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The mood stabiliser lithium suppresses PIP_3 signalling in Dictyostelium and human cells

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SUMMARY

Bipolar mood disorder (manic depression) is a major psychiatric disorder whose molecular origins are unknown. Mood stabilisers offer patients both acute and prophylactic treatment, and experimentally, they provide a means to probe the underlying biology of the disorder. Lithium and other mood stabilisers deplete intracellular inositol and it has been proposed that bipolar mood disorder arises from aberrant inositol (1,4,5)-trisphosphate [IP_3, also known as Ins(1,4,5)P_3] signalling. However, there is no definitive evidence to support this or any other proposed target; a problem exacerbated by a lack of good cellular models. Phosphatidylinositol (3,4,5)-trisphosphate [PIP_3, also known as PtdIns(3,4,5)P_3] is a prominent intracellular signal molecule within the central nervous system (CNS) that regulates neuronal survival, connectivity and synaptic function. By using the genetically tractable organism Dictyostelium, we show that lithium suppresses PIP_3-mediated signalling. These effects extend to the human neutrophil cell line HL60. Mechanistically, we show that lithium attenuates phosphoinositide synthesis and that its effects can be reversed by overexpression of inositol monophosphatase (IMPase), consistent with the inositol-depletion hypothesis. These results demonstrate a lithium target that is compatible with our current knowledge of the genetic predisposition for bipolar disorder. They also suggest that lithium therapy might be beneficial for other diseases caused by elevated PIP_3 signalling.

INTRODUCTION

Bipolar mood disorder (manic depression) is a major, chronic psychiatric disorder with a lifetime prevalence of greater than 1%. Characterised by recurrent episodes of mania and depression, bipolar disorder presents a significant socio-economic burden (Das Gupta and Guest, 2002) and substantially lowers quality of life (Gutiérrez-Rojas et al., 2008). If untreated, it carries a high suicide rate, estimated to be 1 million deaths worldwide per year. The impact of bipolar disorder is not restricted to mental health, as it exhibits co-morbidity with cardiovascular, endocrine and metabolic disorders and is linked to an elevated inflammatory response. The molecular basis of the disorder is unknown. One of the few investigative routes available is to study the mechanism of action of mood stabiliser drugs. Lithium is the most established and commonly used mood stabiliser (Burgess et al., 2001); in addition, preliminary evidence indicates that lithium might suppress the effects of the neurodegenerative disease amyotrophic lateral sclerosis (ALS) (Fornai et al., 2008). In both cases, we lack therapeutic mechanisms. Establishing these mechanisms will lead to a better understanding of the molecular basis of bipolar mood disorder, a rationale for its use in the treatment of neurodegeneration and improved drug design; furthermore, the mechanisms may reveal other therapeutic uses for the existing mood stabilisers.

Inositol depletion is a common outcome of treatment with a number of mood stabiliser drugs (Berridge, 1985; Williams et al., 2002). Lithium inhibits the key inositol synthetic enzymes, inositol monophosphatase (IMPase) and inositol polyphosphate phosphatase (IPP) (Hallcher and Sherman, 1980), whereas an alternative mood stabiliser, valproic acid (VPA), inhibits inositol synthase activity (Shaltiel et al., 2004). A third mood stabiliser, carbamazepine, also causes inositol depletion, but by an unknown mechanism (Williams et al., 2002; Sarkar et al., 2005; Shimshoni et al., 2009). Both lithium and VPA lower the cellular concentration of the second messenger, inositol (1,4,5)-trisphosphate [IP_3, also known as Ins(1,4,5)P_3], leading to the proposition that IP_3 might be the important target in bipolar disorder. However, despite intensive investigation, it has not been possible to directly correlate altered IP_3 signalling and bipolar mood disorder. This raises the possibility that mood stabilisers may suppress alternative forms of inositide signalling.

The social amoeba *Dictyostelium discoideum* provides a good system in which to study the mechanism of action of mood stabiliser drugs and to test drug function. Dictyostelium development is lithium sensitive, but in contrast to other model systems, the two major lithium-sensitive signal pathways, inositol phosphate (IP) and glycogen synthase kinase-3 (GSK-3), are separated into different developmental stages. GSK-3 inhibition leads to mis-patterning of cell specification during multicellular stages of development, whereas inositol depletion substantially retards Dictyostelium cell aggregation (Maeda, 1970; Williams et al., 1999). Here, we demonstrate that phosphatidylinositol (3,4,5)-trisphosphate [PIP_3, also known as PtdIns(3,4,5)P_3] signalling is the major lithium target during Dictyostelium chemotaxis and is suppressed in the human neutrophil cell line HL60.

