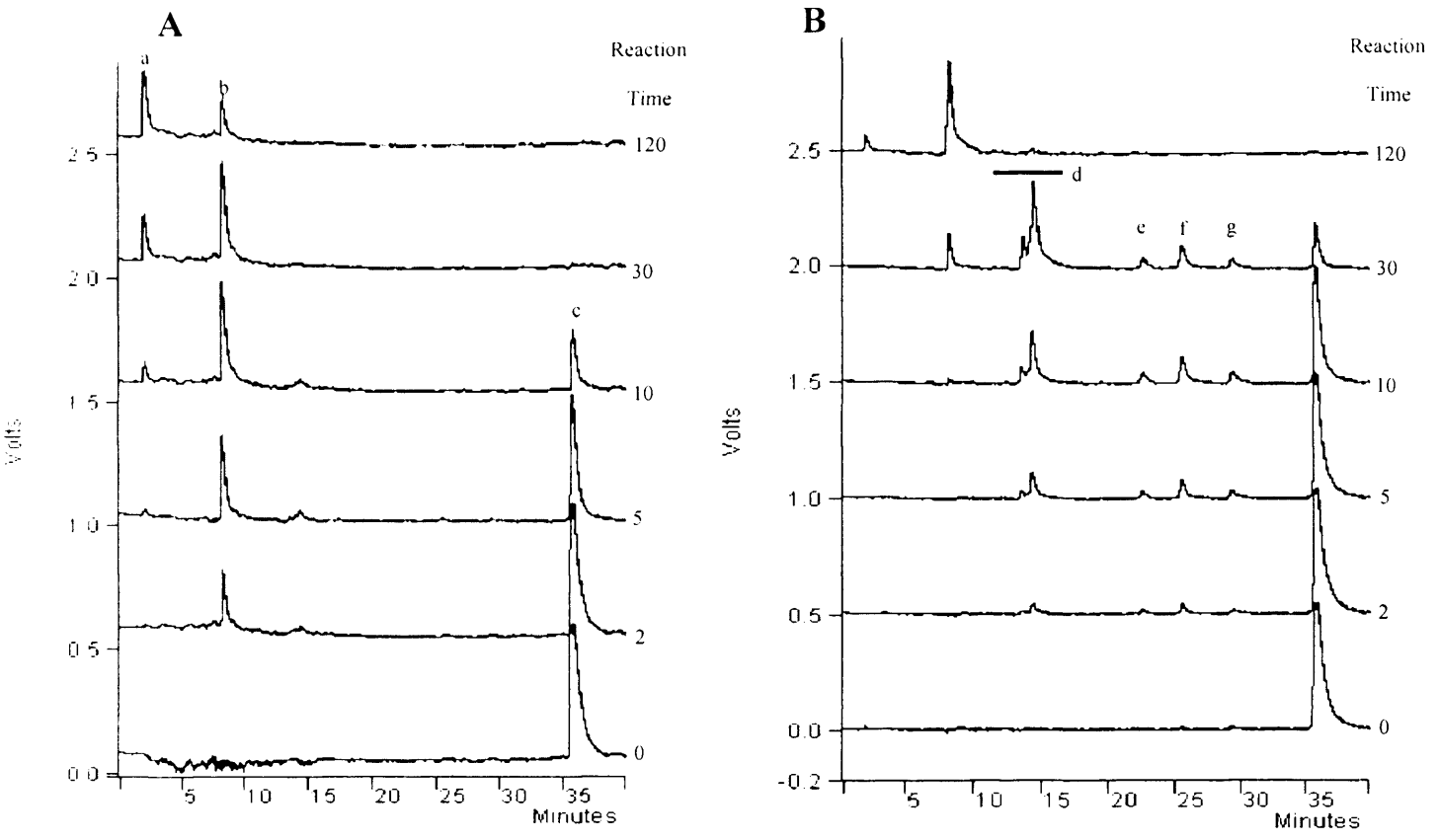


Fig. 3.7: Hydrolysis of $InsP_6$ by MippA. The particulate fraction of cell extracts from MippA overexpressing cells were incubated with $InsP_6$ at either (A) 0.1mM or (B) 1mM. Samples containing the equivalent of 10nmol of initial $InsP_6$ were removed at the times indicated, and analysed by HPLC. Peaks were identified as a – Ins(1,4)P_2; b – $InsP_3$ isomers; c - $InsP_6$; d – $InsP_4$ isomers; e – Ins(1,2,3,4,6)P_5; f – Ins(1,2,3,4,5)P_5; g – Ins(1,2,4,5,6)P_5. Again, the Y-axis refers to the bottom trace and the other traces are offset for clarity.

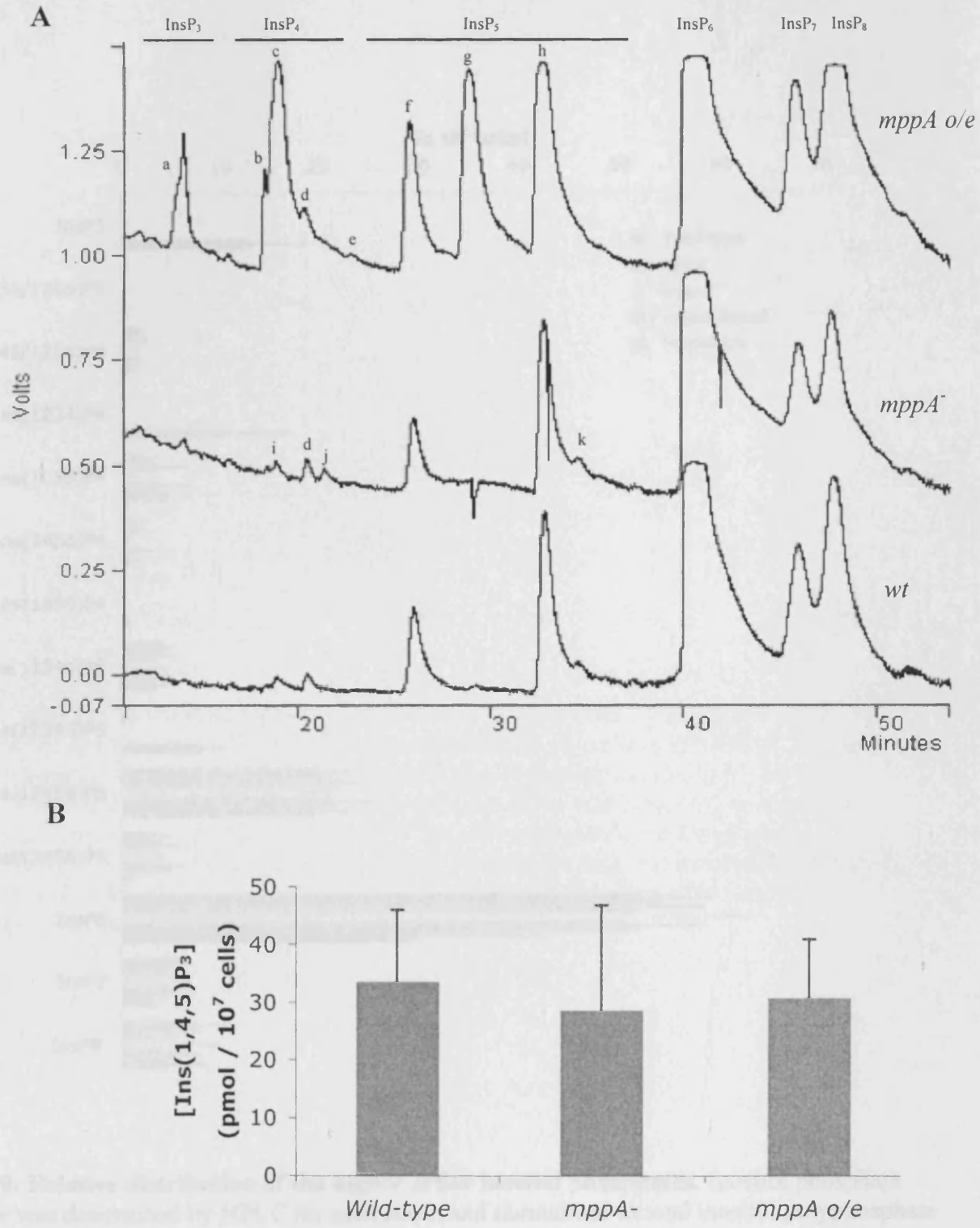


Figure 3.8: Analysis of inositol phosphate abundance in *mppA* mutants. Cells extracts were prepared and analysed by (A) HPLC-MDD, and (B) the Ins(1,4,5)P_3 – specific isotope dilution assay. Isomers separated by HPLC were identified as: a – unresolved $InsP_3$ isomers; b – Ins(1,2,3,5)/(1,2,4,6)P_4; c – Ins(1,2,4,5)P_4; d – Ins(1,2,5,6)P_4; e – Ins(1,4,5,6)P_4; f – Ins(1,2,3,4,6)P_5; g – Ins(1,2,3,4,5)P_5; h – Ins(1,2,4,5,6)P_5; i – Ins(1,3,4,6)/(1,2,3,4)P_4; j – Ins (2,4,5,6)P_4; k- Ins(1,3,4,5,6)P_5.

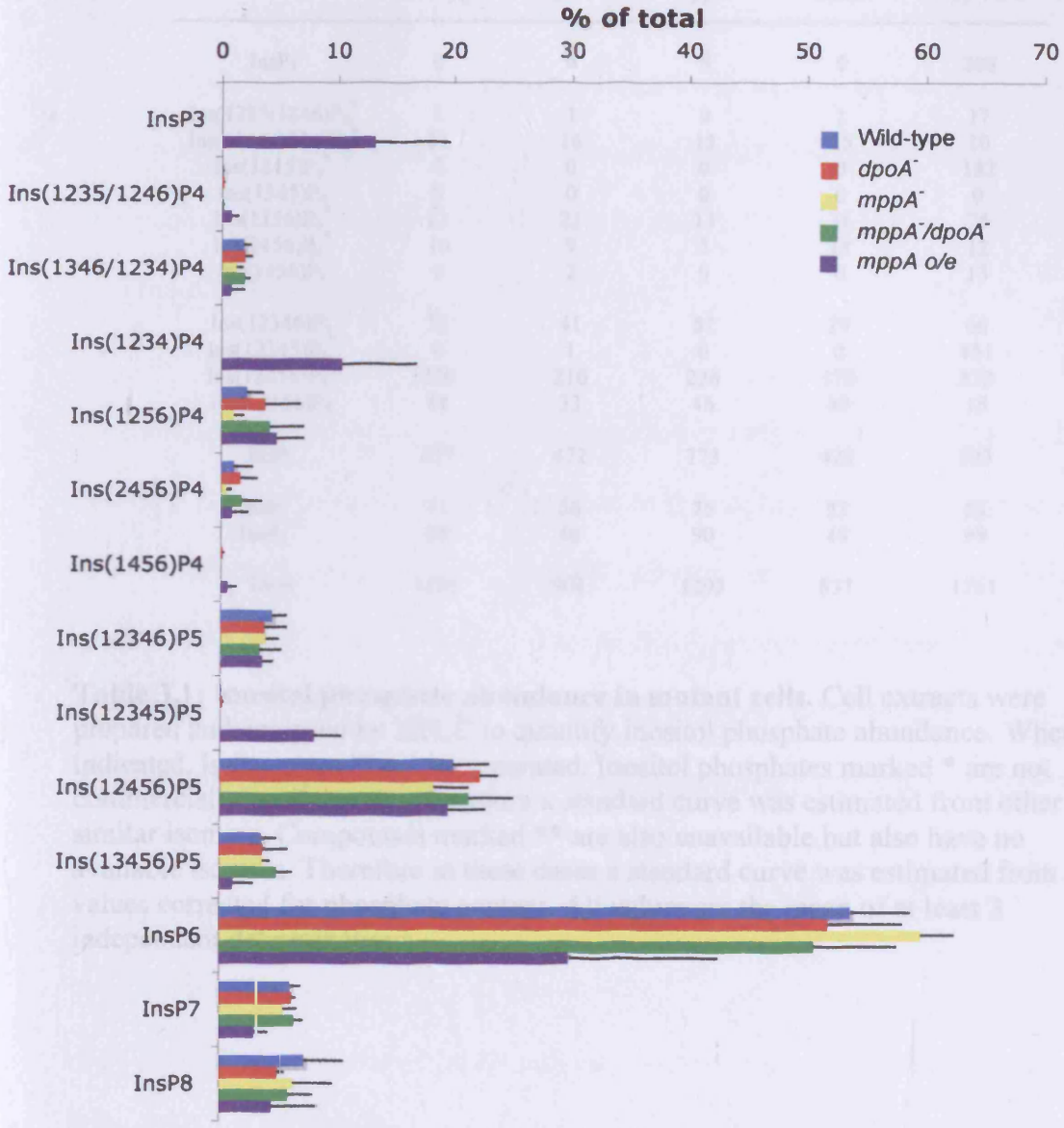


Figure 3.9: Relative distribution of the higher order inositol phosphates. Inositol phosphate abundance was determined by HPLC for each strain and normalised to total inositol polyphosphate levels. Values plotted are the means ± standard deviation of at least 3 independent experiments.

Pmol / 10^7 cells

	Wild-type	*dpoA*⁻	*mppA*⁻	*mppA*⁻ /*dpoA*⁻	*mppA* o/e
$InsP_3$	0	0	0	0	208
Ins(1235/1246)P_4*	1	1	0	1	17
Ins(1346/1234)P_4*	21	16	15	15	10
Ins(1245)P_4*	0	0	0	0	182
Ins(1345)P_4	0	0	0	0	0
Ins(1256)P_4*	23	23	13	31	75
Ins(2456)P_4*	10	9	5	14	12
Ins(1456)P_4	0	2	0	0	13
Ins(12346)P_5	52	41	51	29	66
Ins(12345)P_5*	0	1	0	0	151
Ins(12456)P_5*	236	210	226	178	332
Ins(13456)P_5	44	33	46	40	15
$InsP_6$	637	472	773	422	523
$InsP_7$**	71	56	75	53	58
$InsP_8$**	88	46	90	48	99
Total	1181	901	1293	831	1761

Table 3.1: Inositol phosphate abundance in mutant cells. Cell extracts were prepared and analysed by HPLC to quantify inositol phosphate abundance. Where indicated, isomers could not be separated. Inositol phosphates marked * are not commercially available and therefore a standard curve was estimated from other similar isomers. Compounds marked ** are also unavailable but also have no available isomers. Therefore in these cases a standard curve was estimated from $InsP_6$ values corrected for phosphate content. All values are the mean of at least 3 independent determinations.

After transformation with this vector and re-cloning ~70% of clones were blasticidin sensitive. Recombination was then screened by PCR amplification of the genomic locus, and showed that specific recombination had occurred in all blasticidin sensitive clones to remove the gene, giving a PCR product slightly larger than that of the wild-type gene due to the insertion of multiple stop codons (Fig. 3.10). Loss of MIPP was confirmed by MIPP assay.

To generate a double mutant these cells were then transformed with the plasmid HAD172, used to recapitulate the PO mutant in the original study (Williams et al., 1999). Independent clones were screened by enzyme assay for loss of PO activity and many double mutants isolated (Fig. 3.10). As MIPP activity is increase by the loss of PO, it is possible that the converse may be true. However, when PO activity was measured in *mppA* mutant cells, no reciprocal change in activity was seen (Fig. 3.10).

3.9 Inositide abundance in *dpoA*/*mppA* mutants

dpoA null cells have elevated MIPP activity, and therefore changes in the abundance of higher inositol phosphate species might be expected. However when this was measured by HPLC, no significant changes were detected between either *dpoA* and *mppA* single and double mutants, when compared to wild-type (Table 3.1 and Fig. 3.9).

3.10 The effect of lithium on cell movement

In our standard assay for lithium resistance, *Dictyostelium* mutants are allowed to develop on either agar plates or filters and tested for their ability to form fruiting bodies in the presence of lithium. Whilst this approach is excellent for screening large numbers of clones, to get a more clear understanding of how lithium blocks aggregation and to be able to quantify and compare the sensitivity of different mutants, a more sensitive and detailed study is required. Therefore the effects of lithium on cell motility, both on random amoeboid and directed chemotactic movement were examined. It should be noted that to correct for any non-specific effects cause by salt concentration, all experiments were repeated on the same day in the same concentrations of NaCl and LiCl and normalised, where appropriate, to give a lithium sensitivity index for each parameter (LSI, defined as value in LiCl / value in NaCl).

3.10.1 Random amoeboid movement

To study random cell movement, in the absence of any chemotactic gradient, vegetative cells were simply placed in buffer containing different concentrations of lithium or sodium chloride, left for one hour and then analysed using time-lapse microscopy and image analysis software.

When vegetative wild-type cells were treated in this way, a strong dose-dependant inhibition of their motility with lithium was seen (Fig. 3.11). At 5mM LiCl cell speed is reduced by 42 ± 3% ($P<0.001$). In addition, cell turning was slightly affected, with

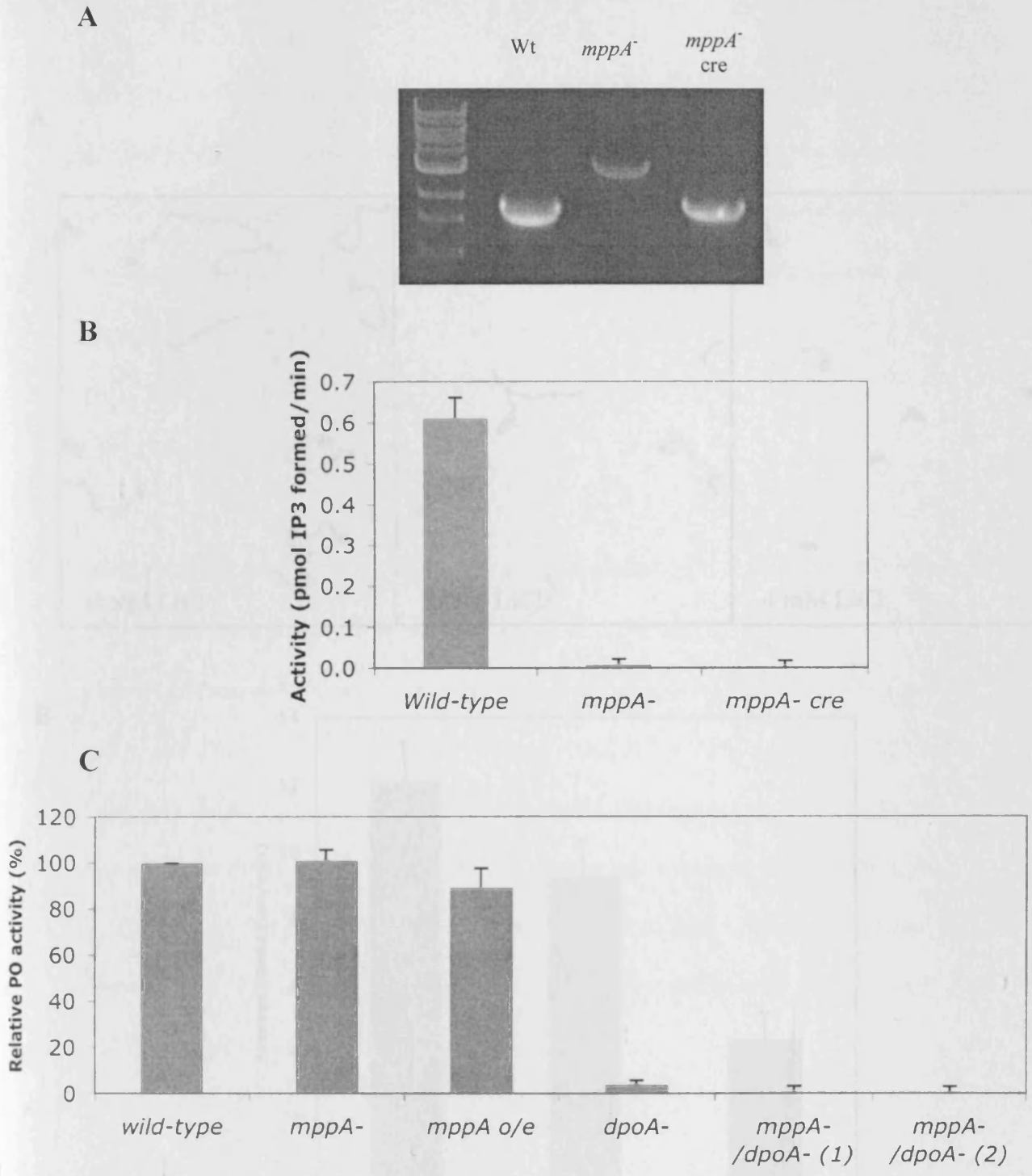
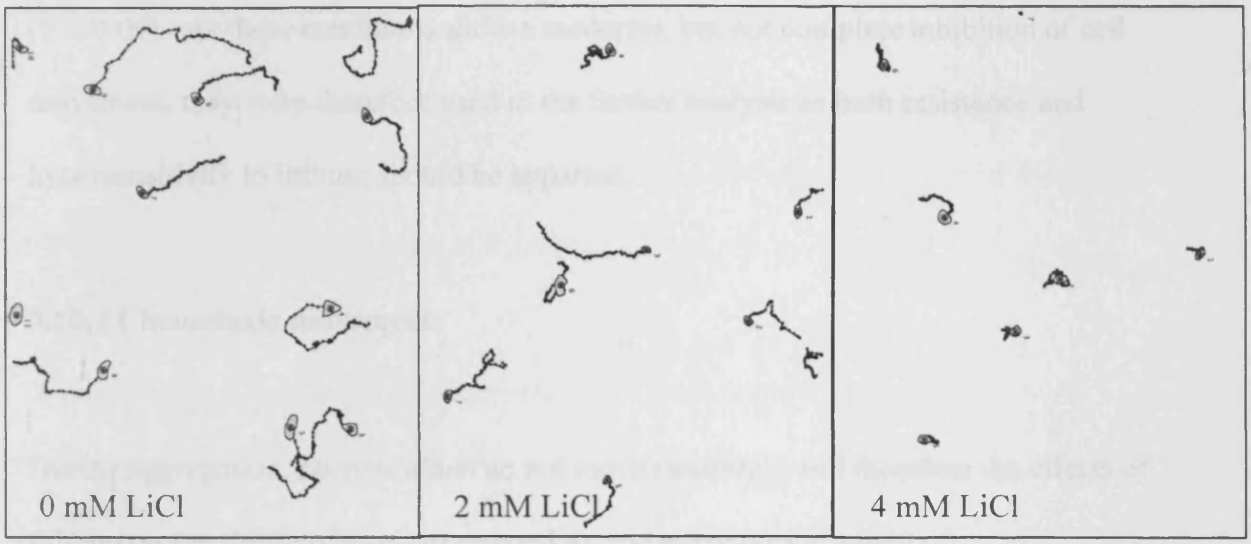


Figure 3.10: Generation of a *mppA*⁻ / *dpoA*⁻ double mutant. (A) PCR analysis of the *mppA* genomic locus after cre-recombination. (B) MIPP activity assay of these cells. (C) PO activity assay of double mutants and PO overexpressors. Results are the mean ± standard deviation of at least 3 independent experiments.

