The inhibition of phosphoinositide synthesis and muscarinic-receptor-mediated phospholipase C activity by Li^+ as secondary, selective, consequences of inositol depletion in 1321N1 cells

Ian H. BATTY* and C. Peter DOWNES

Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

Conditions are described for culture of 1321N1 cells under which cellular inositol is decreased from ~ 20 mM to < 0.5 mM but phosphoinositide concentrations are unaffected. The effects of the muscarinic-receptor agonist carbachol (1 mM) and/or LiCl (10 mM) on phosphoinositide turnover in these or in inositol-replete cells was examined after steady-state [^3H]inositol labelling of phospholipid pools. In both inositol-replete and -depleted cells, carbachol stimulated similar initial (0–15 min) rates of phospholipase C (PLC) activity, in the presence of Li^+ . Subsequently (> 30 –60 min) stimulated PLC activity and [^3H]PtdIns concentrations declined dramatically only in depleted cells. In inositol-depleted cells, carbachol alone evoked increased concentrations of [^3H]inositol, [^3H]Ins P_1 , [^3H]Ins P_2 , [^3H]Ins P_3 and [^3H]Ins P_4 , which were largely sustained over 90 min, and concentrations of [^3H]PtdIns, [^3H]PtdIns P and [^3H]PtdIns P_2 were decreased only to ~ 82 , 84 and 93 % of control respectively. In the presence of Li^+ in these cells, the stimulated rise in [^3H]inositol was prevented and, although accumulation of [^3H]Ins P_1 , [^3H]Ins P_2 and [^3H]Ins P_3 was initially (0–30 min) potentiated, rates of

accumulation of [^3H]Ins P_1 and concentrations of [^3H]polyphosphates later (> 30 –60 min) declined, and concentrations of [^3H]PtdIns, [^3H]PtdIns P and [^3H]PtdIns P_2 were decreased respectively to ~ 39 , 48 and 81 % of control. After 60 min in the presence of both carbachol and Li^+ , stimulated PLC activity was decreased by ~ 70 % compared with the initial rate in depleted cells. This decreased PLC activity was reflected by changes in the stimulated concentrations of [^3H]Ins(1,3,4) P_3 but not of [^3H]Ins(1,4,5) P_3 , but effects of Li^+ on the latter may have been obscured by the demonstrated, concomitant and equal stimulated accumulation of [^3H]inositol 1:2cyclic,4,5-trisphosphate. These data suggest that receptor-mediated PLC activity is selectively impaired by Li^+ as a secondary consequence of inositol monophosphatase inhibition in cells which are highly dependent on inositol re-cycling, but imply that, although Li^+ attenuation of PLC activity correlates closely with parameters indicative of limiting inositol supply, it is not readily attributed to decreased PtdIns P_2 availability. The potential for complex regulation of PLC and PtdIns synthase is discussed.

INTRODUCTION

Many ligands which act via cell-surface receptors exert their effects by regulating the metabolism of inositol phospholipids through the activation of either a phosphoinositide (PI)-specific 3-kinase or phospholipase C (PLC) [1]. The former mechanism is yet to be fully elucidated, although it seems probable that activation of PI 3-kinase results in the phosphorylation of PtdIns(4,5) P_2 to PtdIns(3,4,5) P_3 [2,3], and that this may act as a second messenger [4,5], perhaps by regulating the activity of the Ca^{2+} - and phospholipid-independent form of protein kinase C, PKC ζ [6]. In contrast, receptor regulation of PLC is well characterized, and the hydrolysis of PtdIns(4,5) P_2 by this enzyme is known to release two second messengers, Ins(1,4,5) P_3 , which evokes release of Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C [7]. As the metabolism of Ins(1,4,5) P_3 may also produce additional intracellular mediators [8], it is clear the PtdIns(4,5) P_2 may represent the common precursor for multiple signal molecules.

Cellular concentrations of PtdIns(4,5) P_2 appear to be tightly controlled, as its stimulated hydrolysis by PLC is closely coupled to replenishment from PtdIns4 P and PtdIns [9]. In turn, re-synthesis of PtdIns is accomplished by PtdIns synthase from CMP-phosphatidate (CMP-PA) and free inositol, the ultimate products of the two cycles which function to inactivate diacyl-