RESULTS AND DISCUSSION

When starved, Dictyostelium amoebae enter a developmental programme to generate a multicellular fruiting body (Kessin, 2001).

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To achieve this, cells must aggregate to form a mound of 10^5 cells; a process that requires cell chemotaxis towards cyclic adenosine 3′,5′-monophosphate (cAMP). Lithium treatment had no effect on cAMP synthesis (Fig. 1A), but did have a striking effect on chemotaxis (Fig. 1B; supplementary material Movies 1 and 2). Analysis of individual cells undergoing chemotaxis showed that lithium causes a decrease in cell speed, an increase in cell turning and a small decrease in their chemotactic index (CI), which is a measure of their ability to sense the signal gradient (supplementary material Table S1). A 56% mean decrease in cell speed correlates well with the doubling of aggregation time that has been observed previously for lithium-treated cells (Williams et al., 1999). In the majority of cases, lithium was applied acutely for 1 hour as 10 mM LiCl, however the same effect could be achieved after prolonged treatment in 2 mM LiCl (data not shown). Dictyostelium cells move by local assembly of the F-actin cytoskeleton at their leading edge. Lithium did not interfere with the basic mechanisms of F-actin polymerisation (Fig. 1C), suggesting that the lithium targets an intracellular signalling mechanism.

The effects of lithium treatment on chemotaxis strongly resembled those reported for reduced PIP_3 signalling (Loovers et al., 2006; Takeda et al., 2007). PIP_3 is rapidly induced by cAMP at the leading edge of the cell through translocation of class I phosphoinositide 3-kinases (PI3K) (Funamoto et al., 2002). Although not essential, PIP_3 is required for efficient chemotaxis (Andrew and Insall, 2007; Hoeller and Kay, 2007). Under the conditions used here, we found that treatment with the PI3 kinase inhibitor LY294002 closely matches that seen with lithium (Fig. 1B). A similar defect was seen for mutant cells lacking all five Dictyostelium PI3K genes (*pi3k1-5*, also known as *pikA-C*, *pikF* and *pikG*) (Hoeller and Kay, 2007) (Fig. 1B).

To probe the effect of lithium on PIP_3 signalling, we monitored PIP_3 synthesis in living cells by expression of the PIP_3-specific binding protein PH_{CRAC}-GFP (Parent et al., 1998). When globally stimulated, PIP_3 is generated on the entire plasma membrane causing translocation of PH_{CRAC}-GFP. Lithium treatment suppressed PH_{CRAC}-GFP translocation to 40% of that seen in control cells (Fig. 2A). To confirm these observations, we monitored phosphorylation of the protein kinase B (PKB) homologue, PkbA, an event that is dependent on its membrane translocation through PH domain binding to PIP_3 (Kamimura et al., 2008). In control cells, phospho-PkbA (p-PkbA) could be detected 10 seconds after cAMP stimulation and was lost after 45 seconds (Fig. 2B,C). PkbA phosphorylation was not observed in the *pi3k1-5*-null mutant or in the *pkbA*-null mutant (Fig. 2B). Lithium treatment suppressed PkbA phosphorylation to 45% of control values (Fig. 2C). Finally, we directly measured PIP_3 synthesis following cAMP stimulation and found that new PIP_3 production was suppressed to 38% of that seen following control treatment; this is consistent with the observations on PkbA phosphorylation (Fig. 2C). Together, these results demonstrate that lithium suppresses PIP_3 signalling following cAMP stimulation and can account for the effect of lithium on chemotaxis.

Although PIP_3 signalling was suppressed following cAMP stimulation, other intracellular signalling pathways that are required during chemotaxis were unaffected. Dictyostelium contains two homologues of the protein kinase PKB/AKT1, PkbA and PkgB (also known as PKBR1) (Meili et al., 2000). Both are phosphorylated in response to cAMP stimulation, however only PkbA phosphorylation is dependent on PIP_3 (Kamimura et al., 2008). Consistent with a specific effect on PIP_3, we found that PkgB phosphorylation is unaffected by lithium treatment (Fig. 2B). Cyclic guanosine 3′,5′-monophosphate (cGMP) mediates an additional independent regulator of chemotaxis (Veltman and van Haastert, 2008), however lithium has no effect on cGMP synthesis (Fig. 2D). Finally, ablation of the Dictyostelium PLC (*plc)* and IP_3 receptor (*iplA)* genes does not suppress chemotaxis (Drayer et al., 1994; Traynor et al., 2000) (Fig. 1B and data not shown), indicating that inhibition of IP_3 production or calcium release by lithium is not related to the effects of lithium on chemotaxis.