A



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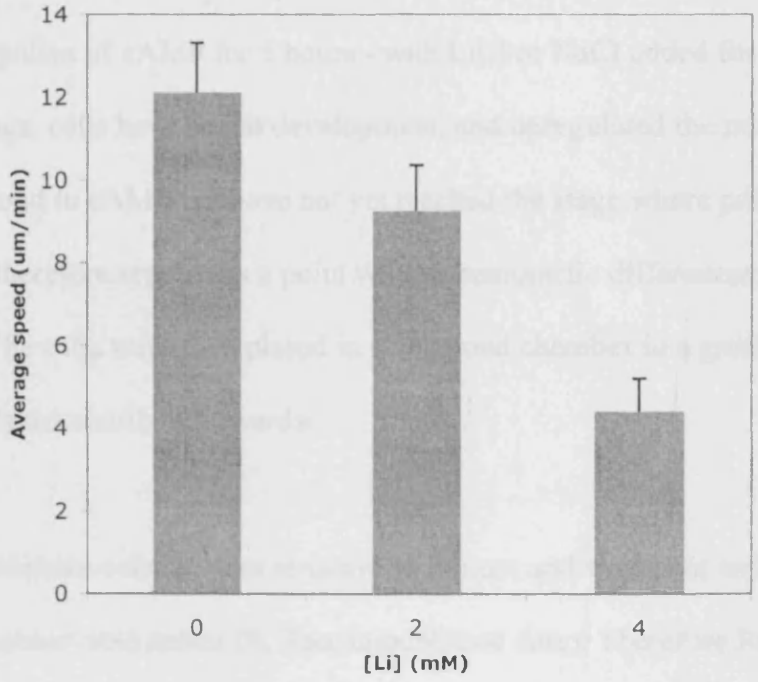


Figure 3.11: Lithium inhibition of cell movement. (A) Vegetative wild-type cells were placed in KK2 buffer containing the concentrations of lithium indicated. Cell movement was then recorded over a 10 minute period by time-lapse microscopy. Cell outlines were then determined for each frame and the path of each cell plotted, using 2D DIAS software. In addition cell speed was calculated for each lithium concentration and plotted in (B). Values are the means ± standard deviation of at least 50 cells.

the amount of direction change increasing by 34 ± 10 %, although this is not as significant and therefore may be simply an artefact of the cells moving more slowly ($P = 0.06$). As these conditions show a moderate, but not complete inhibition of cell movement, they were therefore used in the further analysis as both resistance and hypersensitivity to lithium should be apparent.

3.10.2 Chemotaxic movement

During aggregation, *Dictyostelium* do not move randomly, and therefore the effects of lithium on the ability of a cell to respond to, and move up a gradient of chemoattractant were examined. In this case, cells were used which had been starved and exposed to pulses of cAMP for 5 hours - with LiCl or NaCl added for the final hour. At this stage, cells have begun development, and upregulated the machinery required to respond to cAMP but have not yet reached the stage where polarity is intransient and therefore represents a point where chemotactic differences should be most obvious. The cells were then placed in a Zigmond chamber in a gradient of cAMP, and analysed shortly afterwards.

Under these conditions cells are less sensitive to lithium and treatment with 5mM LiCl caused no observable defect (R. Teo, unpublished data). Therefore for this assay the lithium concentration was increased to 7mM - comparable to the concentration required to inhibit aggregation on agar. At this concentration, the speed of wild-type cells was reduced by 23 ± 7 % ($P<0.005$).

In order to measure how efficiently the cells were moving towards the cAMP source, another parameter was calculated, the directionality. This is defined as the net path length / total path length, and therefore a cell moving in a straight line would have a value of 1 whereas a cell moving in a zig-zag or circle would have a much lower value, independent of speed. In this assay, wild-type cells move with a directionality = 0.7 ± 0.07 in 7mM NaCl, which is reduced by 14 ± 7% in lithium (P=0.02).

In both random, and directed chemotactic movement it seems that lithium has the common effect of inhibiting cell movement. This would clearly lead to a block in aggregation as originally observed and therefore we can use this to study and quantify how the lithium sensitivity of the mutant strains generated above is affected.

3.11 Analysis of the lithium sensitivity of mutant cells

3.11.1 Random movement

To determine whether MippA is required for the lithium resistance seen in *dpoA* null cells, the lithium sensitivity of both single and double knockout strains was examined using the assays described above. In the random movement assay, none of the mutant strains differed from wild-type in the absence of lithium. However, in 5mM LiCl, *dpoA*$^-$ cells are more resistant than wild-type, moving at 7.3 ± 0.5 μm/min as opposed to 6.0 ± 0.5 μm/min (P=0.007). This effect was lost in two independent *mppA/dpoA* double mutant clones which did not differ significantly from wild-type (Fig. 3.12).

The original hypothesis was that the *dpoA*⁻ mutant was resistant to lithium due to elevated MIPP activity. Unexpectedly I found that *mppA* overexpressing cells were hypersensitive to lithium and were almost stationary, moving at 2.9 ± 0.1 μm/min in the presence of 5mM LiCl ($P<0.005$). I measured a lower value for *mppA*⁻ cells than wild-type although this is not as statistically significant ($P=0.01$).

3.11.2 Lithium sensitivity during chemotaxis

When these strains were examined in a cAMP gradient, similar results were obtained. Again, in the presence of 7mM NaCl control, all cell lines were indistinguishable, and the differences only became apparent after lithium treatment. Results were normalized to a NaCl control on the same day to account for day-to-day variation (raw values for the average speed and chemotactic index are included in appendix II).

In this assay, *dpoA*⁻ cells again had reduced lithium sensitivity compared to wild type, with speed reduced to 88 ± 13% compared to 77 ± 7% and although the statistical significance of this was slightly above $P=0.05$ they are also more resistant than *dpoA*⁻ *mppA*⁻ double mutants ($P=0.01$) (Fig. 3.13). Again, *mppA* overexpressors were more sensitive than wild-type ($P=0.01$), unlike *mppA*⁻ cells which were indistinguishable from wild-type. As well as speed, the directionality for these cells was also calculated. In each cell line, this was affected to a similar degree to speed and therefore the two appear to be intimately linked, at least in their lithium sensitivity (fig.3.13).

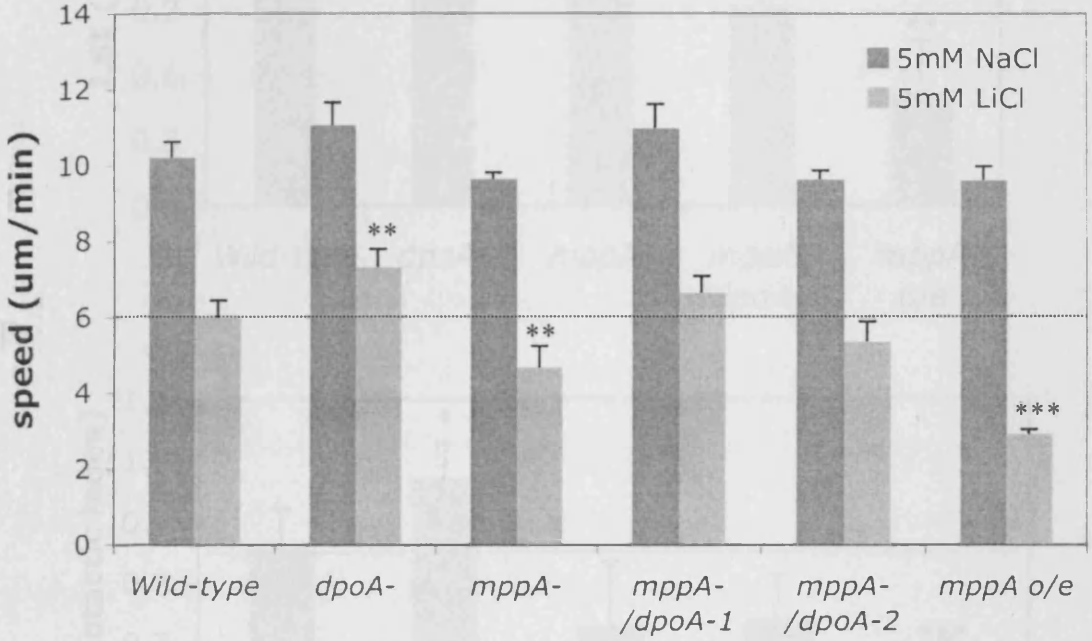
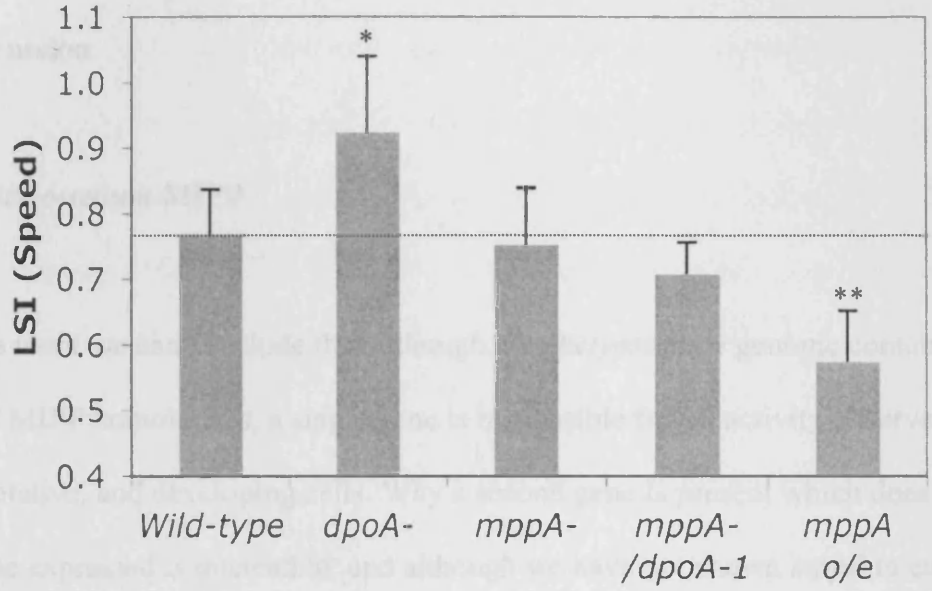


Figure 3.12: Sensitivity of random movement to lithium in mutant strains. Mutant strains were grown vegetatively and treated with either 5mM LiCl or NaCl for one hour. Their movement was then analysed and average speed calculated. Values plotted are the mean ± standard deviation of at least 4 independent experiments, minimum of 50 cells per experiment.** - $P \leq 0.01$, *** - $P \leq 0.005$, Students T-test compared to wild-type.

A



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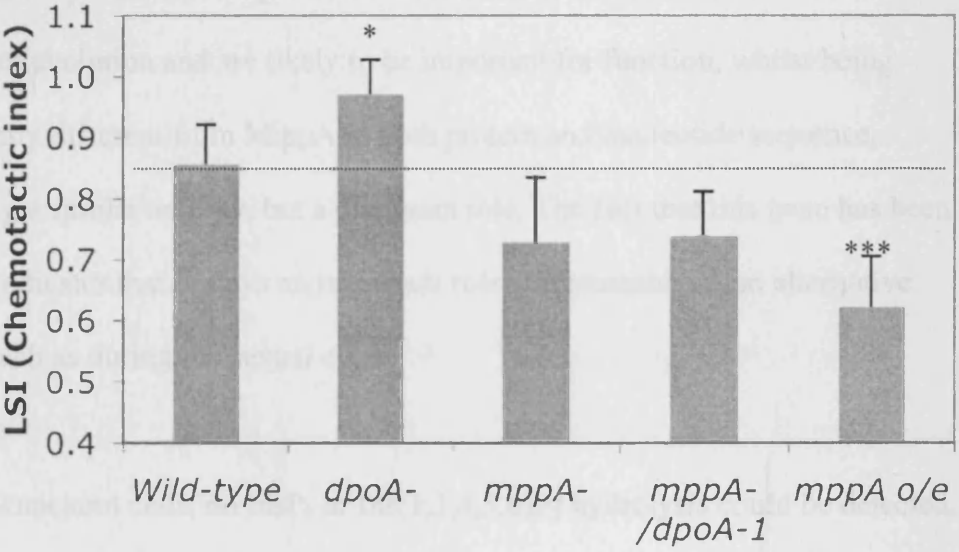


Figure 3.13: Lithium sensitivity in chemotaxing cells. Cells were pulsed for 5 hours with either 7mM LiCl or NaCl added for the final hour. They were then placed in a static cAMP gradient and their movement analysed. Each experiment was normalised to a NaCl control done on the same day to give a lithium sensitivity index (LSI). Results plotted are the means ± standard deviation of four independent experiments with at least 50 cells measured each time. * $P \leq 0.06$, ** $P \leq 0.01$, *** $P \leq 0.005$; Student T-test compared to wild-type.

3.12 Discussion

3.12.1 *Dictyostelium* MIPP

From this work we can conclude that although the *Dictyostelium* genome contains two clear MIPP homologues, a single gene is responsible for all activity observed in both vegetative, and developing cells. Why a second gene is present which does not seem to be expressed is interesting, and although we have not shown *mppB* to encode an active phosphatase, it has preserved all the residues which are conserved throughout evolution and are likely to be important for function, whilst being significantly different from MippA in both protein and nucleotide sequence, suggesting a similar activity, but a divergent role. The fact that this gene has been retained indicates that it plays an important role – presumably in an alternative process such as during the sexual cycle.

In *mppA* knockout cells, no $InsP_6$ or $Ins(1,3,4,5,6)P_5$ hydrolysis could be detected, either in the soluble, or particulate fractions and therefore we conclude that this gene is responsible for the activity described by both Drayer *et al.* in *plc*$^-$ cells, and Williams *et al.* in *dpoA*$^-$ cells (Drayer et al., 1994; Williams et al., 1999).

3.11.2 The activity of *Dictyostelium* MIPP

The original biochemical characterization of MIPP activity in *Dictyostelium* indicated that only $Ins(1,3,4,5,6)P_5$ and $Ins(1,3,4,5)P_4$ / $Ins(1,4,5,6)P_4$ were substrates for

$Ins(1,4,5)P_3$ production and that $InsP_6$ was not (Van Dijken et al., 1995a). In this study however, I have found that $InsP_6$ is a substrate. This discrepancy may be explained by the rather surprising results obtained when different concentrations of $InsP_6$ were used as a substrate. As MIPP is able to differentially dephosphorylate $InsP_6$ at different concentrations, it may be that in the original assay, excessive substrate was used leading to the formation of alternative $InsP_3$ isomers which would not be detected in their assay.

Although it is hard to reconcile the differential dephosphorylation of $InsP_6$ at high and low substrate concentrations with an enzymatic mechanism, previous studies have also hinted at similar peculiarities. Initially MIPP was identified as an $Ins(1,3,4,5)P_4$ 3` –phosphatase, and $InsP_6$ was considered a "potent endogenous inhibitor" as well as a substrate (Hodgson and Shears, 1990; Hughes and Shears, 1990). In a study by Nogimori *et al.*, they show that $InsP_6$ binds the mammalian enzyme with a very high affinity (K_m= 0.3nM) but with a V_{max} of about 350-fold lower than when $Ins(1,3,4,5)P_4$ is the substrate (K_m =130nM). Therefore they propose a mechanism where $InsP_6$ is a strong competitive inhibitor, whilst being a poor substrate (Nogimori et al., 1991). Interestingly, they also observe a very similar pattern of $InsP_6$ dephosphorylation to that seen in this study at high $InsP_6$ concentrations and conclude that it is a non-specific phosphatase when $InsP_6$ is the substrate. My study would indicate that this is not the case, and that $InsP_6$ is actually a good substrate but only at a certain concentrations.

Biochemically it is hard to understand how this mechanism would work and it seems to be without precedent. It may indicate a second low affinity, $InsP_6$ binding site, or

perhaps the ability of $InsP_6$ to bind metal ions can alter the specificity of the reaction. Alternatively, it may be that these 2' phosphorylated inositides are always formed at a low rate, and that only in high $InsP_6$ concentrations do they accumulate sufficiently to competitively inhibit the normal reaction. To study this further requires purified enzyme which we are currently unable to produce.

These observations could have physiological consequences, as they suggest that for optimal activity and the production of a biologically active product, MIPP must be present in a distinct compartment, away from the high concentrations of $InsP_6$ found in the cytosol. In mammalian cells, biochemical studies have placed MIPP activity within the ER whereas its substrates are predominantly cytosolic (Ali et al., 1993; Romano et al., 1998). In contrast, fractionation studies of *Dictyostelium* extracts found that this activity exclusively co-elutes with plasma membrane markers, and not ER (Van Dijken et al., 1997). This activity was further shown to be on the inside of the cell which would mean exposure to the large cytoplasmic phosphoinositide pool.

If this were the case, MIPP would be exposed to the large, high concentration pool of $InsP_6$ and the accumulation of 2' phosphorylated inositides would be predicted. *In vivo* the only detectable 2'phosphorylated species in *Dictyostelium* are Ins(1,2,3,4,6)P_5, Ins(1,2,3,4,5)P_5 and Ins(1,2,5,6)P_4 and as these are unaltered in *mppA*$^-$ cells they are probably formed as non-specific side-products of a different phosphatase due to the exceptionally high $InsP_6$ concentrations. This, and the fact that no differences in phosphoinositide abundance, including $InsP_6$, could be detected in the *mppA*$^-$ cells indicate that it uses a distinct, much smaller pool of substrate, which we cannot separate from the much larger cytoplasmic one. This however is not the

case when *mppA* is strongly overexpressed. In these cells MIPP does not appear to be so tightly contained and some of the enzyme is able to utilize the high concentration pool, leading to the accumulation of 2' phosphorylated species and a complete depletion of Ins(1,3,4,5,6)P_5.

The difficulties found in adding an epitope tag to the full-length protein in all organisms has prevented a clear understanding of the localization and role of MIPP within the cell. We are currently producing an antibody which will tell us more about both MIPP and the subcellular distribution of inositol phosphates *in vivo*.

3.12.3 Inositide abundance in *dpoA* mutants

In *dpoA*$^-$, *mppA*$^-$ and double mutant cells, I detected no significant change in phosphoinositide levels, including no accumulation of 2'phosphorylated species. These observations are in agreement with those made in the original *plc*- mutant, which also had elevated MIPP activity but no major change in phosphoinositide abundance (Drayer et al., 1994) and would indicate that the more subtle increase in MIPP activity in these cells does not significantly alter the metabolism of the majority of the inositol phosphates. Therefore again it is likely that only a small subset of the phosphoinositides are affected which we cannot detect. Indeed, in order to efficiently produce a change, any signaling molecule is required to be at low abundance and therefore in this case, HPLC analysis may not be sensitive enough to detect these changes.

3.12.4 MippA activity is required for lithium resistance

Whether in an assay for random or directed chemotactic movement, lithium has the effect of reducing the cell speed. In tandem, the amount of cell turning is also increased, both factors leading to a developmental block at aggregation. Cells lacking PO activity are more resistant to these effects, when *mppA* is also disrupted, this resistance is lost. This places *mppA* genetically downstream of *dpoA*. This would therefore implicate inositol metabolism and therefore inhibition of IMPase and IPP as the cause of this reduced motility. This raises the question of how increased MIPP activity leads to resistance, and how inhibition of inositol metabolism by lithium leads to defective chemotaxis.

Some clarity may be obtained from observations with *mppA* overexpressing cells. Although these cells are no different from wild-type in control experiments, they are consistently more strongly affected by lithium. This is in contrast to *dpoA*$^-$ cells which also have elevated MIPP activity, but are resistant. The major difference between the two cases is the extent to which MIPP activity is increased – in *dpoA* mutants, it is elevated by about 1.5 fold compared to ~100 fold in the *mppA* overexpressor. This indicates that elevating $InsP_3$ synthesis from of $InsP_6$ dephosphorylation is not sufficient for lithium resistance, and that other factors are also involved. As we have seen, the *mppA* overexpressor has enormous activity - some of which appears to be mis-localised; this may mean that pools of inositol phosphates, not normally regulated by MIPP are altered and could explain the difference between the effects of small and large increases in activity.

Although these experiments show a clear link between PO, MIPP and lithium sensitivity, and is in fact the first mechanism of MIPP regulation to be identified, how PO is able to regulate MIPP is unknown, and as the crystal structure of PO is thought to preclude the use of proteins as a substrate (Hiramatsu et al., 2003), this relationship is likely to be indirect. The fact that alterations in MIPP activity can be shown to alter lithium sensitivity does however represent a novel mechanism by which MIPP and the higher order inositol phosphates can regulate cellular behaviour.