glycerol and Ins(1,4,5) P_3 respectively. The second of these two cycles involves the successive dephosphorylation of Ins(1,4,5) P_3 to inositol via numerous intermediates and is inhibited at several steps by Li^+ (see [10]). This effect of Li^+ is most pronounced at the level of inositol monophosphatase, the enzyme responsible for the dephosphorylation to inositol both of the inositol monophosphates produced by degradation of Ins(1,4,5) P_3 and that produced as an intermediate in the synthesis of inositol *de novo*. Li^+ ions may thus severely decrease cellular inositol concentrations and disrupt PI-dependent signalling as a secondary consequence. As Li^+ acts uncompetitively to inhibit inositol monophosphatase [11], with more profound blockade apparent at higher substrate concentrations, its influence will be most effectively translated in systems with highly or abnormally active PI turnover, and the proposal [12] that the ability of Li^+ to damp signalling in such systems may be the basis of its therapeutic capacity in the treatment of manic-depression has received considerable attention [13–16].

A major feature of Li^+ action implied by its therapeutic usefulness is its apparent cellular selectivity, which is unexpected, given the ubiquity of PI-signalling pathways. Selectivity may arise in part through the uncompetitive mode of action of Li^+ (see [15]), but is also likely to be directed by other factors [16]. Prominent amongst these will be the dependence of different systems on distinct mechanisms of cellular inositol homeostasis,

Abbreviations used: Ins1:2cyclic(4,5) P_3 , inositol 1:2cyclic,4,5-trisphosphate; InsPs, inositol phosphates as specified; PI, phosphoinositide(s); PLC, phospholipase C; CMP-PA, CMP-phosphatidate; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid.

* To whom correspondence should be addressed.

as those cells which rely heavily on $\text{Ins}(1,4,5)\text{P}_3$ degradation and/or synthesis *de novo* for maintenance of inositol concentrations may be particularly vulnerable to the effects of Li^+ , whereas those which can support adequate PtdIns synthesis through their capacity to gain access to extracellular inositol may be protected.

1321N1 astrocytoma cells, a human cell line previously used extensively to characterize receptor-stimulated PI turnover [17–20], have recently been shown to express an active inositol transporter which allows these cells to accumulate a large cellular inositol reserve [21]. In the present study we have taken advantage of this system by culture of these cells in media with high or low inositol concentrations to examine the influence of cellular inositol supply and the relationship between this and the capacity of Li^+ to impair muscarinic-receptor-mediated inositol lipid metabolism.

MATERIALS AND METHODS

Materials

Tissue-culture materials were from Gibco. $\text{D-myo-[2-}^3\text{H]inositol 1,4,5-trisphosphate}$ (21 Ci/mmol), $\text{D-myo-[2-}^3\text{H]inositol 1,3,4-trisphosphate}$ (17 Ci/mmol) and $\text{myo-[2-}^3\text{H]inositol}$ (~ 18 – 24 Ci/mmol) were from New England Nuclear or Amersham. AG1X8 resin (200–400 mesh, formate form) was from Bio-Rad. All other reagents were of analytical grade and obtained from BDH, Aldrich, Sigma or as specified below.

Cell culture

Stock cultures of 1321N1 astrocytoma cells were seeded at ~ 10000 cells/ cm^2 in 80 cm^2 flasks and maintained in 20 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) foetal-calf serum, fungizone (2.5 $\mu\text{g/ml}$) and either gentamycin (10 $\mu\text{g/ml}$) or penicillin + streptomycin (100 units/ml + 100 $\mu\text{g/ml}$ respectively) at 37°C in a humidified atmosphere with CO_2 at 5% [21]. Cells were sub-cultured weekly and medium was replaced at 3–4 day intervals.

Assays of inositol lipid metabolism were performed with cells grown in either 24-multiwell plates or small (3 cm diameter) Petri dishes and cultured either in DMEM containing (replete) or lacking (depleted) inositol. The method for culture of 1321N1 cells under inositol-depleting conditions was based on that described previously [22]. At subculture, growth medium was aspirated and cell monolayers were washed briefly once each with 10 ml of serum-free DMEM with or without inositol and 8 ml of ice-cold trypsin/EDTA in modified Puck's saline A. The latter was aspirated and cells were incubated for ~ 10 min at room temperature. Detached cells were collected by resuspension at $\sim 1 \times 10^6/\text{ml}$ in serum-free DMEM with or without inositol, and then seeded either as previously for maintenance of stocks or diluted to $\sim 1 \times 10^5$ cells/ml in DMEM with or without inositol-containing serum, fungizone and antibiotics as above, except that when inositol-free DMEM was used this was complemented with dialysed serum. After dilution, cells were seeded at 1 ml/well and 4 ml/dish respectively into either 24-well plates or Petri dishes and grown to confluence over ~ 6 days, with one intervening change of medium after 3 days. Confluent monolayers were then incubated in fresh medium supplemented with 1 – $10\text{ }\mu\text{Ci/ml}$ [^3H]inositol for up to 4 days before analysis of [^3H]labelled metabolites or assay of [^3H]inositol lipid hydrolysis.