Following cAMP stimulation, PI3K translocates to the leading edge of the cell, whereas the PIP_3 phosphatase PTEN is lost from

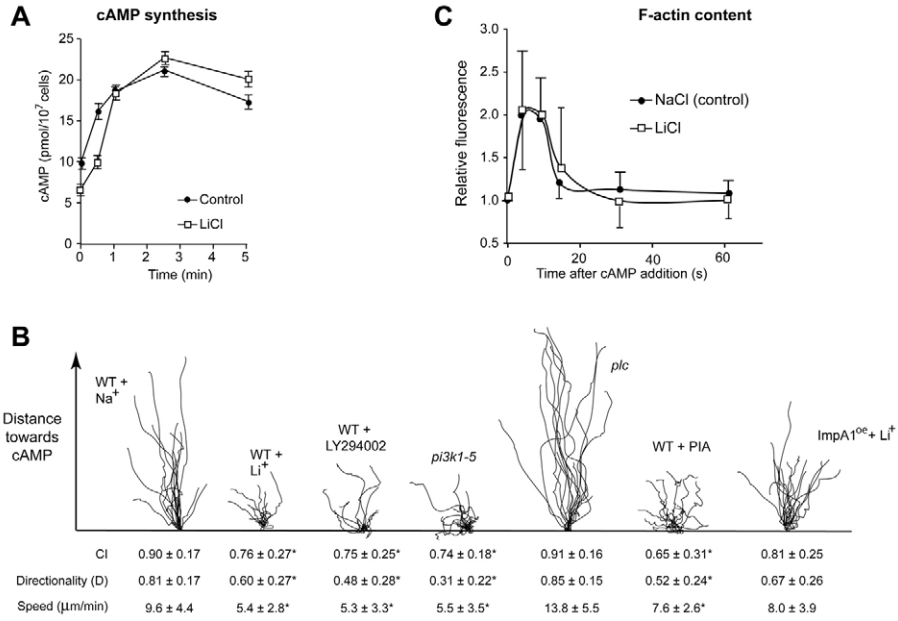

Fig. 1. Effect of lithium on Dictyostelium cells undergoing chemotaxis. (A) Wild-type cells treated with 10 mM LiCl or NaCl (control) were stimulated with 10 μM of 2′-deoxy-cAMP and cAMP production was then measured over 5 minutes. (B) Analysis of chemotaxis for wild-type and mutant cell strains after lithium or control treatment. Representative tracks are shown for each experiment and mean±s.e.m. is shown for the parameters: chemotactic index (CI), directionality (D) (a measure of cell turning where 1 is a straight line) and speed. (C) Wild-type cells were treated with 10 mM LiCl or 10 mM NaCl (control) and the amount of F-actin was measured through TRITC-phalloidin binding. The graph shows the fluorescence at time intervals after cAMP stimulation, displayed as relative values compared with prestimulation fluorescence. All data points are the mean±s.e.m. of three independent experiments.