Chapter 4:

Higher inositol phosphates regulate lithium sensitivity through gene expression in *Dictyostelium*

4.1 Introduction

In the previous chapter I showed that MippA activity is required to mediate the effects of PO loss on lithium resistance to chemotaxis. This implies that alteration in the metabolism of higher inositol phosphates is sufficient to give lithium resistance. One explanation could be that by elevating the production of Ins(1,4,5)P_3 from InsP_6, the inositol depletion effects of lithium treatment can be overcome. However, although this may be able to contribute to a lithium resistant phenotype, it cannot explain why cells overexpressing *mppA* become hypersensitive. In addition, if the disruption of chemotaxis is due to the inhibition of IMPase and IPP, this has been shown to be through a non-competitive mechanism as lithium binds the enzyme-substrate complex (Gee et al., 1988). This means that increased substrate concentration will not be able overcome this, and may actually lead to the inhibition of more enzyme. The only method of overcoming this form of inhibition is therefore to increase the enzyme concentration.

In yeast, the regulation of gene expression by inositol phosphates has been demonstrated a number of times, through a number of mechanisms. These including the direct use of inositol kinases (IPK2/Arg82/IPMK) as transcription factors, regulation of lipid biosynthesis by the Opi1p/Ino2p/Ino4p complex (Hirsch and Henry, 1986), the binding of inositol polyphosphates to chromatin remodelling factors (Odom et al., 2000; Shen et al., 2003; Steger et al., 2003) and the regulation of RNA export and processing (York et al., 1999) - all of which could potentially lead to increased protein levels. Although we do not know whether MippA is present in the nucleus, or is indirectly able to alter the nuclear pool of inositides, it is the only

known enzyme able to dephosphorylate $InsP_6$ and therefore is potentially able to regulate a number of these processes. In this chapter, I investigate whether MippA and other inositol metabolic mutants are able to regulate gene expression by similar processes, and therefore represent a novel mechanism by which lithium sensitivity and chemotaxis may be regulated by the higher order inositol phosphates.

4.2 Identification of *Dictyostelium* IMPase-family genes

Previously, biochemical study has identified three inositol monophosphatase activities in *Dictyostelium*, two of which are lithium sensitive and therefore of the IMPase class. (Van Dijken et al., 1996a). A BLAST search of the *Dictyostelium* genome identified 3 genes containing the conserved domains of the IMPase family of proteins (Fig. 4.1). Of these, one (*impa1*, DDB0204100) is more similar to the IMPase proteins of other organisms, and work by others in this laboratory has shown that it does indeed encode an enzyme with IMPase activity (Keim-Reder, 2006). The other two genes (DDB0167248 and DDB0189923) are respectively more similar to the IPP and PAP-phosphatase proteins and are designated *ippA* and *B*. In other species, however these proteins have been shown to have significant activity against more than one class of substrate and so to some extent these assignments may be arbitrary (Lopez-Coronado et al., 1999).

4.3 Inhibition of PO increases the expression of lithium-sensitive genes

Previous studies have shown that the yeast *ino1* gene, encoding inositol synthase, is upregulated upon inositol starvation (Hirsch and Henry, 1986) and can be regulated

by both the Ino2p/Ino4p complex and several phosphoinositide-sensitive chromatin remodeling complexes (Shen et al., 2003). The *Dictyostelium* genome contains a single homologue, disruption of which leads to inositol auxotrophy and is therefore a possible target for regulation (Fischbach et al., 2006).

When *Dictyostelium* cells were grown overnight with 1.2mM of a PO-specific inhibitor z-Pro-L-prolinal (Goossens et al., 1997; Williams et al., 1999), the expression of *ino1* was increased by 43 ± 6 %. In addition, the expression of all three IMPase family genes was elevated by 60-80% (Fig. 4.2). In contrast, other genes involved in the metabolism of the higher order inositol phosphates (see later) including *mppA* were unchanged, showing that these changes are specific, and that regulation of MIPP activity is at a post-transcriptional level (for the validation of all primer sets used for Q-RT PCR see appendix I).

4.4 MIPP activity is required for regulation of gene expression

To see whether the changes in expression observed above are due to increased MippA activity *mppA* null cells were treated with Z-pro-L-prolinal. When the level of *ino1* and *impa1* expression in untreated *mppA*$^-$ cells was compared with wild-type, no significant differences were seen and therefore loss of MIPP does not seem to affect expression under normal conditions (Fig. 4.3). However, when these cells were treated with PO inhibitor, the increase in *ino1* and *impa1* expression observed in wild-type cells was completely blocked showing that MIPP activity is essential for the up-regulation of gene expression by PO.

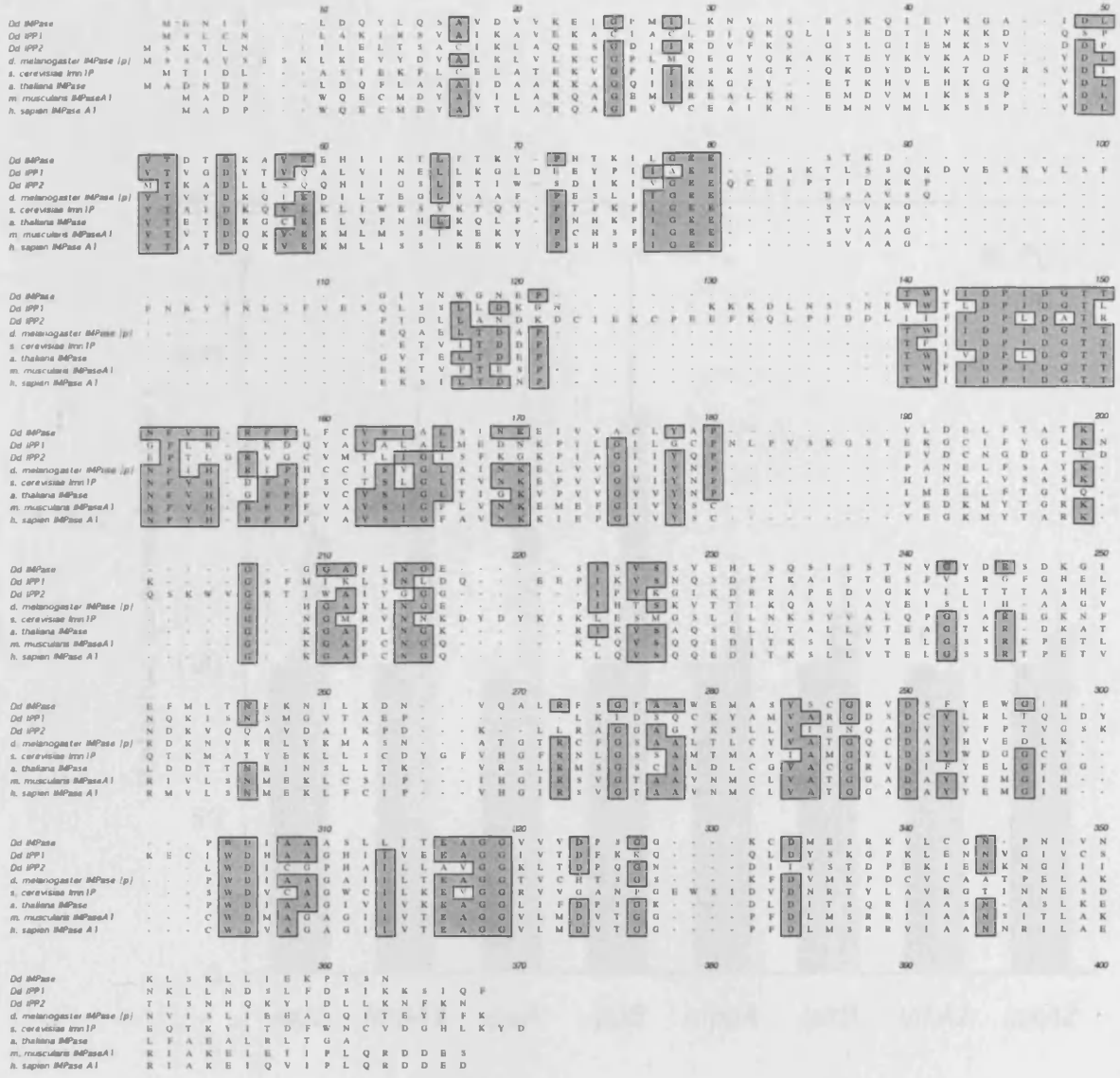


Figure 4.1: Alignment of *Dictyostelium* IMPase family genes against other species.

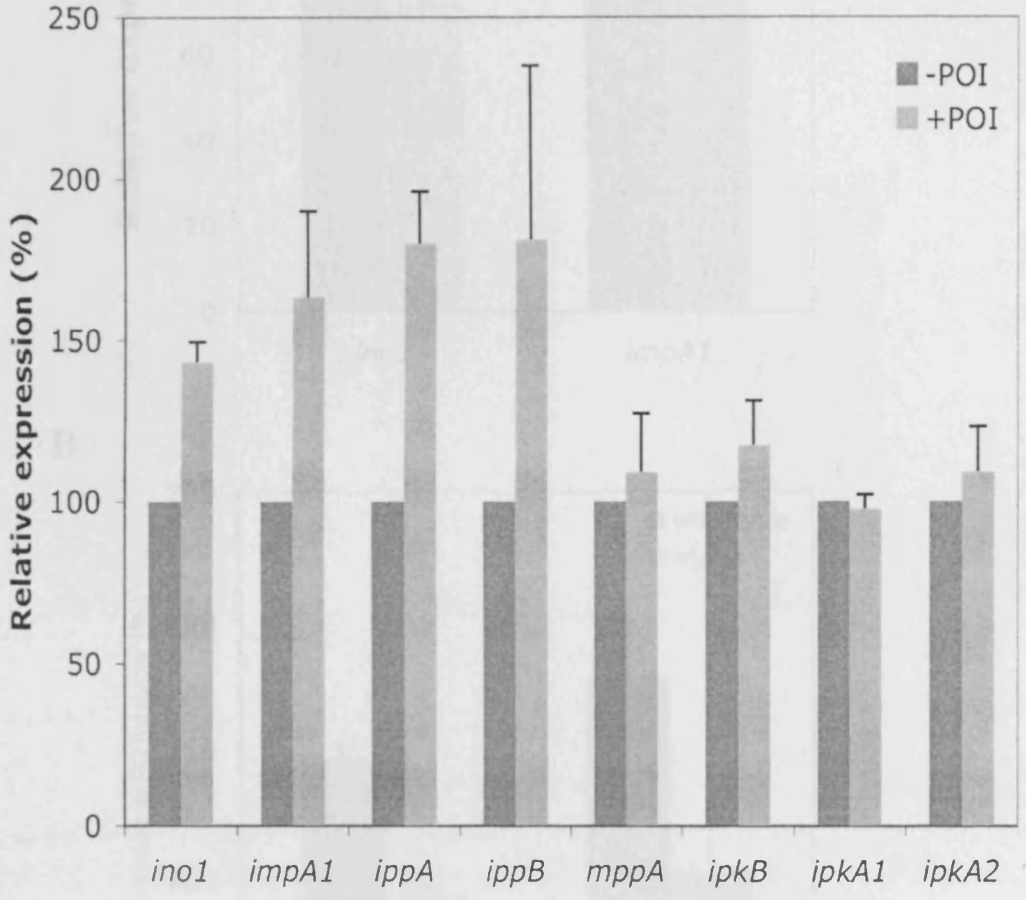


Figure 4.2: The effects of PO inhibition on gene expression. Wild-type cells were grown overnight in the presence of either 1.2mM z-pro-l-prolinal, or an equivalent volume of the DMSO solvent used. RNA levels were then quantified by real-time PCR, and calculated relative to the carrier control. All samples were normalized to Ig7 as a loading control, and values shown are the means of three independent samples. Error bars indicate standard error.

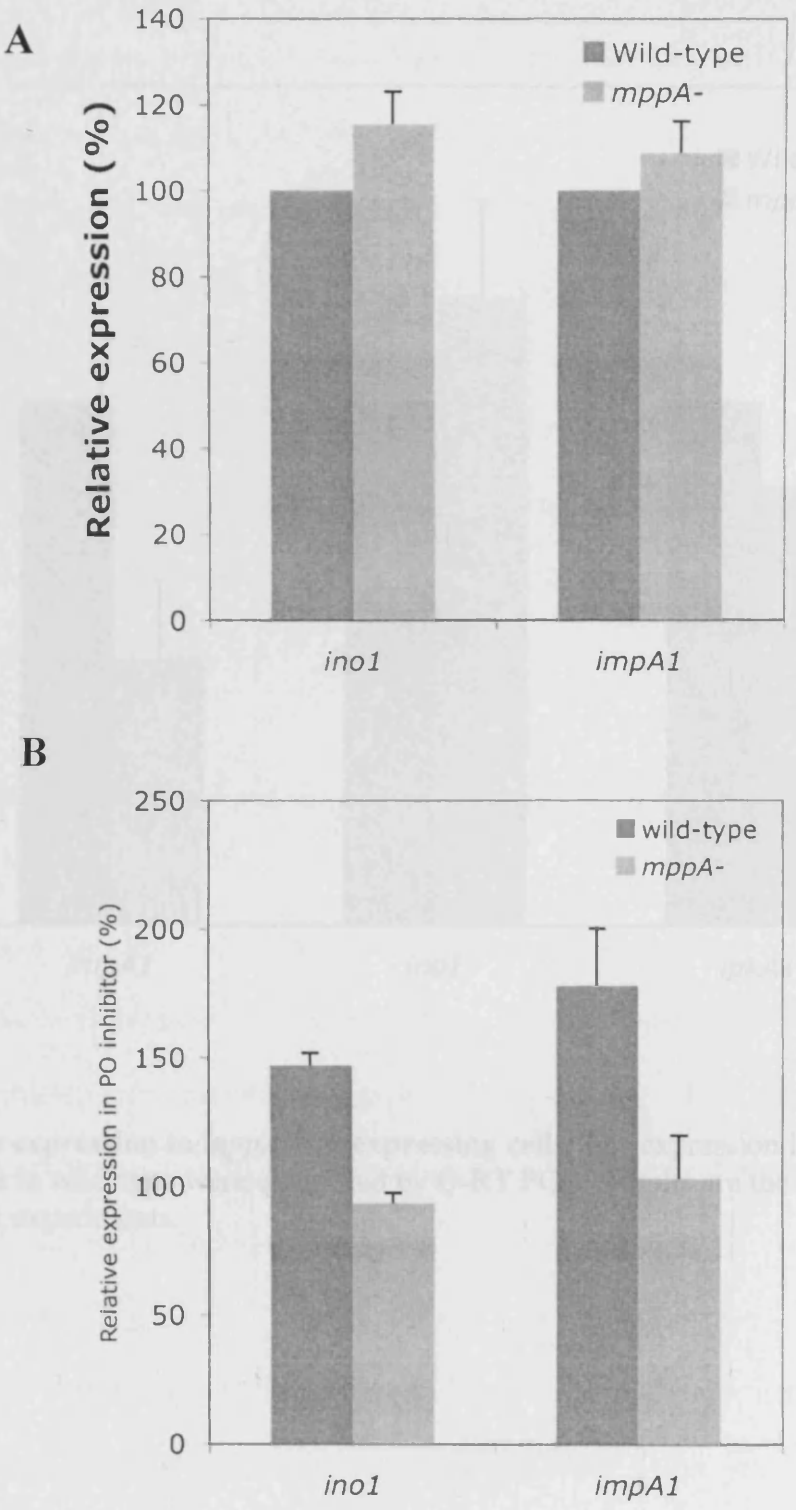


Figure 4.3 : Gene expression in *mppA*⁻ cells. (A) Expression of *ino1* and *impA1* in vegetative *mppA*⁻ cells relative to wild-type. (B) Gene expression of wild-type and *mppA*⁻ cells treated with 1.2mM Z-Pro-L-Prolinal relative to the same cells treated with the DMSO carrier. All results are the mean ± standard error of at least 3 independent experiments.

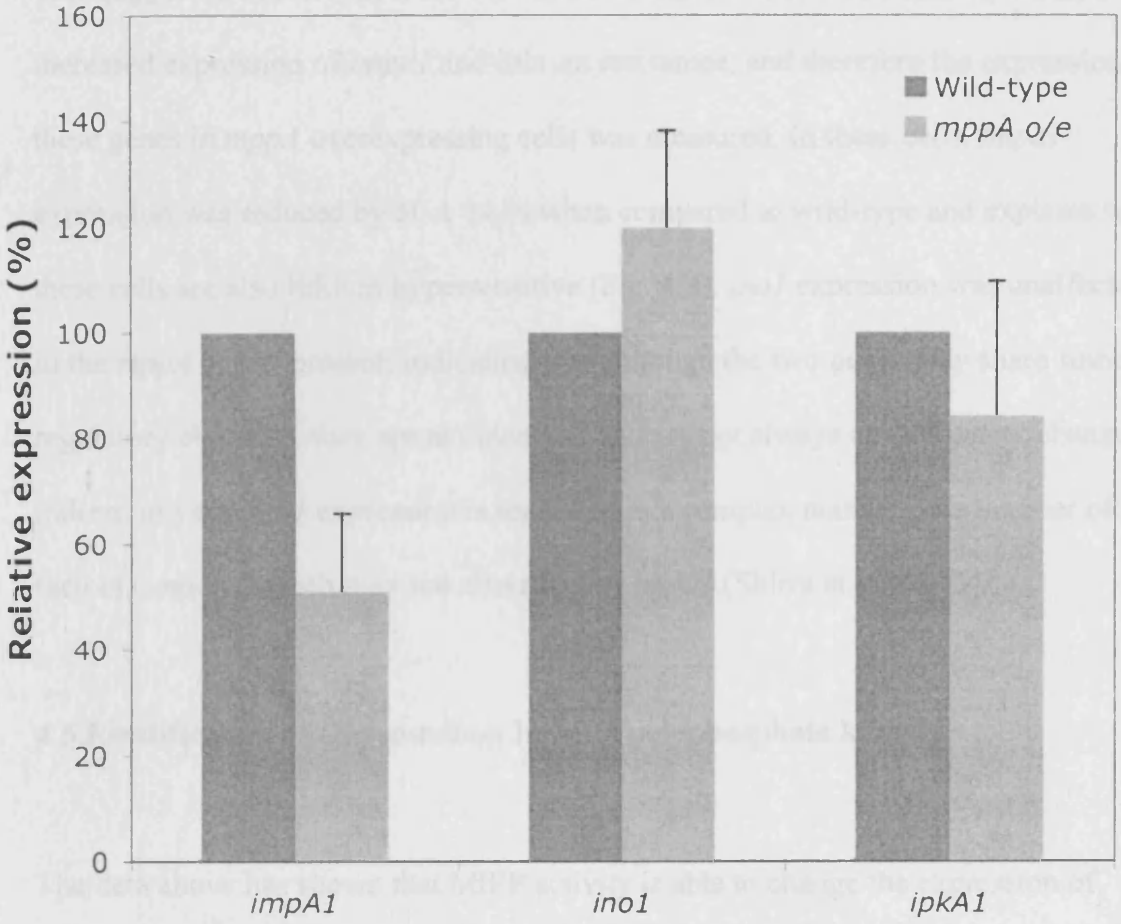


Figure 4.4: Gene expression in *mppA* overexpressing cells. The expression levels of *ino1*, *impA1*, and *ipkA1* relative to wild-type were quantified by Q-RT PCR. Results are the mean ± standard error of three independent experiments.

This suggests a model where the PO-mediated elevation of MIPP activity leads to increased expression of *impa1* and lithium resistance, and therefore the expression of these genes in *mppA* overexpressing cells was measured. In these cells, *impa1* expression was reduced by 50 ± 14 % when compared to wild-type and explains why these cells are also lithium hypersensitive (Fig. 4.4). *ino1* expression was unaffected in the *mppA* overexpressor, indicating that although the two genes may share some regulatory elements, they are not identical and are not always co-ordinately changed; indeed, in yeast *ino1* expression is regulated in a complex manner by a number of factors, some of which may not also regulate *impa1* (Shirra et al., 2005).

4.5 Identification of *Dictyostelium* Inositol polyphosphate kinases

The data above has shown that MIPP activity is able to change the expression of several genes. In order to see whether this is specific to this enzyme or due to a more general mechanism of regulation by inositides, other genes involved in the metabolism of the higher order inositol phosphates were identified to see if they could also induce similar transcriptional changes.

In other organisms, there are two distinct families of enzymes which are able to phosphorylate inositides to produce $InsP_5$. These are the IPMK enzymes (known as IPK2/arg82 in yeast and are also transcription factors) which typically phosphorylate Ins(1,4,5)P_3 at the 3' and 6' positions, and the Ins(1,3,4)P_3 5'/6' kinases which phosphorylate Ins(1,3,4)P_3 at the 5' and 6' positions as well as Ins(3,4,5,6)P_4 at the 1' position (Odom et al., 2000; Stevenson-Paulik et al., 2002; Verbsky et al., 2005b).

The end-product of both enzymes is, however the production of Ins(1,3,4,5,6)P_5 which is subsequently phosphorylated by 2' kinase to produce InsP_6.