Assay of [^3H]inositol lipid hydrolysis

(a) Standard procedure

Routine assays were performed with cells grown in multiwell plates. [^3H]Inositol-labelling medium was aspirated, and cell monolayers were washed briefly with $4 \times 2\text{ ml}$ of Hepes-buffered modified Krebs–Henseleit buffer (118 mM NaCl, 4.69 mM KCl, 1.18 mM MgSO_4 , 1.29 mM CaCl_2 , 1.18 mM KH_2PO_4 , 11.67 mM glucose, 25 mM Hepes, adjusted to pH 7.4 with NaOH) to remove residual extracellular radiolabel. Cells were allowed to equilibrate at 37°C for ~ 30 – 60 min in ~ 0.4 – 0.5 ml of the same buffer, and reactions were then either terminated or continued at 37°C for the times indicated after addition of drugs or buffer vehicle (10–60 μl) to give a final incubation volume of 0.5 ml. Incubations were normally performed in triplicate or duplicate. Reactions were stopped by rapid aspiration of the incubation medium and subsequent addition of 0.6 ml of ice-cold 0.5 M trichloroacetic acid (TCA). Cell debris was collected immediately, and the contents of individual wells, together with a further 0.25 ml wash with ice-cold 0.5 M TCA to collect residual material, were transferred to microfuge tubes and stored on ice until all incubations were completed. Samples were then briefly centrifuged and the resulting supernatants or pellets analysed for [^3H]inositol and phosphates or [^3H]lipids respectively.

Samples (0.8 ml) of supernatant were vigorously mixed with 0.2 ml of 10 mM EDTA (pH 7.5 with NaOH) and 0.8 ml of 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine (1:1, v/v) to remove acid as described previously [23] and centrifuged to aid phase separation. Samples of upper phase (0.9 ml) were then either analysed directly on AG1X8 or pooled from triplicate incubations and the pH adjusted to ~ 6 – 7 by addition of 0.3 ml of 100 mM NaHCO_3 , and samples were stored at 4°C until analysis by Dowex and/or h.p.l.c. anion-exchange chromatography as described below.

Residual supernatant was aspirated from the pelleted cell debris, and pellets were washed with $2 \times 1\text{ ml}$ of 5% (w/v) TCA containing 1 mM EDTA before extraction of [^3H]inositol lipids for ~ 15 – 30 min into 0.75 ml of methanol/chloroform/12 M HCl (80:40:1, by vol.). Aqueous and organic phases were then separated by addition of 0.25 ml of chloroform and 0.45 ml of 0.1 M HCl, followed by brief centrifugation. Portions (0.2–0.4 ml) of lower phase were either counted directly for radioactivity with 5 ml of Optiphase HiSafe 3 (LKB) as a measure of total lipid ^3H or mixed with 100 μg of Folch fraction 1 (Sigma) to act as carrier and then analysed for their separate [^3H]constituents after deacylation as described below.

(b) Modified procedure

For comparative measurements of $\text{Ins}(1,4,5)\text{P}_3$ and inositol 1:2cyclic,4,5-trisphosphate [$\text{Ins}1:2\text{cyclic}(4,5)\text{P}_3$], cells were cultured in individual 3 cm dishes to facilitate rapid sample handling and provide a greater mass of cellular material for analysis, and a modified extraction method, adapted from that described by [24], was used, as $\text{Ins}1:2\text{cyclic}(4,5)\text{P}_3$ is acid-labile. Incubations were performed as described above, except that volumes were increased 4-fold. Reactions were stopped by addition of either (i) 1.5 ml of TCA and immediate transfer on to ice as above or (ii) 1.5 ml of hot water ($> 80^\circ\text{C}$) and immediate transfer to a water bath at $> 80^\circ\text{C}$ for 5–10 min. Portions (50 μl) of sodium phytate (4 mg/ml, adjusted to pH 7.0 with acetic acid) were then added to all samples, which were subsequently cooled on ice for ~ 10 – 15 min. Cell debris was collected plus a further 0.5 ml wash of each dish with TCA or water, then pelleted, and [^3H]lipids were extracted from the pellets as above. Supernatants (2 ml)