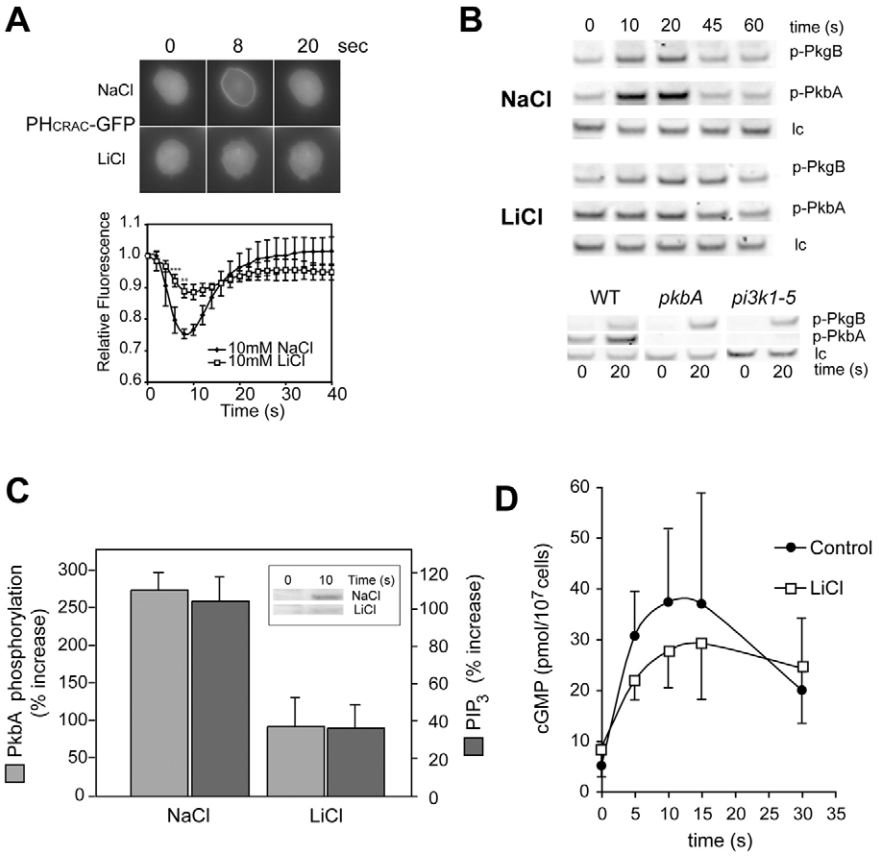

Fig. 2. Lithium suppresses PIP_3 signalling.
(A) Translocation of the PIP_3 marker PH_{CRAC}-GFP to the plasma membrane upon uniform stimulation of control and lithium-treated cells with 1 μM cAMP. The graph shows quantification of PH_{CRAC}-GFP by measuring the cytosolic fluorescence intensity. The values plotted are the means of three independent experiments±s.e.m. (B) Lithium inhibition of PKB. Aggregation-competent cells were treated for 1 hour with 10 mM LiCl or NaCl (control) and then stimulated with 1 μM cAMP. Samples were analysed by western blotting with an antibody that recognises phosphorylation at both threonine 278 on PkbA and threonine 309 on PkgB (Kamimura et al., 2008) (lc is a non-specific band that acts as a loading control). To confirm specificity, the same antibody was used on wild-type cells, *pkbA*-null mutant cells and *pi3k1-5*-null mutant cells that cannot induce PIP_3-dependent phosphorylation. (C) Quantification of PIP_3 synthesis. Cells were stimulated with cAMP under the same conditions as in (B). Phosphorylation of PkbA was measured using an anti-phosphothreonine antibody, as described by Lim et al. (Lim et al., 2001). A direct measurement of the quantity of PIP_3 was made by using a PIP_3 mass ELISA. The results are displayed as percentage increase over the unstimulated level (mean±s.e.m.). (D) Wild-type cells treated with 10 mM LiCl or NaCl (control) were stimulated with 1 μM cAMP and cGMP synthesis was then measured over 30 seconds.

this region. Lithium had no effect on either PI3K translocation to the plasma membrane or PTEN loss from the membrane following cAMP stimulation (Fig. 3E; supplementary material Fig. S1A), indicating that cAMP-stimulated PIP_3 synthesis is not suppressed by loss of PI3K or elevated PTEN activity. We therefore investigated a third possibility, which was whether lithium reduced the amount of the PI3K substrate phosphatidylinositol (4,5)-bisphosphate [PIP_2, also known as PtdIns(4,5)P_2]. We could not detect any lithium-induced changes in the steady-state concentration of PIP_2, however we did detect a 50% and 63% decrease in the rate of PIP and PIP_2 synthesis, respectively (Fig. 3A,B; supplementary material Fig. S1B); this level of reduction closely matches that calculated for cAMP-induced PIP_3 synthesis. These observations suggest that there are two pools of PIP_2. First, there is a slowly metabolised pool that is not involved in cell signalling during chemotaxis and that is insensitive to lithium treatment. Most of this PIP_2 is likely to be bound to proteins, particularly those regulating the actin cytoskeleton (Janmey and Lindberg, 2004). Second, there is a rapidly metabolised pool, which is required for the synthesis of PIP_3 during chemotaxis and is susceptible to lithium inhibition. In Dictyostelium, the amount of PIP_2 within the lithium-insensitive pool is much greater than that involved in cell signalling; we calculated that there was a 50- and 200-fold excess of total PIP_2 concentration over IP_3 and PIP_3, respectively. The presence of a large non-dynamic PIP_2 pool that masks the effects of lithium on PIP_2-mediated signalling might be one reason why, in many systems, it has proven difficult to measure substantial changes in PIP and PIP_2.