4.5.1 The *Dictyostelium* IPMK genes

The IPMK family of enzymes is typified by a tightly conserved PxxxDxKxG phosphoinositide binding motif. A search of the *Dictyostelium* genome identified three genes containing this motif – one of which was the previously identified InsP_6 kinase (Luo et al., 2003).The two other genes (DDB 0203614 and DDB 0218526) were named *ipkA1* and *A2* respectively. In addition a third gene was identified (DDB 0204639, *ipkA3*), which has ~30% identity to the others along its length, but has the proline residue of the inositide-binding domain replaced with an alanine (which was confirmed by sequencing). This residue is absolutely conserved in all other species, and therefore this *Dictyostelium* homologue may either be inactive, or have a different substrate specificity. All three genes do however contain the ATP-binding domain and other regions previously identified as conserved in this class of enzymes (Nalaskowski et al., 2002). All three also lack the calmodulin binding domain which distinguishes the specialized subclass of Ins(1,4,5)P_3 3' kinases found in higher eukaryotes. (Fig. 4.5) (Nalaskowski et al., 2002; Odom et al., 2000). Therefore we conclude that, with the possible exception of the third gene, these encode IPMK homologues and when tested by RT-PCR, all three are expressed in vegetative cells (Fig. 4.6).

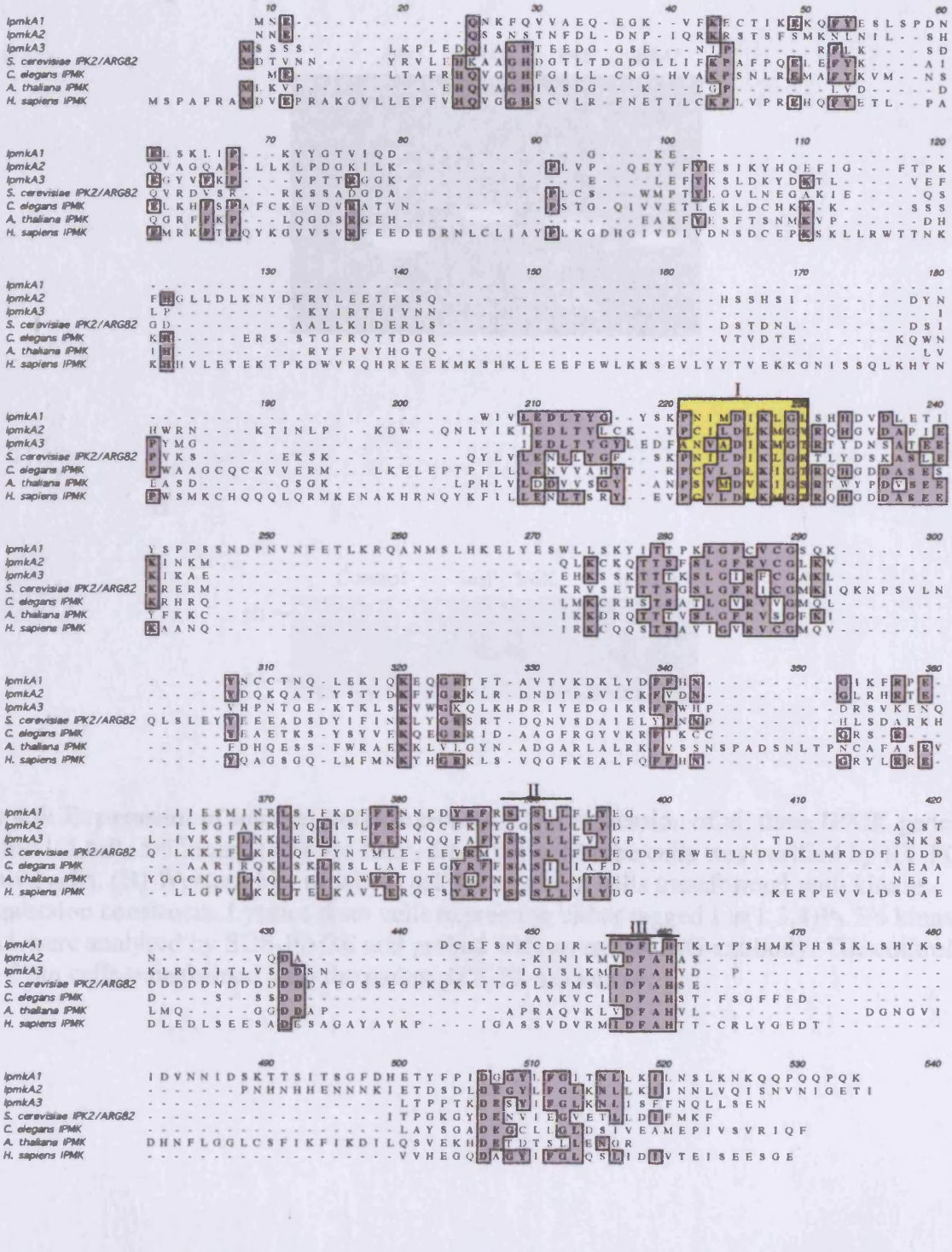
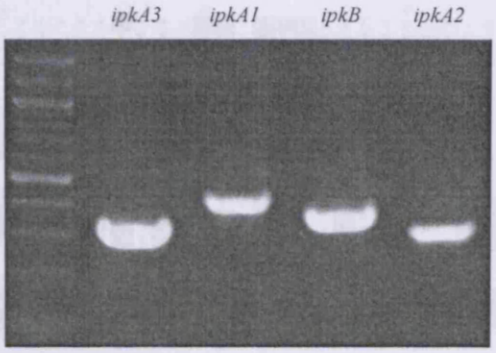


Figure 4.5: Alignment of *Dictyostelium* IPMK genes. The three putative *Dictyostelium* homologues were aligned with those identified from other specied. The boxed motif I indicates the conserved catalytic motif as are the other characteristic motifs II (unknown function) and III (ATP-binding).

A



B

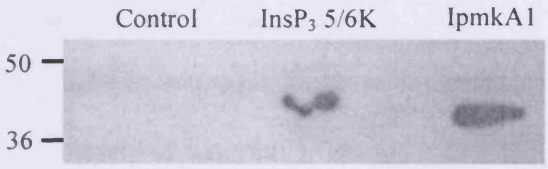


Figure 4.6: Expression of inositol polykinases. (A) The expression of all three IPMK genes and the single Ins(1,3,4)P_3 5/6 kinase (labelled InsP_3 5/6 K) in wild-type cells was verified by RT-PCR of vegetative cells. (B) Western blot of whole cell lysates from cells transformed with kinase overexpression constructs. Lysates from cells expressing either tagged Ins(1,3,4)P_3 5/6 kinase, or IpmkA1 were analysed by SDS-PAGE and probed with an anti-FLAG antibody. The control lane is an extract from cells transformed with the parent pTX-FLAG vector.

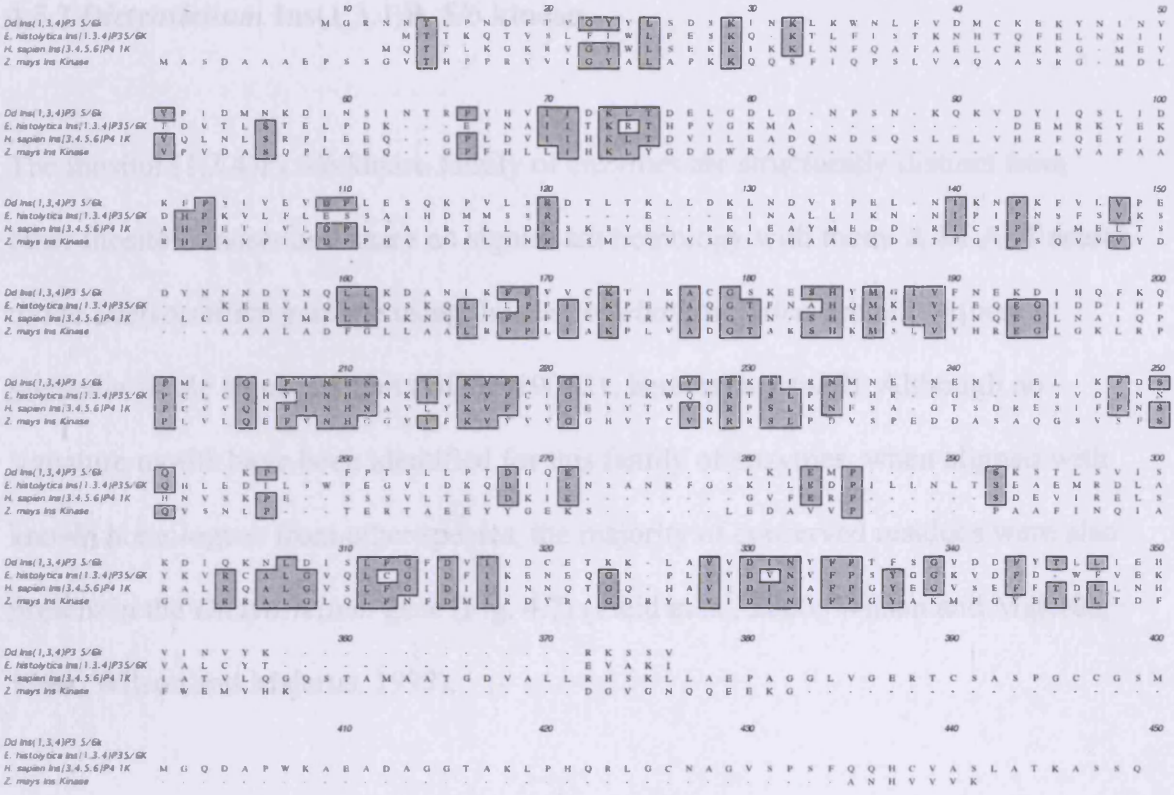


Figure 4.7: Alignment of *Dictyostelium* Ins(1,3,4)P_3 5/6 kinase with other species. A clustal alignment of the single *Dictyostelium* homologue against those previously identified through evolution.

4.5.2 *Dictyostelium* Ins(1,3,4)P_3 5/6 kinase

The inositol (1,3,4)P_3 5/6 kinase family of enzymes are structurally distinct from other inositol kinases and share no significant homology with them. A BLAST search of the *Dictyostelium* genome using the *Entamoeba histolytica* protein sequence yielded a single significant hit (DDB 0190521, here named *ipkB*). Although no signature motifs have been identified for this family of enzymes, when aligned with known homologues from other species, the majority of conserved residues were also present in the *Dictyostelium* gene (Fig. 4.7) (Field et al., 2000; Wilson and Majerus, 1996; Wilson and Majerus, 1997).

4.6 The generation of Inositol kinase overexpressors

To see whether these genes were able to regulate gene expression, cell lines overexpressing a member of each class of protein were generated. Full-length cDNAs of both *ipkA1* and *ipkB* were cloned into constructs for expression in *Dictyostelium*, under the control of the constitutive actin 15 promoter. In addition, as both proteins are soluble, we were able to add a FLAG-epitope tag to the N-terminus of both and after transformation into cells, a protein product of the correct size could be identified by western blot (Fig. 4.6).

As I could express tagged fusion proteins in *Dictyostelium*, I examined their subcellular localization. When cells expressing either kinase were fixed and stained, both proteins had a predominantly nuclear localization - similar to that reported in

other organisms and by biochemical studies in *Dictyostelium* which placed the conversion of Ins(1,4,5)P_3 to InsP_6 in the nucleus (Van der Kaay et al., 1995). In addition, both proteins were also present in numerous small punctate spots throughout the cell - which are presumably either vesicles or protein aggregates (Fig. 4.8). Whether these spots are real, or an artefact of overexpression is impossible to say although such structures have not previously been reported in similar experiments, expressing GFP fusion protein in human and Arabidopsis cells (Nalaskowski et al., 2002; Qin et al., 2005). All other studies with these enzymes have been purely biochemical and therefore it may have been missed. Indeed, such a similar pattern for both kinases does perhaps suggest that they may be co-localised on vesicles although again, it is impossible to tell with these constructs.

4.7 The inositol polyphosphate abundance in inositol kinase overexpressors

In order to assess the effect of inositol kinase overexpression, we extracted the inositol phosphates from these cells and quantified them by HPLC-MDD. As with many of the other mutants, very little difference between cells overexpressing either kinase, and control cells could be detected (Table 4.1 and Fig. 4.9). The only significant change seen was that cells overexpressing IpmkA1 contained 2.5 fold more InsP_7 than control ($P<0.005$, Student's T-test). Whether this is physiological product of the enzyme or not is unclear, but as the enzyme shares a common catalytic domain with the InsP_6 kinase which would normally synthesise InsP_7, it may simply be an artefact due to excessively high levels of expression.

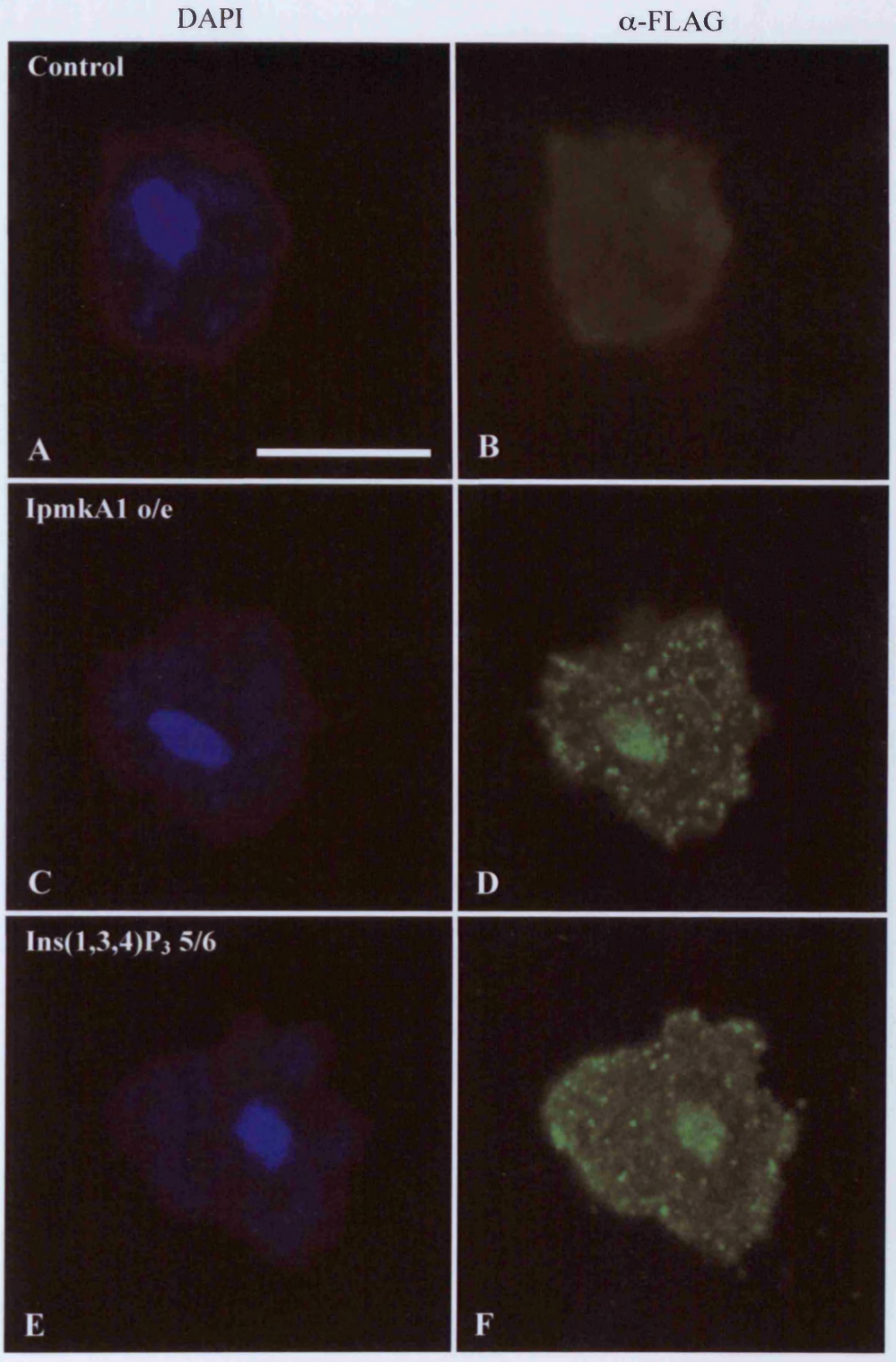


Figure 4.8: The subcellular localisation of IpmkA1 and Ins(1,3,4)P_3 5/6 kinase. Cells overexpressing either IpmkA1 (C & D), or Ins(1,3,4)P_3 5/6 kinase (E & F) or control cells transformed with pTX-FLAG (A & B) were fixed and stained with stained with both DAPI (A, C and E) and anti-FLAG antibody (B, D and F). All images are confocal sections through the middle of the cell. Bar = 10μm.

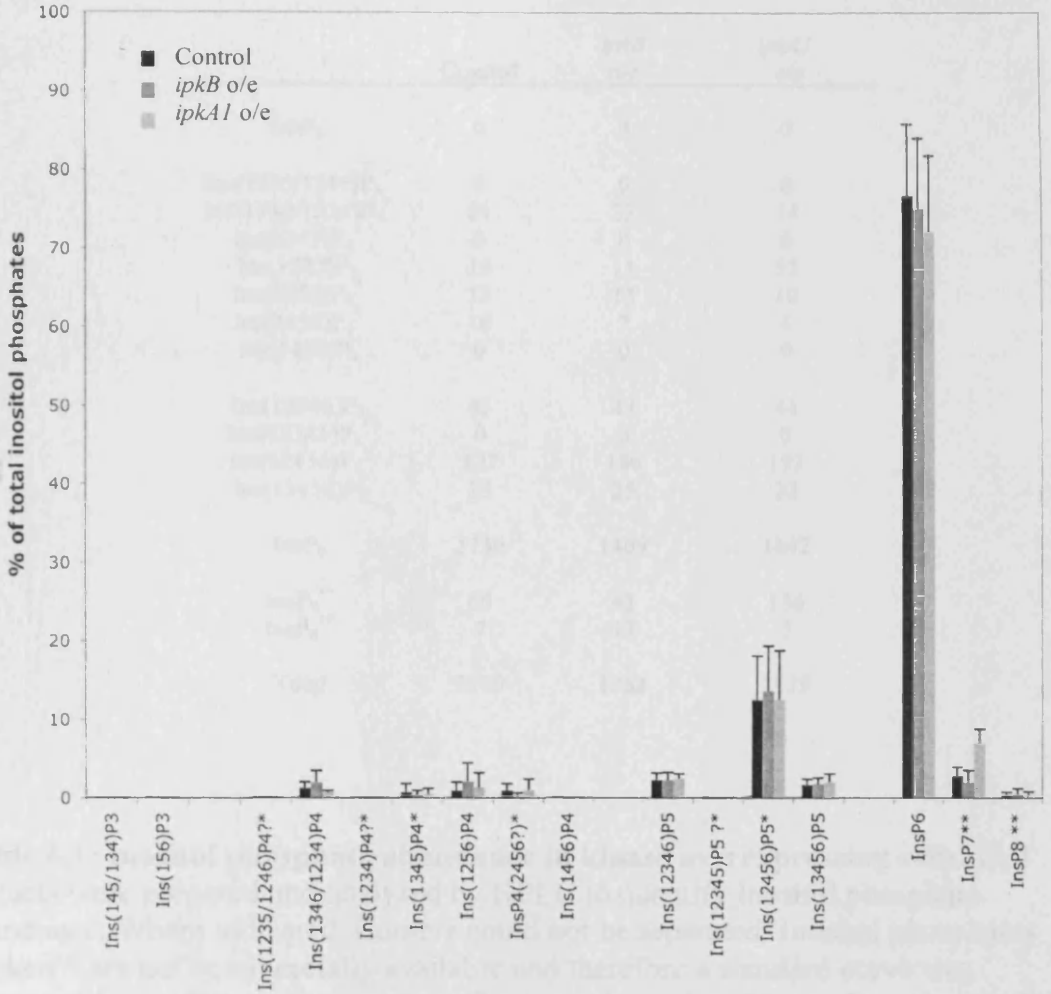


Figure 4.9: Relative distribution of the higher order inositol phosphates in kinase overexpressing cells. Inositol phosphate abundance was determined by HPLC for each strain and normalised to total inositol polyphosphate levels. Values plotted are the means ± standard deviation of at least 3 independent experiments.

Pmol / 10^7 cells

	Control	*ipkB* o/e	*ipkA1* o/e
$InsP_3$	0	0	0
$Ins(1235/1246)P_4$*	0	0	0
$Ins(1346/1234)P_4$*	21	22	14
$Ins(1245)P_4$*	0	0	0
$Ins(1345)P_4$	19	11	15
$Ins(1256)P_4$*	13	15	10
$Ins(2456)P_4$*	16	7	6
$Ins(1456)P_4$	0	0	0
$Ins(12346)P_5$	43	43	44
$Ins(12345)P_5$*	0	0	0
$Ins(12456)P_5$*	227	186	193
$Ins(13456)P_5$	33	25	32
$InsP_6$	1730	1409	1642
$InsP_7$**	60	42	156
$InsP_8$**	7	13	7
Total	2169	1762	2119

Table 4.1: Inositol phosphate abundance in kinase overexpressing cells. Cell extracts were prepared and analysed by HPLC to quantify inositol phosphate abundance. Where indicated, isomers could not be separated. Inositol phosphates marked * are not commercially available and therefore a standard curve was estimated from other similar isomers. Compounds marked ** are also unavailable but also have no available isomers. Therefore in these cases a standard curve was estimated from $InsP_6$ values corrected for phosphate content. All values are the mean of at least 3 independent determinations.