The behaviour of Dictyostelium PTEN offers a means to directly monitor the rapidly metabolised, lithium-sensitive PIP_2 pool. In Dictyostelium, PTEN localisation to the plasma membrane is dependent on PIP_2, and a reduction in PIP_2 leads to a reduced rate of PTEN binding (Vazquez et al., 2006). Lithium treatment of unstimulated cells reduced the amount of PTEN-GFP associated with the plasma membrane (Fig. 3C), consistent with a reduction of PIP_2. In agreement with these observations, lithium treatment of unstimulated cells increased PIP_3 and p-PkbA compared with control-treated cells (Fig. 3D).

Although a good marker for changes in the PIP_2 signalling pool, the change in PTEN behaviour following lithium treatment is unlikely to have a major effect on cell signalling. This is because cAMP stimulation removes PTEN from the plasma membrane and hence stimulated production of PIP_3 arises solely from PI3K activation. As lithium treatment only alters the amount of bound PTEN and not its relocation from the membrane (Fig. 3E), the reduced amount of newly synthesised PIP_3 following cAMP stimulation is probably the result of suppressed PIP_3 generation. During chemotaxis, cells generate a steep gradient of PIP_3, with high concentrations at the leading edge of the cell, and lithium treatment will act to flatten this gradient. The effects we observe following lithium treatment match the phenotype of *pi3k1-5*-null mutants and not that of *pten*-null mutants (Iijima and Devreotes, 2002; Hoeller and Kay, 2007; Kortholt et al., 2007), suggesting that a failure of PIP_3 synthesis, rather than reduced breakdown of the protein, gives rise to the effect of lithium.

A number of experiments suggest that the effects of lithium on phosphoinositide signalling may have arisen through inositol depletion. First, propylisopropylacetic acid (PIA), a specific inhibitor of myo-inositol synthesis (Shimshoni et al., 2007), has a similar effect on chemotaxis to lithium (Fig. 1B). Second, IMPase is inhibited by lithium through an uncompetitive mechanism (Atack et al., 1995), which means that the inhibition can be reversed by increasing the concentration of the enzyme. Consistent with such a mechanism, lithium sensitivity is suppressed by overexpression of the Dictyostelium IMPase gene (*impa1*) (Fig. 1B). This lithium resistance can also be observed at the molecular level where *impa1* overexpression counteracts the effect of lithium on PkbA phosphorylation and reverses the inhibition of PIP/PIP_2 synthesis (Fig. 3B,F).

These observations indicate that PIP_3 is the major target of lithium in Dictyostelium chemotaxis. To examine whether this is unique to Dictyostelium, we investigated the effect of lithium on the human cell line HL60 following their differentiation into neutrophil-like cells that are capable of chemotaxis (Collins et al., 1979). These differentiated HL60 cells undergo chemotaxis to the peptide fMLP in a process that involves PIP_3 (Cui et al., 2000). Global stimulation using a chemoattractant generates PIP_3 on the plasma membrane, which we monitored by plasma membrane translocation of the PIP_3-binding protein PH_{Akt}-GFP (Servant et al., 2000) (Fig. 4A). Consistent with our Dictyostelium observations, PH_{Akt}-GFP translocation was attenuated following lithium treatment (Fig. 4B). This indicates that the effects of lithium on PIP_3 signalling are conserved in human cells, extending the repertoire of the effects of lithium on inositide signalling.

In conclusion, we have shown that lithium suppresses PIP_3 signalling. These results have implications beyond the regulation of chemotaxis and could explain the therapeutic efficacy of lithium in the treatment of bipolar disorder. PIP_3 signalling in the nervous system is important for axonal guidance, synaptogenesis and synaptic transmission (Zhou et al., 2004; Ramsey et al., 2005; Chadborn et al., 2006; Martin-Pena et al., 2006; Xu et al., 2007), and mice with elevated PIP_3 levels suffer seizures (Backman et al., 2001). Lithium might, therefore, alter these processes through an

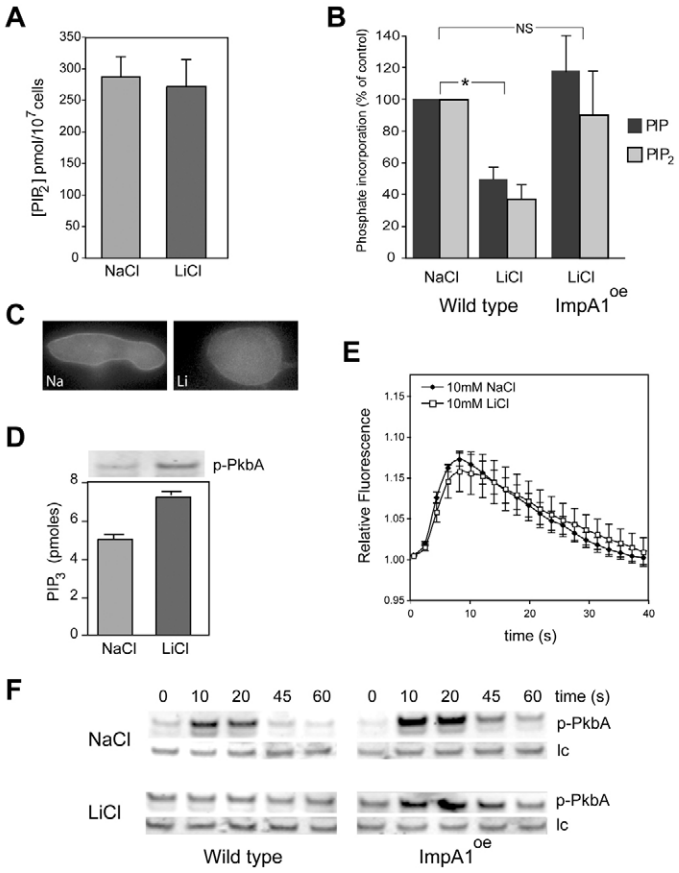

Fig. 3. Lithium effects on PIP synthesis. (A) The total mass of PIP_2 in Dictyostelium cells was measured by alkaline hydrolysis of membrane lipids followed by measuring the released IP_3 (Chilvers et al., 1991). Dictyostelium cells contain between 270-290 picomoles of PIP_2 and lithium-treated cells showed no detectable difference compared with control (NaCl) cells. (B) Measurement of PIP and PIP_2 following treatment with 10 mM LiCl. Wild-type cells show a significant ($P<0.01$, paired *t*-test) decrease in both PIP and PIP_2 following lithium treatment compared with NaCl control treatment. This decrease is reversed by increased expression of the ImpA1 protein. (C) Lithium treatment decreases the amount of PTEN-GFP associated with the plasma membrane of cells undergoing chemotaxis. Images show typical cells. (D) Lithium treatment increases the basal level of PIP_3 and p-PkbA in unstimulated cells. (E) Wild-type cells expressing PTEN-GFP were pulsed for 5 hours and treated with 10 mM NaCl or LiCl for 1 hour. Translocation following uniform stimulation with 1 μM cAMP was quantified by measuring the change in cytosolic fluorescence intensity. Values plotted are the means of three independent experiments and error bars indicate the s.e.m. Note that the fluorescence intensity measurements are the relative changes compared with the unstimulated level that has been normalized for each cell. This means that although lithium-treated cells had less PTEN on their membrane in the unstimulated state, the kinetics of loss from the membrane remained the same as that seen for the NaCl control-treated cells. (F) A cDNA encoding ImpA1 was expressed in wild-type Dictyostelium cells from the *actin15* gene promoter. Samples were analysed for p-PkbA content as in Fig. 2B.

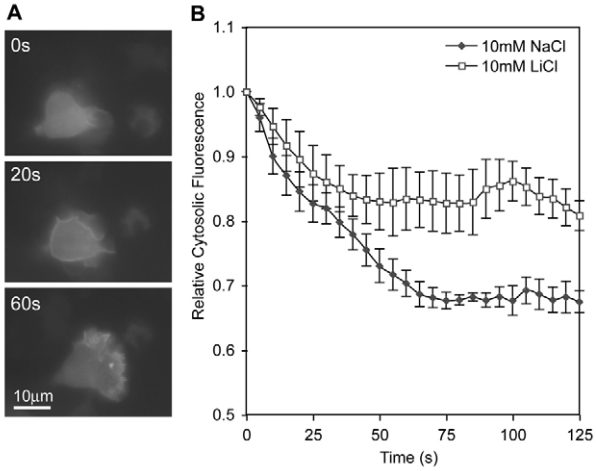

Fig. 4. Lithium suppresses PIP_3 signalling in HL60 cells. (A) HL60 cells expressing PH_{Akt}-GFP were differentiated to neutrophils and then globally stimulated with fMLP. PH_{Akt}-GFP translocates from the cytosol to the plasma membrane within 20 seconds. At 60 seconds, cells have returned to a polarised state with localised at the leading edge. (B) Translocation of the PH_{Akt}-GFP is measured by the same method used in Dictyostelium. Graph shows quantification by measuring the cytosolic fluorescence intensity. Values plotted are the means of three independent experiments ± s.e.m.