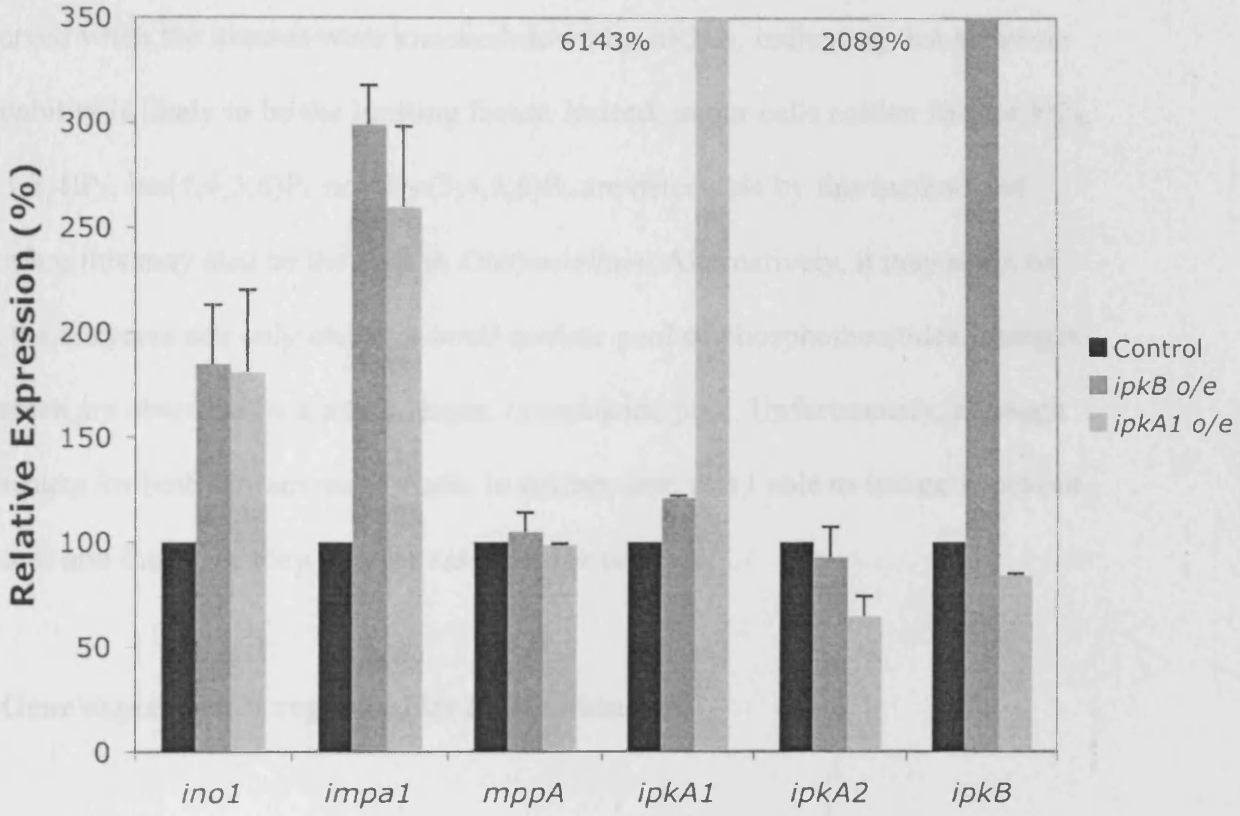


Figure 4.10: Gene expression in inositol kinase overexpressing cells. The effect of inositol kinase overexpression on the expression of other inositol metabolic genes was analysed by Q-RT PCR. RNA was extracted from cells transformed with either *ipkA1* or *ipkB* overexpression constructs and expressio levels calculated relative to cells transformed with the pTX-FLAG parent plasmid. Values plotted are th mean ± standard error of 3 independent experiments.

To our surprise, overexpression of neither kinase significantly affected the total concentration of Ins(1,3,4,5,6)P_5. Others have however reported similar results in mammalian cells where overexpression of IPMK in HEK293 cells also made no difference, although Ins(1,3,4)P_3 5/6 kinase did increase Ins(1,3,4,5,6)P_5 to some degree (Verbsky et al., 2005b). In their study, much more dramatic effects were observed when the kinases were knocked-down by siRNA, indicating that substrate availability is likely to be the limiting factor. Indeed, in our cells neither Ins(1,4,5)P_3, Ins(1,3,4)P_3, Ins(1,4,5,6)P_4 nor Ins(3,4,5,6)P_4 are detectable by this method and therefore this may also be the case in *Dictyostelium*. Alternatively, it may again be that the enzymes can only utilize a small nuclear pool of phosphoinositides, changes of which are obscured by a much larger, cytoplasmic pool. Unfortunately, although constructs for both kinases were made, in neither case was I able to isolate knockout mutants and therefore they may be essential for survival.

4.8 Gene expression is regulated by inositol kinases

The overexpression of these two classes of inositol kinase allows us to test whether the changes in *ino1* and *impa1* expression due to MippA activity are specific, or due to a general mechanism of regulation by inositol phosphates. When the expression of these genes in cells overexpressing either kinase were measured, both *ino1* and *impa1* RNA levels were increased, by ~90% and 160% respectively (Fig. 4.10). Again this seems to be specific to these enzymes, as the kinases do not affect the expression of each other, or *mppA*.

4.9 Discussion

4.9.1 Loss of PO gives lithium resistance through transcriptional regulation

In our model, inhibition of the IMPase family of proteins by lithium leads to the reduced motility described in the previous chapter. Here we show that inhibition of proly oligopeptidase leads to a significant increase in the mRNA levels of all three IMPase family members. If these increases in mRNA levels are representative of changes in protein levels this gives a mechanism for the lithium resistance seen in the *dPOa* null mutant. Indeed, *Dictyostelium* cells overexpressing IMPase have been shown to become lithium resistant (Keim-Reder, 2006). Also, it is interesting that *ino1* is also regulated, as its product, inositol synthase, is an indirect target of valproic acid. This would therefore also explain the observation that *dpoA* null cells are cross-resistant to both drugs (Williams et al., 2002).

The changes in gene expression upon PO inhibition are also specific, as it only appears to be those genes directly involved in the recycling of Ins(1,4,5)P_3 and *de novo* inositol synthesis that are affected. This argues that the physiological role of this response is to adjust the metabolism of the soluble inositol phosphates feeding into phospholipid synthesis. In yeast, *ino1* expression is up-regulated by inositol starvation, and *IMN1* (encoding the yeast IMPase) is up-regulated by the addition of inositol (Hirsch and Henry, 1986; Murray and Greenberg, 1997; Murray and Greenberg, 2000). If this is also the case in *Dictyostelium* it may only be necessary for the PO to regulate *ino1* expression as this would lead to increased intracellular inositol and consequentially the up-regulation of *impa1* – a hypothesis which may

now be easily tested using the *ino1* knockout which has recently been isolated (Fischbach et al., 2006).

4.9.2 Regulation of gene expression by MippA

When *mppA* null cells are treated with a PO inhibitor, expression of *ino1* and *impa1* is unchanged showing that MippA activity is absolutely required for PO-induced changes in gene expression. This explains why the *mppA* null mutation is a suppressor of the *dpoA*$^{-}$ phenotype. Although we still do not know how PO and MIPP are connected, it must be post-transcriptional as *mppA* expression remains constant throughout.

How MIPP alters gene expression in the nucleus is an interesting problem, as all the evidence from other organisms places it within the endoplasmic reticulum. Although the evidence in *Dictyostelium* is inconsistent with this, with MIPP activity co-fractionating with plasma membrane markers (Van Dijken et al., 1997), my data in this study does indicate that it is restricted within a compartment, away from the bulk of its substrates. There is however, no reason to believe that it is in the nucleus (Ali et al., 1993) and thus it would seem that it generates a signal which is transported there. Whether this signal is in the form of an inositol phosphate or not is unclear, but as $InsP_6$ and inositol kinases are present in the nucleus, if $InsP_6$ is to be metabolized by dephosphorylation it must be able to reach MIPP somehow - again indicating either nuclear MIPP or transport between compartments.

An alternative explanation, may be that MIPP is able to regulate other factors which are able to signal to the nucleus. For example, in yeast the membrane lipid composition is sensed by Opi1p which is localized to the ER by its association with the vesicle-associated-membrane protein-associated protein (VAP) and phosphatidic acid (Loewen et al., 2004; Loewen et al., 2003); upon addition of inositol, this protein is released and translocates to the nucleus to reduce the transcription of UAS_{INO}-containing genes, including *ino1* by repression of the Ino2p/Ino4p transcription activator complex (Jesch *et al.*, 2005). In addition to this, inositol addition is also able to supress the unfolded protein response (UPR) pathway which senses ER secretory stress (van Anken and Braakman, 2005), and regulate Ino2p/Ino4p target gene expression independently of Opi1p (Jesch et al., 2006). Therefore, if MIPP is located in the ER, it is possible that it is able to regulate either of these pathways and intersect other transcriptional regulators in the nucleus.

Although small increases in MippA activity are able to generate an increase in *impa1* expression, a large increase in MippA activity due to overexpression from a strong, actin promoter has the opposite effect - correlating with lithium sensitivity. This discrepancy could be down to a number of reasons, most likely due to the mis-localisation of the enzyme when strongly overexpressed. One possible reason is that the 2' phosphorylated species that accumulate in *mppA* overexpressing cells are potentially competitive substrates for other enzymes involved in inositol metabolism. This has previously been shown for $Ins(1,2,4)P_3$ which is able to inhibit the $Ins(3,4,5,6)P_4$ 1' kinase activity of the $Ins(1,3,4)P_3$ 5/6 kinase enzyme with an IC_{50} of 9μM (Adelt et al., 2001). In these cells the concentration of $InsP_3$ isomers can be estimated as ~50 μM (using an average cell volume of $40\mu m^3$, www.dictybase.org)

and therefore it may actually be this effect, rather than increased MIPP activity which leads to the decrease in gene expression. Alternatively in cells overexpressing *mppA* Ins(1,3,4,5,6)P_5 is completely absent and therefore gene expression may also affected by the chromatin remodeling mechanisms described previously.

4.9.3 Transcriptional regulation by the higher inositol phosphates

In addition to MippA, I have shown that the expression of *ino1* and *impa1* can be regulated by two other classes of enzymes involved in the metabolism of the higher order inositol phosphates. This would therefore argue that the effects seen are due to changes in inositide abundance rather than any enzyme-specific activity such as the regulation of arginine-responsive genes by IPMK/Arg82. Although MIPP is unlikely to directly affect nuclear inositides, I have demonstrated that both IpmkA1 and Ins(1,3,4)P_3 5/6 kinase are localized to the nucleus indicating that there is a specific nuclear pool and this is therefore likely to be crucial for the regulation of gene expression.

With the exception of $InsP_7$, we were unable to detect any significant changes in overall phosphoinositide abundance in the kinase overexpressors. These measurements are again most likely hampered by the presence of a large, reasonably static cytoplasmic pool. However, the predicted activities of these kinases would suggest increased rates of inositol phosphorylation and therefore elevated $InsP_5$ and potentially $InsP_4$. As this leads to an increase in expression it would correlate well with the chromatin remodeling model suggested by Shen *et al.* and Steger *et al.* (Shen et al., 2003; Steger et al., 2003), whereby Ins(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 are able

to activate Swi/Snf chromatin remodeling complex activity, increasing promoter accessibility. An increase in MIPP activity might have a similar effect, as $InsP_6$ was also shown to be inhibitory to other chromatin remodeling activities. However, without a way of specifically measuring the nuclear inositol phosphates we cannot be sure, but this data implies that it may have significance *in vivo*.

Ultimately, it may be a combination of mechanisms that lead to the observed changes in expression, but this study does however demonstrate *in vivo* that gene expression can be regulated by PO via the higher order inositol phosphates. These data also directly correlate with the sensitivity to lithium observed in the previous chapter and therefore indicates a novel pathway by which both transcription and chemotaxis can be regulated by PO, and may have a broad physiological significance.

Chapter 5:

Lithium inhibits chemotaxis through phospholipid depletion

5.1 Introduction

The previous chapters have shown that lithium inhibits cell movement and that this may be overcome by increased expression of the IMPase family of genes in response to the loss of PO activity. This indicates that inhibition of movement is due to the reduced recycling of Ins(1,4,5)P_3 to inositol, which leads to the depletion of both inositol phospholipids and Ins(1,4,5)P_3 and it may therefore affect the signaling properties of both.

As discussed previously, whether calcium signaling plays a direct role in chemotaxis is debatable as is the role of Ins(1,4,5)P_3. Indeed, *Dictyostelium* cells lacking all phospholipase C (PLC) activity have no chemotactic defect and develop normally, therefore questioning the role of receptor-activated PLC in chemotaxis (Drayer et al., 1994). In these cells it was also observed that although no Ins(1,4,5)P_3 could be generated from PtIns(4,5)P_2, the intracellular concentration was unchanged due to compensatory upregulation of an InsP_5 phosphatase (Van Dijken et al., 1995a) – identified as MippA in this study. As a similar increase in MippA results in lithium resistance in *dpoA*$^-$ cells we might therefore expect to see a similar result in *plc*$^-$ cells.

However, PLC is also the only known enzyme which is capable of hydrolysing inositol phospholipids and therefore a knockout would be expected to also have a more stable pool of PtIns(4,5)P_2. If it is the depletion of phospholipids which is responsible for lithium action then this would also lead to resistance but in a *mppA*-independent manner. Therefore these mutants allow us to investigate the mechanism of lithium action on chemotaxis and genetically test the inositol depletion hypothesis

to understand more about the role of inositol signaling and recycling in cell movement.

5.2 Generation of PLC knockout strains

Previously it has been shown that the *Dictyostelium* genome contains a single *plc* gene, of the delta family, and that disruption of this is sufficient to stop all $Ins(1,4,5)P_3$ production from $PtIns(4,5)P_2$ (Drayer et al., 1994). Although it was reported that this mutation had no effect on chemotaxis when compared to a random integrant, when this mutant was compared with our laboratory wild-type strain it was found to move significantly faster (Fig. 5.1). In addition, the published *plc* knockout was made in the Ax3 genetic background whereas our laboratory uses the Ax2 wild-type strain and therefore it was necessary to generate a new knockout in order to make comparisons with our existing mutants.

As previously, a knockout construct was made consisting of two homologous regions separated by the FLOX-flanked blasticidin cassette and therefore inside the cell, a homologous recombination event would lead to both insertion of the blasticidin cassette and a deletion of ~100bp of coding sequence from the middle of the PLC locus, inactivating the gene (Fig. 5.2). Our laboratory wild-type cells were therefore transformed with this construct and clones screened by PCR of the genomic PLC locus. After this screen, positive clones were tested for the presence of PLC protein by western blot using an antibody raised to the N-terminus of the *Dictyostelium* protein (a gift from P. van Haastert) (Drayer et al., 1994); in all cases,

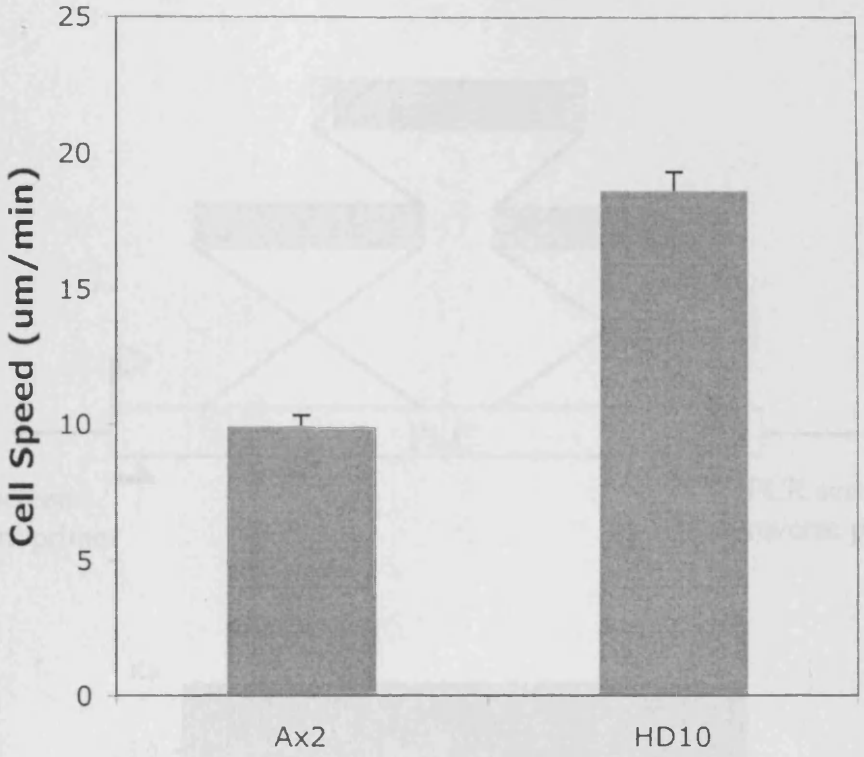


Figure 5.1: The published PLC null cells are not comparable to our laboratory strains. The wild-type strain from our laboratory (Ax2) and the published Ax3-background PLC null cell line (HD10) were analysed in the random movement assay described previously. The results are representative of two independent experiments.

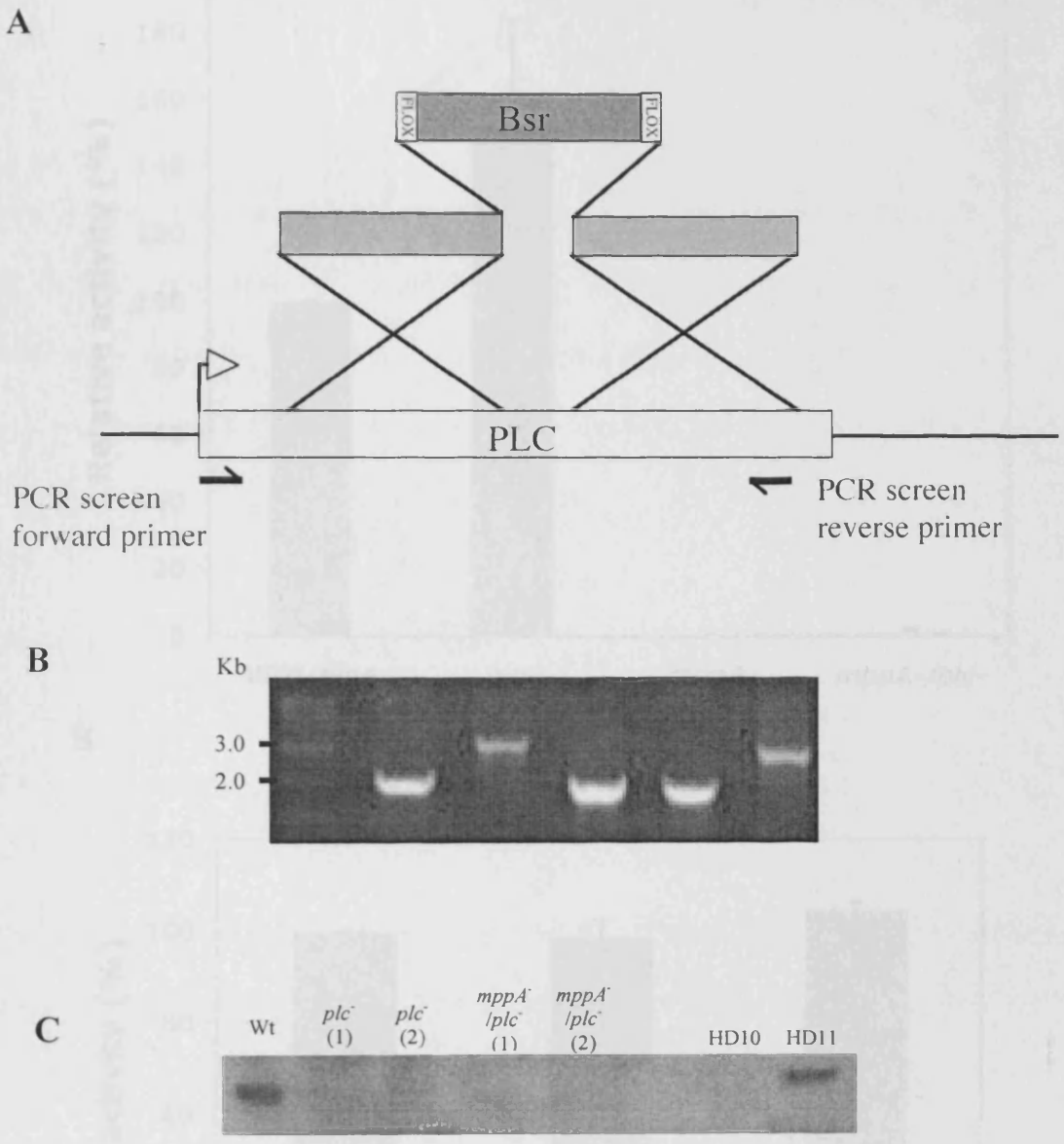


Figure 5.2: Generation of a new PLC knockout. (A) Schematic of knockout construct design. (B) Confirmation of recombination by PCR from genomic DNA using the primers indicated above, positive KO clones give a product which is 1.3 Kb larger due to the insertion of the blasticidin cassette. Clones where the construct integrated elsewhere produce a product of 1.8Kb. (C) Western blot of extracts from knockout clones probed with a specific α-PLC antibody. HD10 is the published mutant and HD11 is the published random integrant control.