effect on PIP_3 signalling. Furthermore, bipolar mood disorder has a strong genetic predisposition but is a complex genetic trait that probably involves combinations of multiple loci (Craddock and Jones, 1999). As yet, no susceptibility gene has been unambiguously identified but, interestingly, many of the candidate genes currently under investigation converge on the PIP_3- and PKB-mediated signal pathway (Silverstone et al., 2005; Carter, 2007). The results presented here provide a mechanism that might directly couple current genetic studies with a target of lithium therapy.

Conversely, suppression of PIP_3 signalling might open up therapeutic uses for lithium. In human patients, aberrant PIP_3 signalling is linked to macrocephaly, mental retardation, cerebellar hypertrophy, ataxia, seizures, epilepsy and autism (Li et al., 1997; Yates, 2006). Beyond the nervous system, regulators of PIP_3 signalling are frequently mutated in tumours (Leslie and Downes, 2004; Barber and Welch, 2006) and familial PTEN mutations are associated with a number of syndromes that cause hamartomas, such as Cowden, Bannayan-Riley-Ruvalcaba and Proteus syndromes (Eng, 2003). The discovery that suppression of PIP_3 signalling is a major consequence of lithium treatment offers both an insight into the mechanism of lithium action within the nervous system and suggests therapeutic opportunities for this well-established drug, such as in the control of tumour progression.

TRANSLATIONAL IMPACT

Clinical issue

Bipolar mood disorder, often called manic depression, is a long-term psychiatric illness that causes dramatic changes in energy, behaviour and mood. The underlying cause, or causes, of this disorder are unknown and there is no cure for this illness. In most cases, the disease is treated with mood-stabilising medications. Of these, lithium is the most common and possibly the best; however, despite over 50 years of clinical use, its therapeutic mechanisms remain unknown.

At the cellular level, lithium causes depletion of myo-inositol. One prevalent hypothesis is that this reduces inositol (1,4,5)-trisphosphate [IP_3, also known as Ins(1,4,5)P_3], an important intracellular signalling molecule. So far, extensive investigation has failed to establish whether inhibition of IP_3 signalling is the therapeutic mechanism for lithium or whether changes in IP_3 are associated with the disorder.

Results

This study provides the first demonstration that lithium has a major effect on phosphatidylinositol (3,4,5)-trisphosphate [PIP_3, also known as PtdIns(3,4,5)P_3], an alternative inositol-based signalling molecule. Lithium suppresses PIP_3 signalling in both the social amoeba Dictyostelium and cultured human neutrophil cells. It does this by suppressing synthesis of the phosphoinositide precursors of PIP_3. Increased expression of the enzyme inositol monophosphatase (IMPase) reverses the effects of lithium, suggesting that lithium acts through inositol depletion. Interestingly, in the absence of lithium, elevated IMPase leads to elevated PIP_3 signalling.

Implications and future directions

These findings have important implications in lithium therapy and neurochemistry. PIP_3 is a major signalling molecule in neurons, and suppression of PIP_3 signalling may have significant neuromodulatory effects in bipolar mood disorder patients. There is a strong familial predisposition for bipolar mood disorder suggesting a genetic component. These findings support further investigation of genes associated with PIP_3 signalling, such as the IMPase gene *IMPA2*. The ability of lithium to target PIP_3 might also lead to new therapeutic uses for lithium. Aberrant PIP_3 signalling is linked to a range of neurological diseases, including macrocephaly, epilepsy and autism. Beyond the nervous system, regulators of PIP_3 signalling are frequently mutated in tumours and inheritable diseases. Furthermore, the mood stabilisers valproic acid and carbamazepine also deplete inositol and may be used to suppress PIP_3 signalling. Unlike lithium, these drugs can be chemically modified, presenting a route to new generations of PIP_3 inhibitors.