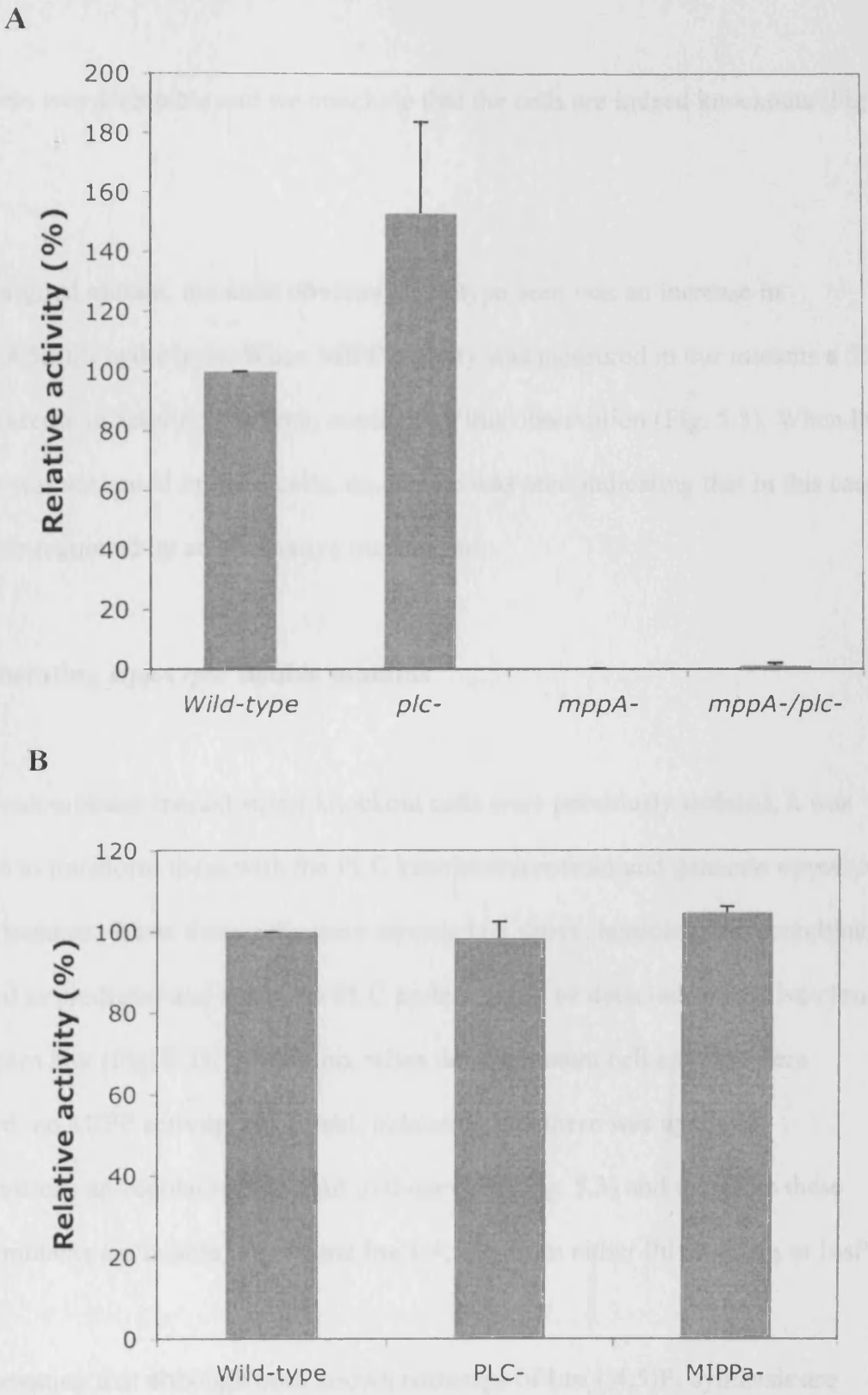


Figure 5.3: MIPP and PO activity in *plc* null cells. (A) The particulate fractions from cell extracts were incubated with $InsP_6$ and activity measured as the rate of $Ins(1,4,5)P_3$ production/minute. Results are calculated relative to wild-type cells and are the mean ± standard deviation of three independent experiments. (B) PO activity in the soluble fraction of mutant cell extracts was measured relative to wild-type.

no protein was detectable and we conclude that the cells are indeed knockouts (Fig. 5.2).

In the original mutant, the most obvious phenotype seen was an increase in Ins(1,3,4,5,6)P_5 hydrolysis. When MIPP activity was measured in our mutants a 50 ± 25 % increase in activity was seen, confirming this observation (Fig. 5.3). When PO activity was measured in these cells, no change was seen indicating that in this case, MippA is regulated by an alternative mechanism.

5.3 Generating *mppA*$^-$/*plc*$^-$ double mutants

As cre-recombinase treated *mppA* knockout cells were previously isolated, it was possible to transform these with the PLC knockout construct and generate *mppA*$^-$/*plc*$^-$ double mutants. When these cells were screened as above, homologous recombination occurred as predicted and again, no PLC protein could be detected in positive clones by western blot (Fig. 5.2). In addition, when double mutant cell extracts were prepared, no MIPP activity was found, indicating that there was again no compensatory up-regulation of *mppB* in these cells (Fig. 5.3) and therefore these double mutants are unable to generate Ins(1,4,5)P_3 from either PtIns(4,5)P_2 or InsP_6.

It is interesting that although both known pathways of Ins(1,4,5)P_3 synthesis are disrupted, these cells have no obvious growth defect and are still able to undergo development and form normal fruiting bodies (Fig. 5.4). In fact, when Ins(1,4,5)P_3 was measured in these cells, it was not significantly different from either wild-type, or

the *mppA*$^-$ parent (Fig. 5.5), indicating that a third, alternative route of synthesis must exist.

If in the absence of PLC and MIPP activity, Ins(1,4,5)P_3 levels are maintained by the upregulation of a third pathway, we might expect to see changes in the levels of the inositol polyphosphates. However when quantified by HPLC, again no significant differences were seen between the mutants, although this may again be due to the limitations of this technique as previously discussed (Table 5.1)

5.4 The secretion of inositol phosphates in *mppA* and *plc* null cells

In the course of measuring the Ins(1,4,5)P_3 content of cells, it became apparent that it was also rapidly accumulating in the medium such that when placed in fresh medium, after 5 hours the amount of Ins(1,4,5)P_3 in the medium is as much as 10 fold the amount inside the cells, accumulating at 4 pmol/min/10^7 cells (Fig. 5.6). Unfortunately due to the large volumes of media required to extract sufficient secreted inositol polyphosphates we were unable to use our HPLC system and therefore cannot tell if other inositol polyphosphates are also released. Consequently we do not know whether it is released as Ins(1,4,5)P_3 or secreted in a different form and formed by extracellular kinases or phosphatases. This phenomenon has been previously reported in *Dictyostelium* and it was estimated that as much as 10% of the intracellular Ins(1,4,5)P_3 is released per minute (Van Haastert, 1989).

If Ins(1,4,5)P_3 is actively secreted, it may give an indication of the rate of turnover within the cell as, in order to maintain intracellular levels, both synthesis and

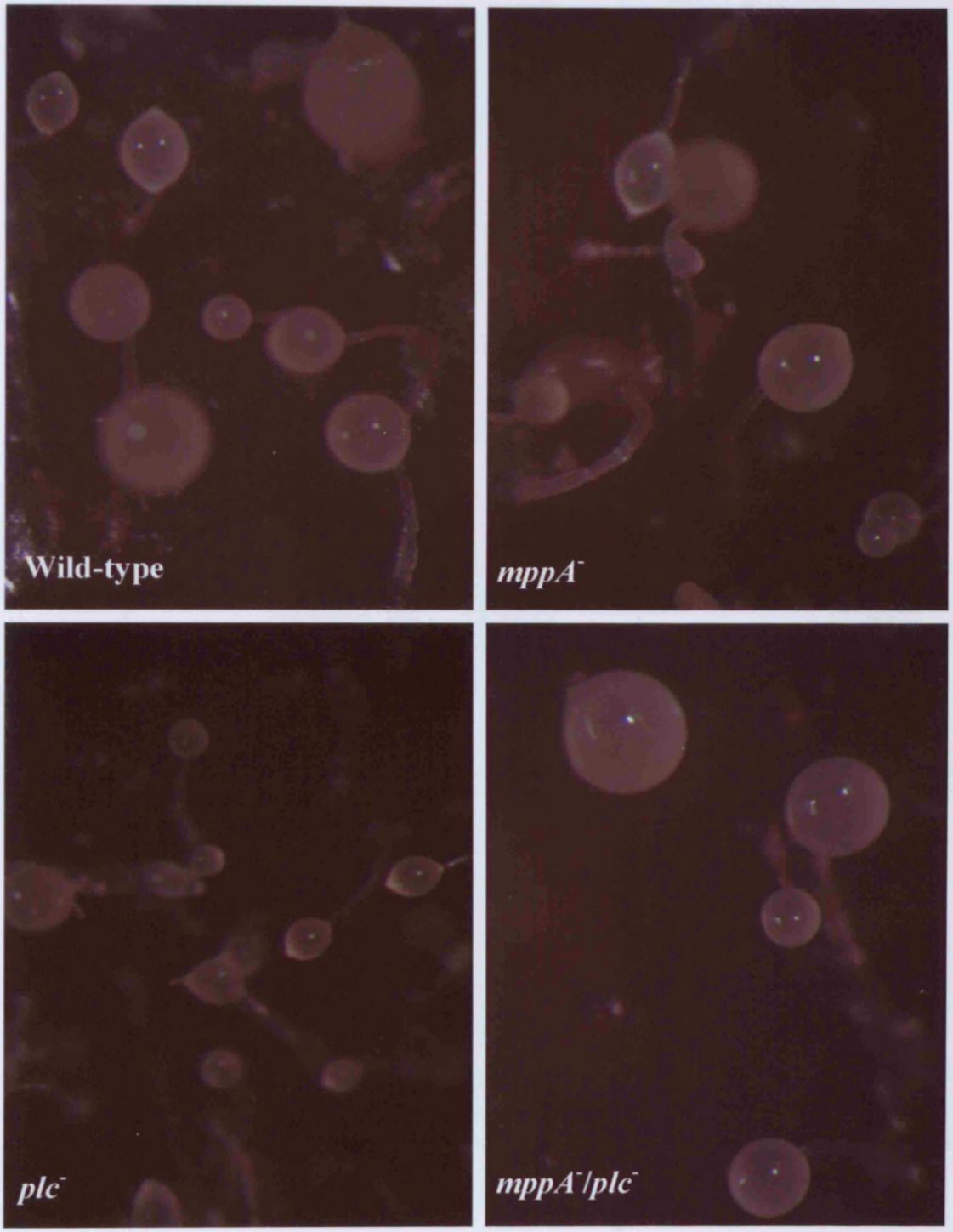


Figure 5.4: The development of *mppA* and *plc* null cells. Cells were plated clonally on SM agar plates with a lawn of bacteria. Images were taken from the centre of the plaques which were formed after one week.

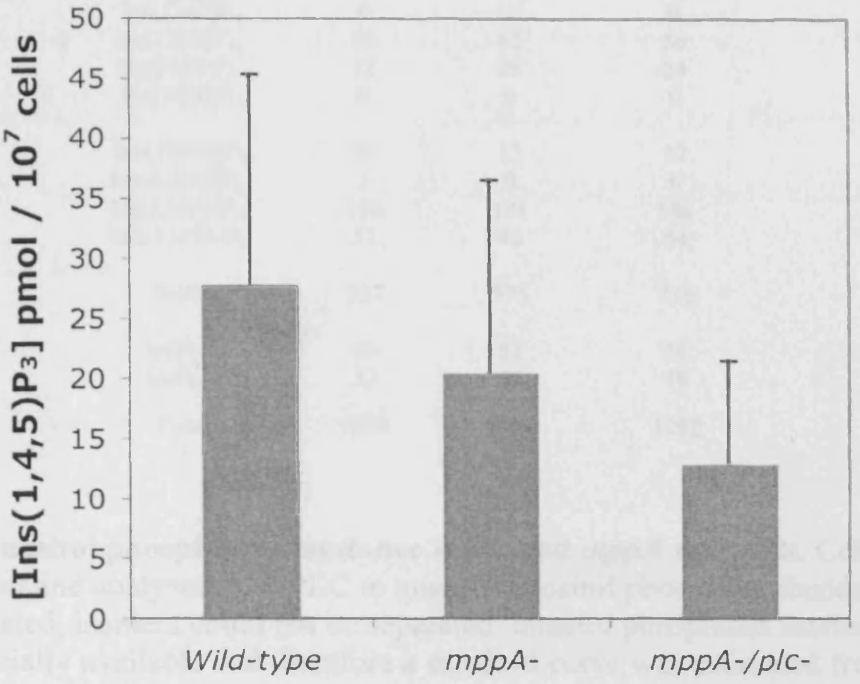


Figure 5.5: Ins(1,4,5)P_3 levels in *mppA/plc* double knockout cells. Inositol phosphates were extracted from vegetative cells grown in the absence of exogenous inositol and Ins(1,4,5)P_3 levels quantified using the isotope dilution assay. Values plotted are the means ± standard deviation of three independent determinations.

Pmol / 10^7 cells

	Wild-type	*plc*$^-$	*mppA*$^-$/*plc*$^-$
$InsP_3$	0	0	0
Ins(1235/1246)P_4*	1	1	0
Ins(1346/1234)P_4*	24	10	16
Ins(1245)P_4*	0	0	0
Ins(1345)P_4	0	0	0
Ins(1256)P_4*	60	42	56
Ins(2456)P_4*	12	29	24
Ins(1456)P_4	0	0	0
Ins(12346)P_5	20	15	17
Ins(12345)P_5*	1	0	0
Ins(12456)P_5*	192	181	186
Ins(13456)P_5	52	46	54
$InsP_6$	527	575	719
$InsP_7$**	65	53	56
$InsP_8$**	33	47	48
Total	1009	1057	1292

Table 5.1: Inositol phosphate abundance in *plc* and *mppA* null cells. Cell extracts were prepared and analysed by HPLC to quantify inositol phosphate abundance. Where indicated, isomers could not be separated. Inositol phosphates marked * are not commercially available and therefore a standard curve was estimated from other similar isomers. Compounds marked ** are also unavailable but also have no available isomers. Therefore in these cases a standard curve was estimated from $InsP_6$ values corrected for phosphate content. All values are the mean of at least 3 independent determinations.

import/export would have to remain balanced. When the rate of Ins(1,4,5)P_3 secretion was measured in *plc*$^-$ and *mppA*$^-$ mutants it was found to be reduced by ~50% in both cases, indicating that extracellular Ins(1,4,5)P_3 is not produced exclusively by either enzyme (Fig. 5.6). In *mppA*$^-$/*plc*$^-$ double mutants however the rate of Ins(1,4,5)P_3 accumulation was reduced even further, to 40% of wild-type demonstrating that although intracellular levels of Ins(1,4,5)P_3 do not hugely change, these mutants have significant differences in inositol polyphosphate turnover resulting in a decreased rate of extracellular accumulation.

5.5 *plc*$^-$ and *mppA*$^-$/*plc*$^-$ cells are lithium resistant

Although disruption of the single *Dictyostelium plc* gene has been reported to have no effect on chemotaxis, our previous experiments would predict that the changes in inositol metabolism that occur in this mutant might lead to altered lithium sensitivity. When *plc*$^-$ cells were treated with 7mM lithium chloride and placed in a gradient of chemoattractant, movement was not significantly inhibited, compared with wild-type where speed was reduced to 77% (Fig. 5.7) and therefore *plc*$^-$ cells are lithium resistant ($P=0.03$).

To determine whether this resistance is due to elevated MippA activity and therefore by a similar mechanism as *dpoA* null cells, the assay was repeated with the *mppA*$^-$/*plc*$^-$ double mutant. Again, these cells were more resistant than wild-type, and speed was only moderately reduced to 87% ($P\leq0.05$). This data would however suggest that in *plc*$^-$ cells, lithium resistance is independent of MippA and therefore is likely to be due to altered lipid, rather than soluble inositide metabolism and signalling.

5.6 *ipma1* and *ino*1 expression is not regulated by PLC

Although lithium resistance in *plc*⁻ cells appears to be MippA-independent, the mechanism of resistance may still be due to changes in gene expression. However, when the levels of *ipma1* and *ino1* expression were quantified in *plc*⁻ cells, they were not significantly changed relative to wild-type, despite increased MippA activity (Fig. 5.8). This therefore demonstrates that *plc*⁻ cells are resistant to the effects of lithium by a different mechanism than *dpoA*⁻ cells, independent of a simple up-regulation of its target IMPase family genes.

5.7 Discussion

5.7.1 Pathways of Ins(1,4,5)P_3 synthesis

In order to make a reasonable comparison with existing mutant strains from our laboratory, new PLC null cells were generated. As previously reported, these mutants again have no obvious growth or developmental defects and maintain normal levels of Ins(1,4,5)P_3 levels due to up-regulation of MippA. As all known pathways of Ins(1,4,5)P_3 synthesis require the activity of either PLC or MIPP it was a surprise to find that in *mppA*⁻/*plc*⁻ double mutants, when both of these pathways are disrupted, significant amounts of Ins(1,4,5)P_3 remain. As these cells have no detectable $InsP_6$ phosphatase activity, Ins(1,4,5)P_3 must therefore be due produced by either another phospholipase, or a novel route – possibly by the phosphorylation of inositol.

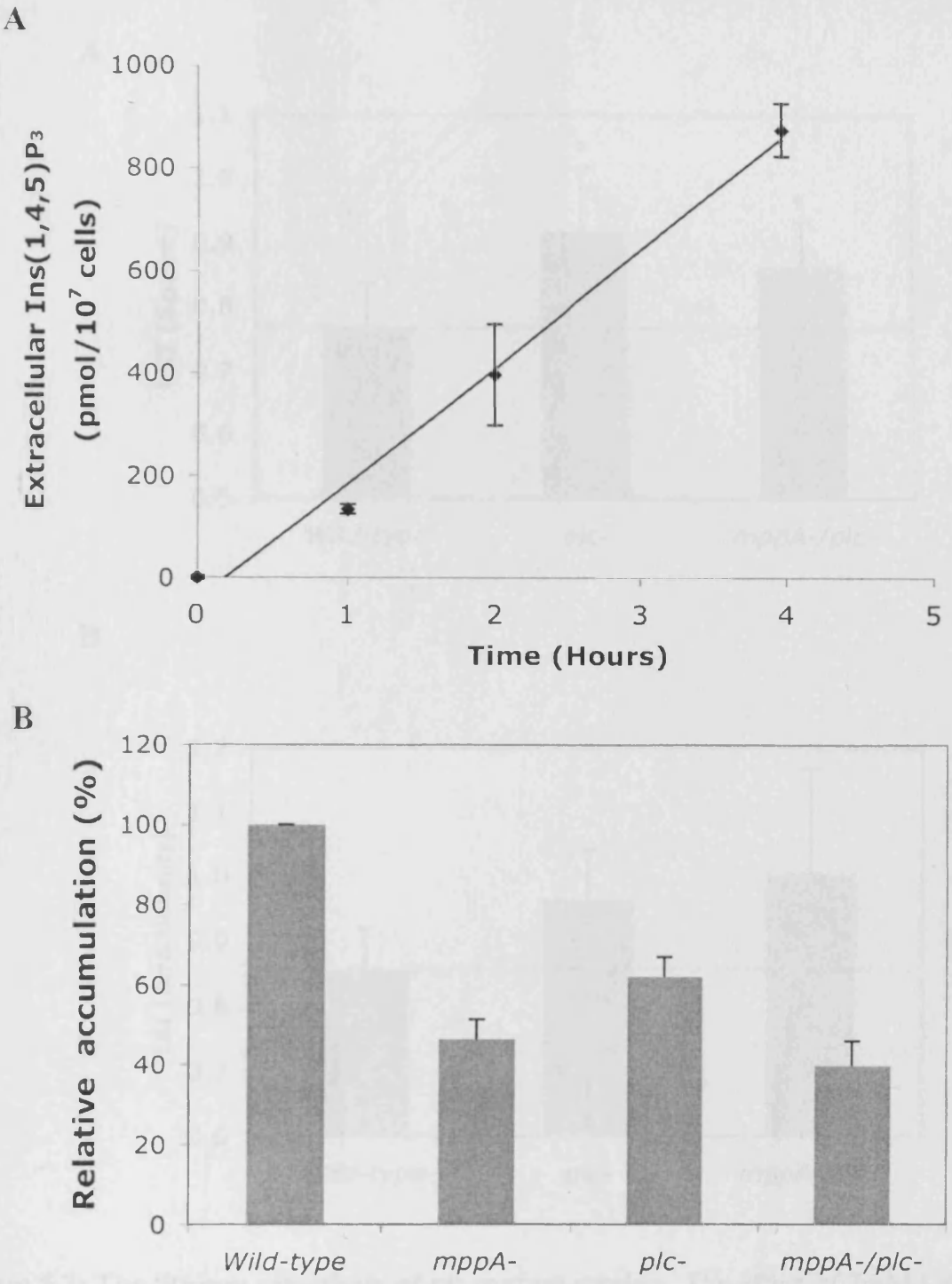
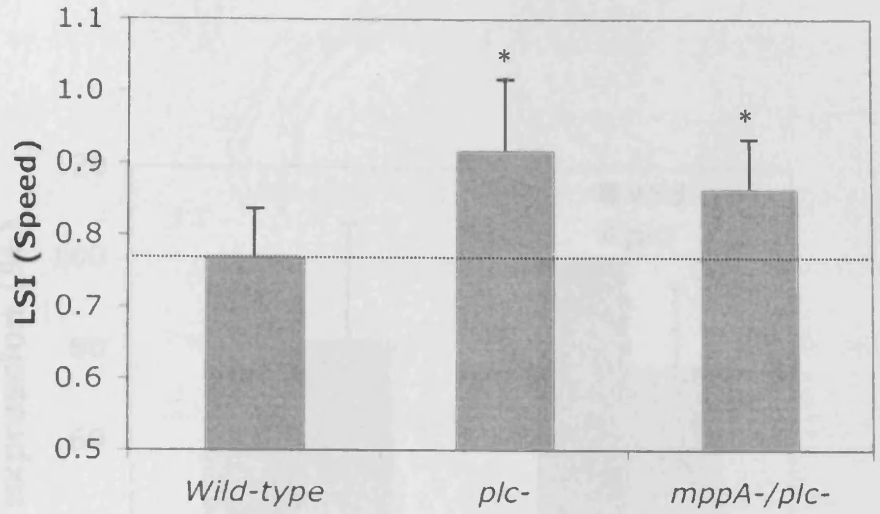


Figure 5.6: The extracellular accumulation of Ins(1,4,5)P_3. (A) Wild-type cells were placed in fresh medium and grown in shaking suspension. At the times shown, samples were removed, the cells removed by centrifugation, and the amount of Ins(1,4,5)P_3 in the medium measured by the isotope dilution assay. The results of a typical experiment are shown. (B) The rate of extracellular Ins(1,4,5)P_3 accumulation in *mppA* and *plc* null cells was measured relative to wild-type. Values plotted represent the mean ± standard deviation of at least two independent experiments.

A



B

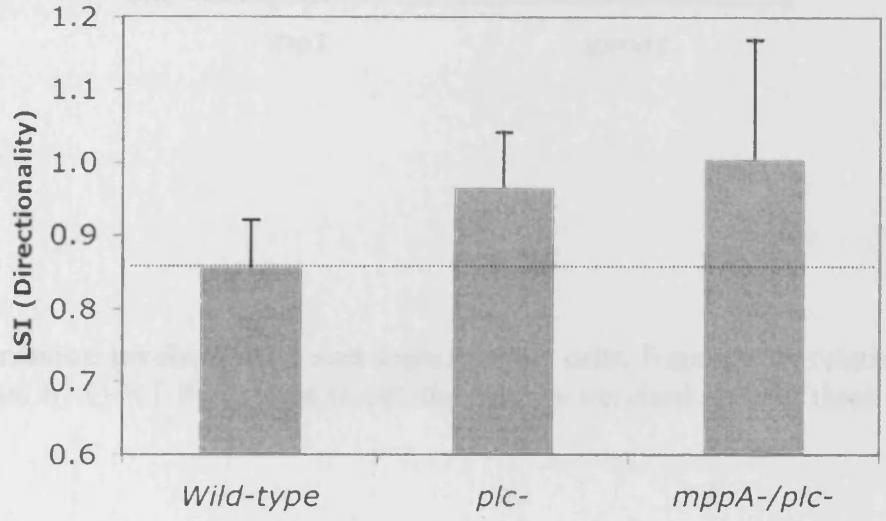


Figure 5.7: The lithium sensitivity of *plc* mutant strains. The effect of 7mM LiCl on chemotaxing mutant cells was measured as previously using a chemotaxis chamber. Both speed and chemotactic index were normalised to control values obtained in 7mM NaCl on the same day. Results plotted are the means ± standard deviation of four independent experiments. * $P \leq 0.05$, Student's T-test.

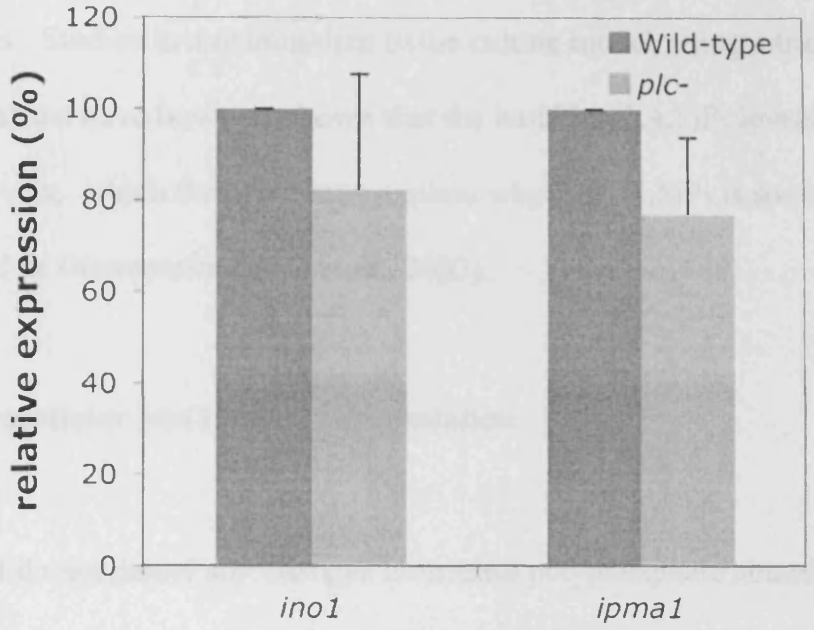


Figure 5.8: Expression levels of *ino1* and *impa1* in *plc*⁻ cells. Expression, relative to wild-type cells was measured by Q-RT PCR. Results are the mean ± standard error of three independent experiments.

Although we do not fully understand the physiological role of Ins(1,4,5)P_3 in *Dictyostelium*, the fact that disruption of two of its synthetic pathways does not affect movement indicates that it does not play a significant role as a second messenger for chemotaxis. Studies in a mammalian tissue culture model, using a truncated cytosolic MIPP construct have however shown that the basal Ins(1,4,5)P_3 levels can affect basal calcium levels, which therefore may explain why Ins(1,4,5)P_3 is so robustly maintained in *Dictyostelium* (Yu et al., 2003).

5.7.2 Extracellular Ins(1,4,5)P_3 accumulation

Although I do not detect any changes in inositol polyphosphate abundance between any of these mutants, the rate of Ins(1,4,5)P_3 accumulation outside of the cell is significantly changed. Why cells secrete such large amounts of inositol polyphosphates is an interesting question. As the rate of extracellular accumulation is so significant, it is unlikely to simply be due to cell leakage, and as inositol polyphosphates are highly charged molecules they are unlikely to be able to cross membranes passively. Unfortunately, as analysis by HPLC was not possible it is unclear whether Ins(1,4,5)P_3 is itself secreted, or is released in another form and converted by an extracellular enzyme but the fact that this accumulation is reduced but still present in both *mppA* and *plc* null cells suggests that this is not the case and that it is connected to the rate at which Ins(1,4,5)P_3 can be synthesized internally. In the experiments described here, we have effectively removed all the extracellular inositol phosphates at the start and therefore whether these cells will continue to accumulate extracellular inositol phosphates or reach an equilibrium is unknown; if an equilibrium is reached, the level of this may be related to the intracellular levels of

Ins(1,4,5)P_3 and therefore may ultimately be unchanged in both PLC and MIPP null cells when growing normally.

Althought inositol phosphatate secretion is relatively unknown, it does not appear be restricted to *Dictyostelium*. The presence of extracellular Ins(1,4,5)P_3 was first identified in the interstitial fluid of the rat hippocampus and was shown to be increased upon activation of the muscarinic receptor by pharmacological agonists, reflecting changes in the intracellular levels (Minisclou et al., 1994). This report was subsequently confirmed by a study in both cortical brain slices and a human neuroblastoma cell line, showing that $InsP_2$ and $InsP_4$ were also exported - although they did not look for $InsP_5$ or $InsP_6$ (Roberts et al., 1997). In both these studies however, an increase in extracellular inositol phosphate levels was only seen after stimulation of the internal levels, indicating that the rate of export was directly linked to the intracellular metabolism. This would agree with these observations in *Dictyostelium* where it appears that the rate of extracellular accumulation is also related to the rate of inositol phosphate turnover.

Why cells might do this is unclear - in mammalian cells the amount exported only represented about 2-4% of the amount induced by agonist stimulation so it is unlikely that its purpose is to attenuate the signal (Roberts et al., 1997). An alternative explanation might be that extracellular inositides are involved in intercellular signaling. However, as these *Dictyostelium* mutants have reduced export but no obvious developmental defect this may not be the case, unless the extracellular levels reach a stable equilibrium concentration which is unchanged in these cells. What is clear however, is that these extracellular inositides represent a significant pool and the

rate of accumulation appears to be representative of the rate of intracellular synthesis, and not simply the basal level of Ins(1,4,5)P_3.

5.7.3 The effects of lithium on chemotaxis, and resistance in *plc*⁻ cells

This study has shown that, although they have no chemotactic phenotype under normal conditions, *plc*⁻ cells are resistant to the effects of lithium. In addition, this resistance is independent of MippA activity and is not accompanied by an increase in *IMPase* expression indicating an alternative mechanism of resistance that is based upon metabolic changes.

When cells are treated with lithium, the levels of *myo*-inositol are reduced which has been shown to lead to a reduced rate of inositol lipid biosynthesis and PtIns levels in several organisms (Ciapa and Maggio, 1993; Ding and Greenberg, 2003; Joseph et al., 1987). This also produces a reduction in the basal levels of Ins(1,4,5)P_3 and therefore both phospholipid, and Ins(1,4,5)P_3 signalling may be affected. As PLC represents the sole link between inositol phospholipid and soluble inositide signaling manipulation of this gene allows us to genetically separate the two and the observation that *mppA*⁻ /*plc*⁻ double mutants remain resistant to lithium shows that the PLC null mutation is genetically epistatic and therefore it is a disruption of lipid hydrolysis, and not soluble inositide metabolism that is crucial for the determination of lithium sensitivity.

This data implies that by inhibiting the recycling of inositol, lithium is able to deplete the levels of inositol phospholipids and block movement. In cells lacking PLC activity, the pool of inositol phospholipids cannot be depleted by hydrolysis and is

therefore more stable and less sensitive to lithium. This model would also predict that, although increased MIPP activity may help, this resistance is largely independent of higher inositol phosphate metabolism and Ins(1,4,5)P_3 production as indeed was observed.

5.7.4 A role of PLC in chemotaxis?

If mutation of PLC is able to confer lithium resistance by stabilization of the pool of phospholipids, the regulation of this pool may be part of its physiological role. Indeed, PLC may play a more significant role by the removal of PtIns(4,5)P_2 than by the formation of Ins(1,4,5)P_3. As the recycling of Ins(1,4,5)P_3 back to PtIns requires the action of multiple enzymes as well as transport between compartments, this will be a relatively slow process and therefore, although it may not play a role in direct signalling, PLC activity may act as a general attenuator of inositol lipid signaling.

As PLC activity has been shown to decrease thoughout early development this may also partly explain the increase in both inositol lipids and cell polarity which are observed at the same time (Cubitt and Firtel, 1992; Ellingson, 1974). Although similar effects are not observed in a PLC knockout it should be noted that in these cells, both the rate of PtIns(4,5)P_2 synthesis and the total amount of PtIns(4,5)P_2 are unchanged (Drayer et al., 1994). For this to be the case there must be an up-regulation of an alternative pathway for the removal of inositol phospholipids from the membrane, which would suppress inositol lipid accumulation . Indeed, functional redundancy would seem to be a common property of inositol metabolism in general, and the data

presented throughout this study all indicates the importance of the rate of inositide turnover, in both the action of lithium and of chemotaxis in general.

Chapter 6:

Discussion

6.1 The aims of this work

The main aim of this study is to clarify and understand the effects of lithium on cell movement and chemotaxis. Previous work in this laboratory has identified a mutation of the *dpoA* gene which confers lithium resistance and also has altered inositol metabolism. It has previously been suggested that the theraputic action of lithium is by inhibition of inositol signaling (Berridge et al., 1989) and therefore an aim of this project was to determine whether it is these changes in inositol metabolism that are responsible for lithium resistance in this mutant. In addition, by determining the mechanism of lithium resistance in this and other inositol metabolic mutants, I hope to understand both the mechanism of lithium action, and the role of inositol metabolism in cell motility.

6.2 Lithium as a tool for studying cell movement

In this work I have shown that lithium is able to inhibit cell movement in a dose-dependant manner, explaining how lithium treatment is able to block *Dictyostelium* aggregation and development. Furthermore, as this inhibition is seen in both chemotaxing and randomly moving cells, the action of lithium is not to block chemotactic signal transduction, but to inhibit the underlying fundamental cell movement. This is a very robust system, indicative of its fundamental role, and none of the mutants generated in this study have any significant movement or developmental phenotype unless the overall level of inositol is lowered by lithium treatment. As these mutants are affected to a different extent by lithium this allows us

to gain insight into the role of the affected genes and signaling pathways, providing a useful tool for the study of this basic motility and cell behaviour.

6.3 The mechanism of lithium resistance in *dpoA*⁻ cells

Previous work has shown that in *dpoA* null cells, lithium resistance is accompanied by an increase in the rate of Ins(1,4,5)P_3 production from $InsP_6$. I have identified and cloned the *Dictyostelium* homologue of MIPP, the enzyme responsible for this activity and shown that it is required for lithium resistance in *dpoA* null cells. This demonstrates that resistance is due to the altered metabolism of the higher order inositol phosphates. Although an increase in MIPP activity and Ins(1,4,5)P_3 synthesis may be able to overcome some of the inositol-depleting effects of lithium, cells expressing very high levels of MippA actually become hypersensitive and therefore the changes in lithium sensitivity must be due to a different mechanism.

In cells treated with a PO-specific inhibitor, the expression of all three IMPase-family genes as well as *ino1* were up-regulated in a MippA dependant manner. Therefore, in this case, lithium sensitivity is likely to determined by transcription, where increased RNA (and therefore presumably protein) levels of IMPase and IPP1 and 2 coincide with lithium resistance, and decreased expression of IMPase reflects the lithium hypersensitivity observed in *mppA* overexpressing cells. Work in our laboratory has previously demonstrated that overexpression of IMPase is sufficient to give lithium resistance (Keim-Reder, 2006) and therefore, if these changes in RNA levels are representative of protein levels, when prolyl oligopeptidase activity is disrupted, cells become more resistant to lithium due to a MIPP-mediated increase in IMPase and IPP

levels providing strong evidence that it is indeed the inhibition of these enzymes that is responsible for inhibition of cell movement.

Although we know little about the physiological role of PO, this represents a novel signaling pathway where PO is able to act as a master regulator of inositol metabolism through the control of gene expression. Although we do not know how MIPP activity is regulated, other than it does not seem to be at a transcriptional level or even any of the biological substrates of PO, the fact that MIPP activity is modified in both PO and in PLC-null cells (independent of PO) implies that it may have a signaling role and this may be important in a number of processes. The challenge now is therefore to understand how this happens and to determine the physiological role of both MIPP and PO.

6.4 Inositol phosphate metabolism and transcriptional regulation

This study shows that MIPP activity is required for the transcriptional regulation of *ino1* and *impa1* by PO. As overexpression of two independent nuclear inositol kinases also produces a similar change in expression, it appears that the same pathway is involved and that the level of transcription is related to the inositide status of the cell. As it is specifically only the genes directly involved in the production of *myo*-inositol, and not higher order inositol metabolism that are regulated, the purpose of this regulation is likely to be the maintenance of phospholipid levels comparable to that extensively studied in yeast. The regulation of these genes is also complex, as in *plc*$^-$ cells the expression of both *ino1* or *impa1* is unchanged, despite having a comparable increase in MIPP activity to *dpoA* null cells. This, and the observation in *mppA*

overexpressing cells that *impa1* expression is down-regulated independently of *ino1*, indicates that there are multiple pathways involved and that the actual level of expression is due to the integration of a number of signals.

In yeast, *ino1* expression can be regulated by a number of different processes, using numerous sensors of inositide status, including inositol itself as well as the higher order inositol phosphates, via chromatin remodeling (Hirsch and Henry, 1986; Kelley et al., 1988; Loewen et al., 2004; Shen et al., 2003; Steger et al., 2003). In addition, the UAS_{INO} containing genes, including *ino1,* are negatively regulated by the Opi1p transcriptional regulator which itself can be regulated by a number of mechanisms including phosphorylation by protein kinases A and C and retention in the ER by binding to phosphatidic acid and vesicle-associated-membrane-protein-associated protein (VAP) (Loewen et al., 2004; Loewen et al., 2003; Sreenivas and Carman, 2003; Sreenivas et al., 2001). As some of these factors are able to regulate the level of expression independantly, the ultimate read-out of RNA levels is an integration of several signals. This therefore may explain the unchanged expression of *ino1* in PLC null cells, as even if elevated MIPP activity affects chromatin remodeling to facilitate transcription, other changes in the lipid metabolism may lead to repression through factors such as Opi1p, canceling this out. Indeed, this would also make metabolic sense, as if a pathway of phospholipid hydrolysis is blocked, a cell is unlikely to try and upregulate inositol lipid synthesis.

The transcriptional regulation of *impa1* is considerably less well understood. In yeast, expression of *IMN1*, the gene encoding IMPase, is increased by growth in inositol and decreased by inositol starvation although the mechanism is unknown (Murray and

Greenberg, 1997; Murray and Greenberg, 2000). In addition *IMN1* is also down-regulated by lithium and VPA treatment, probably due to decreased inositol levels. Therefore as up-regulation of *ino1* will lead to increased inositol availability, it is likely that *impa1* expression is regulated as a direct consequence.

Although these experiments show that higher inositol phosphate metabolism is able to regulate gene expression, it is impossible to say from this study which of these mechanisms are involved. However, these observations in *Dictyostelium* do fit with the more detailed transcriptional studies in yeast so it appears that at least some of these regulatory pathways are conserved. Whatever pathways are involved, these experiments demonstrate that the higher order inositol phosphate metabolism can affect the transcription of lithium-sensitive genes, and alter the ability of the cell to tolerate treatment which may have implications for the understanding of both lithium treatment, and bipolar disorder.

6.5 Inositol phosphate metabolism and chemotaxis

Much of the research into the role of inositol signaling in chemotaxis has concentrated on the activation of PI-3' kinase and PTEN, leading to the localized production of PtIns(3,4,5)P_3 and the recruitment of PH-domain containing proteins to the leading edge (Haugh et al., 2000; Parent et al., 1998; Servant et al., 2000). Although a recent study has questioned whether this itself is responsible for directional sensing, or provides a supplementary increase in local actin polymerization, the production and regulation of PtIn(3,4,5)P_3 clearly plays an important role, and is crucial for normal aggregation (Loovers et al., 2006). In contrast, both Ins(1,4,5)P_3 and calcium

signaling appear to be dispensible, as cells lacking either *plc* and *iplA* activity are still able to chemotax normally (Drayer et al., 1994; Traynor et al., 2000). In this study I have shown that lithium treatment is able to block cell movement due to inhibition of IMPase and IPP and therefore although $Ins(1,4,5)P_3$ may not be a signal, the recycling of inositol phosphates is clearly important for maintaining cell movement.

If the production of inositol lipids is important for chemotaxis, as PLC is able to remove them it may also play an important role. Although the concentration of $PtIns(4,5)P_2$ is too high to itself be used as a signal by cAMP-activated PLC, PLC activity may act as a general attenuator of inositol lipid signaling, regulating the turnover of the lipid pool by removing the inositol head group and forcing it to undergo a slow multi-step recycling process. As the rate of $PtIns(4,5)P_2$ synthesis remains constant in *plc* null cells (Drayer et al., 1994) there are clearly other routes by which inositol lipids are lost, but as the action of IMPase is essential for both recycling and *de novo* synthesis, lithium allows us to effectively block both pathways. As this leads to inhibition of cell movement, the efficient replenishment of the dynamic lipid pool plays an important role in the maintenance of motility.

6.6 Summary

In this study, I have shown that lithium is able to block aggregation due to inhibition of the IMPase family of genes. Inhibition of IMPase and IP reduces both the recycling and *de novo* synthesis of inositol lipids and it is therefore this process that is crucial for basic cell movement. In addition, I have demonstrated that this process is subject to regulation by PO, via the metabolism of the higher inositol phosphates, with

implications both in basic cell biology, and for the understanding and treatment of bipolar disorder.

The metabolism and signaling of the inositol polyphosphates and lipids, represents an enormously complex system, which due to this complexity, is often studied at the individual enzyme level. In this study however, most of the enzymes involved do not themselves produce an important second messenger signal and therefore it is more appropriate to think in terms of metabolic flow. Indeed, in soluble inositol phosphate metabolism, such as the production of $Ins(1,4,5)P_3$, there appears to be a significant amount of redundancy indicating that in this case, it is the flow, rather than the specific pathway which is important. Therefore, metabolic flow is responsible for maintaining cell movement, and it is by the inhibition of this that lithium exerts its effects on cell movement.

Appendix I:

Design and Validation of Quantitative

Real-Time PCR Assays

A1.1 Design of Primers

For each gene of interest, specific primers were designed to produce a product of approximately 100-200bp and to have a melting temperature of approximately 57°C (see Table A1.1). As an internal loading control primers were also designed for the Ig7 gene encoding the mitochondrial large subunit rRNA which should remain constant. To accurately check that each primer set generated a single, clean product cDNA synthesized from wild-type cells was used as the template in real-time PCR reactions using the Sybr green fluorescent dye, which fluoresces upon binding double-stranded DNA. After the reactions had completed 35 cycles, the reaction products were analysed by slowly increasing the temperature so that as the melting point of each product is reached, it will dissociate leading to a drop in fluorescence. As different products will have different melting temperatures, multiple products or primer dimers can easily be identified. This is mostly clearly seen by plotting the rate of change in fluorescence against temperature such that the dissociation of a product is seen as a peak.

When the primer sets were tested in this way, using cDNA from vegetative wild-type cells as template, a single peak was observed in each case indicating a good, discrete product suitable for quantative analysis (Fig. A1.1). Control reactions, with no template present gave no significant signal, with the exception of the *ino1* and *ipkA1* primer sets where a peak was seen at a lower temperature, indicating primer dimers. These however are not a problem in the presence of template due to the preferential formation of the true product.