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METHODS

Analysis of Dictyostelium chemotaxis

D. discoideum strain AX2 was used in all assays. Washed log-phase cells were developed in shaking suspension in KK2 buffer for 5 hours at 5×10^6 cells/ml with 6-minute pulses of 100nM cAMP. All drug and control treatments were added only for the final hour, except for PIA, which is required throughout pulsing. Pulsed cells were placed in a Z02 Zigmond chamber (Neuro Probe) (Zigmond, 1977) with 1 μM of cAMP/KK2 as the source and KK2 as the sink. All solutions contained the appropriate drugs to ensure continued treatment. Cells were recorded after 20 minutes using video microscopy with a 20× objective and by taking DIC images every 6 seconds. Cell movement was analysed using the Dynamic Image Analysis System (DIAS) (version 3.4.1, Soll Technologies, Iowa City, IA) (Soll et al., 2001). For statistical analysis, we used Kruskal-Wallis and Mann-Whitney *U* tests, with a post-hoc Dunn test for multiple comparison.

Cell signalling assays

cAMP and cGMP were measured as described previously (Snaar-Jagalska and Van Haastert, 1994) using isotope dilution assay kits (GE healthcare). Actin polymerisation was measured using a modified version of the method of Hall et al. (Hall et al., 1988). Pulsed cells (2.5×10^7 cells/ml) were stimulated with 1 μM cAMP and 100 μl aliquots were fixed at the set time intervals by addition of 900 μl of stop solution (3.7% formaldehyde, 0.1% Triton X-100, 0.5 μM TRITC-phalloidin, 10 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.8). Cells were resuspended in 800 μl of methanol and F-actin was assayed as fluorescence intensity at 540/590 nm. Wild-type cells were transformed by electroporation with the plasmids WF38 (PH_{CRAC}-GFP) (Parent et al., 1998), PTEN-GFP (Iijima and Devreotes, 2002), pOH30 (YFP-PI3K2N) or pPH_{PLC} (PLC-δ1-PH-GFP) (Hoeller and Kay, 2007). Pulsed cells were stimulated by the addition of 1 μM of cAMP. Protein translocation was recorded by fluorescence video microscope with a 60× objective, and the fluorescence intensity of the cytosol of each cell in each frame was measured using ImageJ software and normalised to the prestimulated level of the same cell. For PKB phosphorylation, pulsed cells were stimulated with 1 μM of cAMP and samples were lysed directly into 100 μl of 2×LDS loading buffer (Invitrogen) supplemented with 100 mM NaF, 0.4 mM Na_3VO_4 and 3 mM EDTA on ice. Samples were separated by electrophoresis using 3-8% NuPage tris-acetate gels (Invitrogen), blotted onto nitrocellulose membrane (Amersham) and then probed with a rabbit anti-phospho PKC (pan) antibody (190D10, Cell Signalling Technology) or, for quantification, with an anti-phosphothreonine antibody (Lim et al., 2001). PIP_3 was measured directly by PIP_3 mass ELISA (Echelon). Phosphoinositide turnover was monitored by incubating chemotactic-competent cells in internal buffer [139 mM sodium glutamate, 5 mM glucose, 5 mM EGTA, 20 mM PIPES at pH 6.6, 1 mM magnesium sulphate, 0.03% saponin, 0.005 volume of

protease inhibitor cocktail 1 and 2 (Sigma), and 5 μCi of γ-^{32}P-labelled adenosine triphosphate (ATP)] for exactly 6 minutes, followed by lysis in acidified methanol, extraction in water-methanol-chloroform (1:2:1), and separation by thin-layer chromatography on silica gel 60 TLC plates (VWR) in a solvent mix (chloroform-acetone-methanol-acetic acid-distilled water in a ratio of 40:15:13:12:7). ^{32}P lipid incorporation into lipids was measured using a Typhoon phosphoimager (Pawolleck and Williams, 2009).

Culture and stimulation of HL60 cells

HL60 cells were grown at 37°C in RPMI 1640 medium supplemented with 25 mM HEPES, 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine. Before stimulation, the cells were differentiated into neutrophil-like cells in 8-well chamber slides (Lab-Tek) by the addition of 1.3% DMSO to the medium for 5 days. Cells were stimulated by pipetting fMLP (Sigma) directly into each chamber, to a final concentration of 1 μM. Translocation of the GFP marker to the membrane was quantified as for Dictyostelium cells, with images taken at 5 second intervals.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

A.J.H., J.K., R.T. and R.S.B.W. conceived and designed the experiments; J.K., R.T., J.V.R., O.P., B.O. and J.R. performed the experiments; O.H. contributed strains and plasmids; A.J.H., J.K. and R.T. wrote the paper.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
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