Gene	Orientation	Sequence	Melting Temperature (°c)	Product Size (bp)
ino1	Forward	GACACCGTCGTCGTTATGTGGTCCG	67.9	437
	Reverse	GCAATCATATCATCGACAACATTTG	58.1	
impa1	Forward	GGTAATGAACCAACATGGGTAATTG	59.7	465
	Reverse	GCTGTACCTGAGAATCTTAATGCTTGAAC	63.9	
ippA	Forward	AGAGGAACAATGTGAAATACCAAC	57.6	421
	Reverse	TTGAACTTTATCATTGAAATGAGAGGC	58.9	
ippB	Forward	GATCAAGAGGAACCAATTAAGGTTTC	60.1	323
	Reverse	CCTTTTGAGTAATCTAATTGTTGTTT	55.3	
mppA	Forward	ACCAATGAAATACAAACCACAATCC	58.1	124
	Reverse	TCCATCCACTCCCAATGAACC	59.8	
ipkB	Forward	TTACAGTTGGCTACTACTTGTCAGATTCC	63.9	198
	Reverse	ATGGATTATCAAGGTCACCTAACTCATCA	62.4	
ipkA1	Forward	GCAGGTTCAACACCATTCAAAAAATC	60.1	176
	Reverse	TCCAACACTATCCATTCCTTACCATC	61.6	
ipkA2	Forward	TGGTAGTTTTTTGAGTGTCAGCCC	61.0	174
	Reverse	TGATGATGTTGTTGTTGTTGTTGTAGTG	60.7	
ig7 long	Forward	GTACTTAAACCGCTGGTTAATTG	57.1	343
	Reverse	CGCTACCTTAGGACCGTCATAGTTAC	64.8	
ig7 short	Forward	TCCAAGAGGAAGAGGAGAACTGC	62.4	137
	Reverse	TGGGGAG\GTCGTTACACCATTC	62.1	

Table A1.1: Real – time PCR primer details

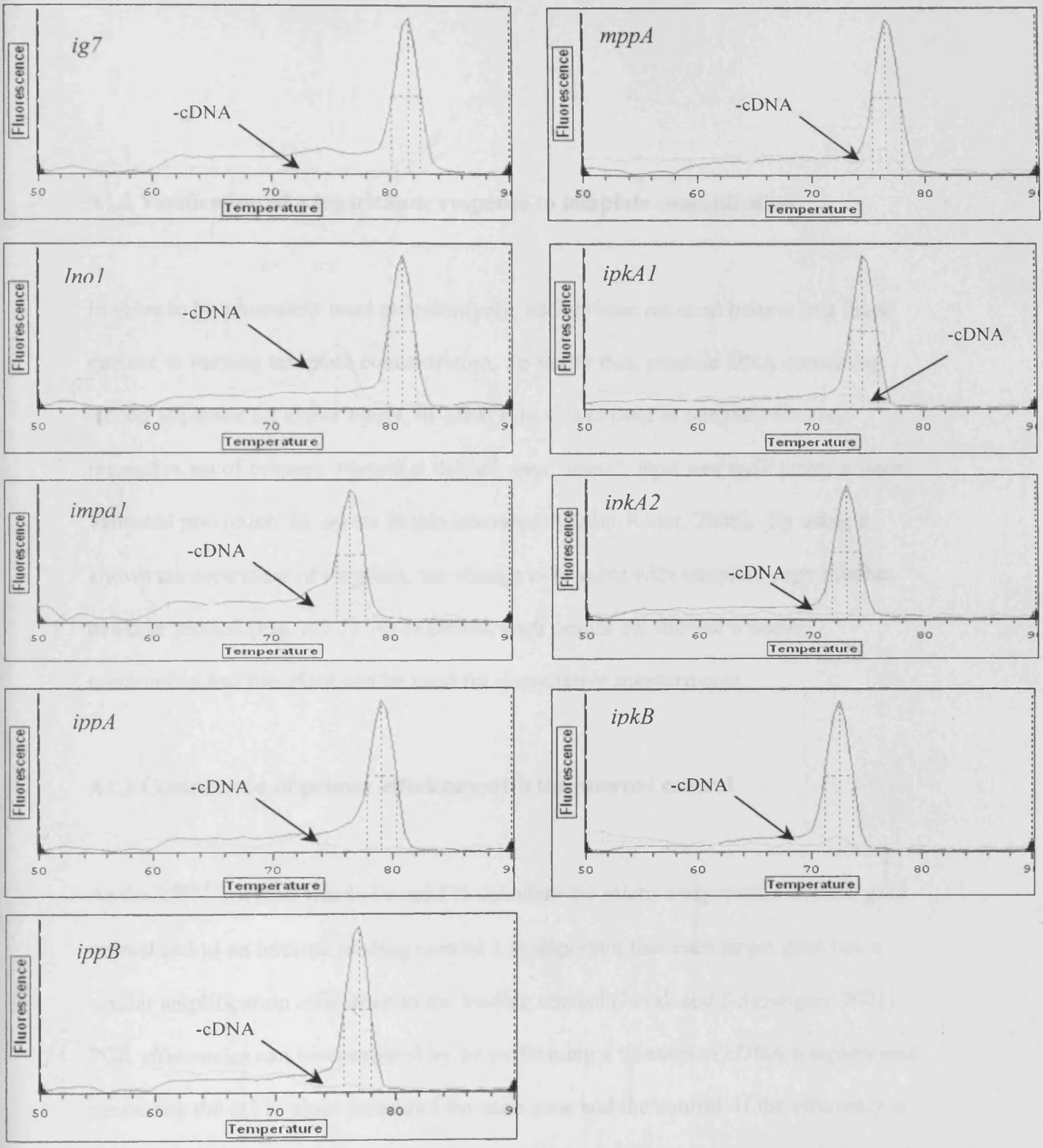


Figure A1.1: Melting curve analysis of real time PCR products. Typical examples of the melting curves for each primer set used. The rate of change in fluorescence is plotted and therefore peaks indicate a large change in fluorescence due by the dissociation of any double stranded products formed. Control reactions with no cDNA template are also plotted

A1.2 Verification of a logarithmic response to template concentration

In order to be accurately used quantitatively, each primer set must behave in a linear manner to varying template concentration. To verify this, plasmid DNA containing cloned sequence for either *mppA*, or *ipkA, B* or *C* was used as template for each respective set of primers. Note that the *ig7, ino1, impa1, ippA and ippB* primers were validated previously by others in this laboratory (Keim-Reder, 2006). By using a known concentration of template, the change c(T) value with template copy number could be plotted (Fig. A1.2). As expected, each primer set showed a linear relationship and therefore can be used for quantitative measurments.

A1.3 Comparison of primer efficiency with the internal control

As the $2^{-\Delta\Delta c(T)}$ method was to be used to calculate the relative expression of each gene normalized to an internal loading control it is important that each target gene has a similar amplification efficiency to the loading control (Livak and Schmittgen, 2001). PCR efficiencies can be compared by by performing a titration of cDNA template and measuring the c(T) values measured for each gene and the control. If the efficiency is similar for both genes, the difference between the two c(T) values at each dilution will remain constant. This was seen to be largely the case for all the primer sets (Fig. A1.3). Although the *mppA* and *ipkB* reactions do seem to give a slight upwards gradient, over the range of change in expression levels observed in this study this would prove insignificant. This therefore indicates a single product and comparable

efficiency for each primer set and validation that they may be used for quantiation using the $2^{-\Delta\Delta c(T)}$ method (Livak and Schmittgen, 2001).

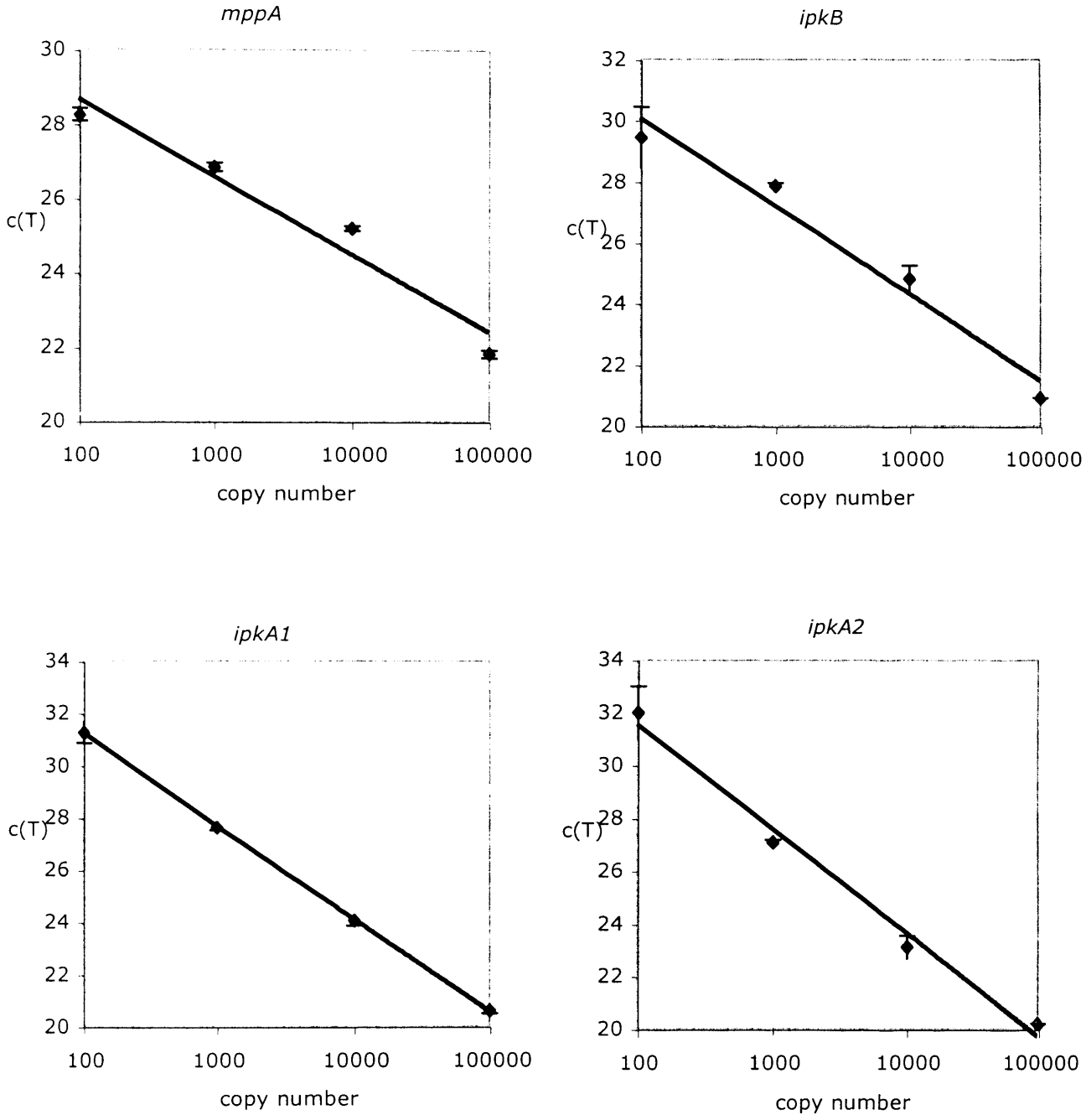
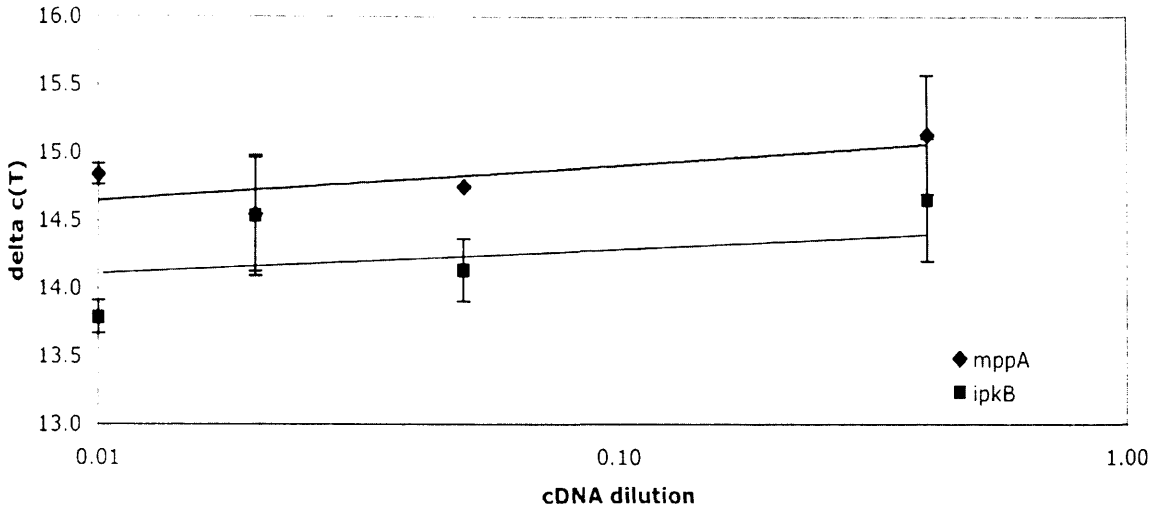


Figure A1.2: c(T) value has a linear response to increasing template. Plasmid DNA of known concentration containing each gene was used as template for real-time PCR reactions to verify a linear relationship between copy number and c(T) value. Reactions were all done in triplicate.



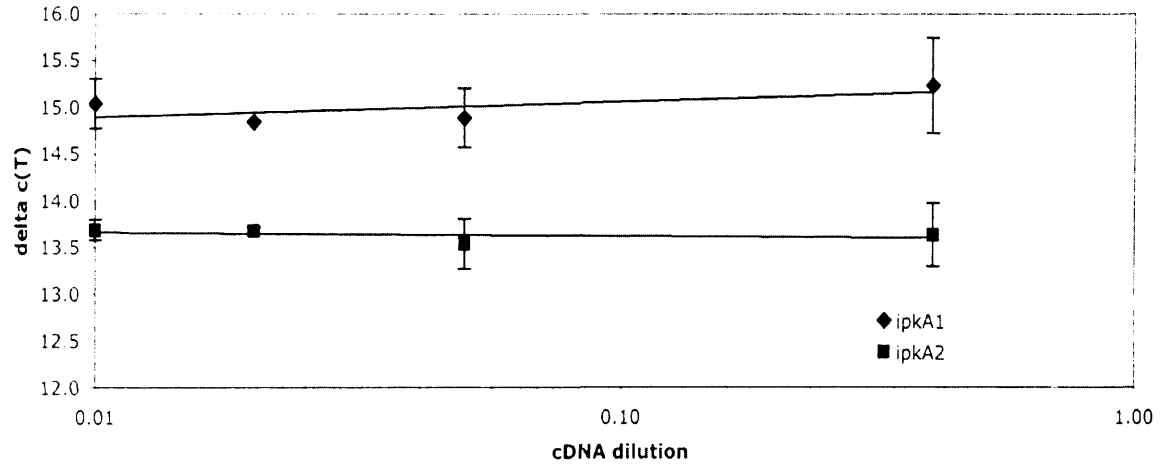


Figure A1.3: Comparison of PCR efficiencies. To compare amplification efficiency of the product for each gene with the Ig7 internal control a cDNA serial dilution was analysed by real-time PCR. Each dilution was tested with each set of gene specific primers as well as the Ig7 loading control in triplicate. The values plotted are the difference between the c(T) values of the gene-specific primer and Ig7 at each cDNA dilution: therefore a horizontal line indicates an equal response to template dilution for both sets of primers.

Appendix II:

Unprocessed chemotaxis data

Speed (μm/min)

		Experiment 1	2	3	4	Mean	±
Wild-type	NaCl	9.78	8.50	7.95	10.90	**9.28**	**0.66**
	LiCl	8.00	5.68	6.40	8.52	**7.15**	**0.67**
	LSI	0.82	0.67	0.81	0.78	**0.77**	**0.07**
dpoA⁻	NaCl	8.09	8.45	10.03	8.36	**8.73**	**0.44**
	LiCl	8.57	7.07	8.22	8.20	**8.02**	**0.33**
	LSI	1.06	0.84	0.82	0.98	**0.92**	**0.12**
mppA⁻	NaCl	8.02	8.34	8.89		**8.42**	**0.25**
	LiCl	6.46	6.67	5.81		**6.31**	**0.26**
	LSI	0.81	0.80	0.65		**0.75**	**0.09**
mppA⁻/dpoA⁻	NaCl	9.83	9.23	10.35	11.12	**10.13**	**0.40**
	LiCl	6.68	7.12	6.86	7.96	**7.16**	**0.28**
	LSI	0.68	0.77	0.66	0.72	**0.71**	**0.05**
mppA o/e	NaCl	8.48	8.61	7.23	7.48	**7.95**	**0.35**
	LiCl	4.41	5.90	3.74	4.28	**4.58**	**0.46**
	LSI	0.52	0.69	0.52	0.57	**0.57**	**0.08**
plc⁻	NaCl	9.82	8.02	9.59	7.80	**8.81**	**0.52**
	LiCl	7.90	10.69	8.35	10.22	**9.29**	**0.69**
	LSI	0.80	1.00	0.87	1.00	**0.92**	**0.10**
mppA⁻/plc⁻	NaCl	8.02	9.13	11.57	12.83	**10.39**	**1.10**
	LiCl	6.57	8.68	10.27	10.31	**8.96**	**0.88**
	LSI	0.82	0.95	0.89	0.80	**0.87**	**0.07**

Directionality

		Experiment 1	2	3	4	Mean	±
Wild-type	NaCl	0.81	0.69	0.67	0.72	**0.72**	**0.03**
	LiCl	0.68	0.54	0.63	0.62	**0.62**	**0.03**
	LSI	0.84	0.78	0.94	0.86	**0.86**	**0.07**
dpoA⁻	NaCl	0.65	0.68	0.69	0.66	**0.67**	**0.01**
	LiCl	0.60	0.67	0.64	0.69	**0.65**	**0.02**
	LSI	0.92	0.99	0.93	1.05	**0.97**	**0.06**
mppA⁻	NaCl	0.68	0.71	0.70		**0.70**	**0.01**
	LiCl	0.56	0.53	0.43		**0.51**	**0.04**
	LSI	0.82	0.75	0.61		**0.73**	**0.11**
mppA⁻/dpoA⁻	NaCl	0.70	0.70	0.76	0.71	**0.72**	**0.01**
	LiCl	0.59	0.50	0.55	0.48	**0.53**	**0.02**
	LSI	0.84	0.71	0.72	0.68	**0.74**	**0.07**
mppA o/e	NaCl	0.66	0.71	0.53	0.68	**0.65**	**0.04**
	LiCl	0.38	0.48	0.28	0.48	**0.41**	**0.05**
	LSI	0.58	0.68	0.53	0.71	**0.62**	**0.08**
plc⁻	NaCl	0.67	0.69	0.68	0.68	**0.68**	**0.00**
	LiCl	0.63	0.68	0.60	0.72	**0.66**	**0.03**
	LSI	0.94	0.99	0.88	1.06	**0.97**	**0.07**
mppA⁻/plc⁻	NaCl	0.61	0.71	0.64	0.58	**0.64**	**0.03**
	LiCl	0.53	0.61	0.75	0.65	**0.64**	**0.05**
	LSI	0.87	0.86	1.17	1.12	**1.01**	**0.16**

Table A2.2: Raw DIAS data for chemotaxing cells. Speed and chemotactic index was measured in a number of independent experiments for each mutant. Values shown are the mean for each independent experiment. LSI indicates the ratio of the LiCL to the NaCl value on each day. Errors represent the standard error for absolute speed and C.I., and standard deviation for LSI.

Appendix III:

Gene, Protein and Strain Nomenclature

Nomenclature for *Dictyostelium* proteins used in this work

Gene name	Protein Name	Activity/notes	Dictybase ref.
mppA	Multiple Inositol Polyphosphate Phosphatase A (MippA)	$InsP_6$ 2'/3'/6' phosphatase / phytase	DDB0186447
mppB	MippB	Not expressed in vegetative of developing cells	DDB0218876
ipkA1	Inositol Polyphosphate Kinase (IpkA1)	$Ins(1,4,5)P_3$ 3'/6' kinase	DDB0203614
ipkA2	IpkA2	As above	DDB0218526
ipkA3	IpkA3	Lacks conserved proline residue of IpkA1 and A2	DDB0204639
ipkB	IpkB	$Ins(1,3,4)P_3$ 5'/6' kinase / $Ins(3,4,5,6)P_4$ 1' kinase	DDB0190521
ipkC	IpkC	$Ins(1,3,4,5,6)P_5$ 2' kinase	DDB0187902
impa1	Inositol MonoPhosphatase (IMPase)	InsP phosphatase	DDB0204100
ippA	Inositol Polyphosphate Phosphatase (IppA)	$InsP_?$ phosphatase	DDB0167248
ippB	IppB	$InsP_2$ 1' phosphatase / PAP phosphatase?	DDB0189923
plc / pipA	PhosphoLipase C (PLC)	Delta class inositide-specific $PtIns(4,5)P_2$ lipase	DDB0201656
dpoA	Prolyl Oligopeptidase A (DpoA)	Cleaves peptides after proline residues	DDB0185041
pisA	Phosphatidyl Inositol Synthase (PisA)	Generates PtIns from myo-inositol and CDP-DAG	DDB0218645

Strain nomenclature

Strain Name	Parent	Genotype	Notes
HAD230	Ax2	*mppA⁻ / Bsr^r*	Clone 1
HAD231	Ax2	*mppA⁻/ Bsr^r*	Clone 2
HAD234	HAD230	*mpp⁻ / Bsr^s*	Cre-lox treated *mppA* KO
HAD236	Ax2	*plc⁻ / Bsr^r*	Clone 1
HAD237	Ax2	*plc⁻/ Bsr^r*	Clone 2
HAD243	HAD234	*mppA⁻/ plc⁻/ Bsr^r*	Clone 1
HAD244	HAD234	*mppA⁻/ plc⁻/ Bsr^r*	Clone 2
HAD246	HAD234	*mppA⁻/ dpoA⁻/ Bsr^r*	Clone 1
HAD247	HAD234	*mppA⁻/ dpoA⁻/ Bsr^r*	Clone 2
HD10	Ax3	*plc⁻/ G418^r*	Published *plc* KO (Drayer *et al.*, 1994)
HD11	Ax3	*plc^wt/ G418^r*	Published *plc* random integrant

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