Figure 1 Comparative labelling of PI in inositol-replete and -depleted cells

Inositol-replete (●) and -depleted (○) cells were grown as described in the Materials and methods section, and then incubated in medium supplemented with [³H]inositol (2 μCi/ml) for the indicated times before extraction and analysis of [³H]lipids. The data are presented as the relative proportion of radiolabelling of each [³H]lipid achieved in inositol-replete or -depleted cells by day 4. Values representing 100% for PtdIns, PtdInsP and PtdInsP₂ in replete or depleted cells respectively are 140729 ± 7566, 7741 ± 639 and 6228 ± 335, or 2711639 ± 103342, 169516 ± 14462 and 168256 ± 11546 d.p.m./well of cells, and are means ± S.E.M. of 3 paired experiments. Where not indicated, S.E.M. is within the symbol shown. A significant increase from labelling at the previous point in replete or depleted cells respectively is denoted by * or **.

were treated as in (a) with volumes increased proportionately, except that neutral extracts were mixed with 1,1,2-trichlorotrifluoroethane without tri-*n*-octylamine. Neutralized samples were stored at 4 °C until analysis by h.p.l.c.

Deacylation

[³H]Lipids were deacylated by a modification of the method described by [25]. Briefly, dried extracts were dissolved in 0.75 ml of methanol/chloroform (4:1, v/v), and 25 μl of 100 mM EDTA (pH 7.5 with NaOH) and 40 μl of 1.2 M NaOH in 50% methanol were added. Samples were incubated at 37 °C for 15 min, then neutralized with 50 μl of 1.0 M acetic acid. Phases were partitioned by addition of 1.5 ml of methanol/chloroform (1:9, v/v), 0.75 ml of 2-methylpropan-1-ol and 1.5 ml of water. After brief centrifugation, samples (2 ml) of upper phase were diluted with 5 ml of water and analysed on AG1X8 resin as described below.

Mass measurement of inositol phospholipid

Lipid extracts prepared in the absence of carrier were hydrolysed overnight at 4 °C with 50% (v/v) HF, and the free inositol released was separated and quantified respectively by g.l.c. and mass spectrometry as described previously [26].

Chromatography

(a) On Dowex AG1X8

[³H]Inositol phosphates and [³H]glycerophosphoinositol phosphates were separated on columns comprising 1 ml of 50% (w/v) slurry of AG1X8 resin as described previously [27,28]. In some experiments a combined [³H]InsP₁₋₄ fraction was collected by elution as for [³H]InsP₄. Samples (10 ml) of relevant fractions were counted for ³H radioactivity after addition of 10 ml of scintillant prepared by dissolving 15 g of 2,5-diphenyloxazole ('PPO') and 0.375 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene ('POPOP') in 2 litres of xylene, followed by dilution to 4 litres with Triton X-100.

(b) By h.p.l.c.

Separation of [³H]InsP₃ isomers was achieved on a 25 cm Partisphere (5 μM) SAX column equipped with matching guard cartridge (LaserChrom) eluted with the following gradient to 1.0 M (NH₄)H₂PO₄, adjusted to pH 3.8 with H₃PO₄ (B), at a flow rate of 1.0 ml/min: 0 min, 0%B; 10 min, 0%B; 115 min, 100%B; 120 min, 100%B. Samples (1.5–2.0 ml) were routinely spiked with nucleotides as a guide to column performance and reproducibility as previously described [29], and the column eluate was monitored continuously at 254 nm. Fractions (0.33 ml) were collected over the appropriate section of the gradient and counted for ³H radioactivity with 3 ml of FloScint IV (Packard). ³H-labelled metabolites are identified by comparison of retention times for samples and standards determined in separate runs. Standard [³H]Ins(2,4,5)P₃ was prepared in mixture with [³H]Ins(1,4,5)P₃ from a total lipid extract from [³H]inositol-labelled 1321N1 cells by deacylation and alkaline hydrolysis as previously described [30].

Expression of data

Results are expressed as ³H-labelled metabolites per well or dish of cells. One well of cells corresponds to ~ 300–350 μg (inositol-replete) or ~ 250–300 μg (inositol-depleted) protein and is equivalent to a cell volume of ~ 1.5–2.0 μl. Protein and cell volume were measured as described previously [21]. Where indicated, data were analysed by comparison of the paired values obtained over the indicated number of experiments by using Student's *t* test. Values were taken as significant at *P* ≤ 0.05.

RESULTS

Effects of low-inositol medium on cellular inositol and PI concentrations

Figure 1 compares the [³H]inositol labelling of phosphoinositide pools in cells cultured in DMEM containing (replete) or lacking

Figure 2 Comparative ability of inositol-replete and -depleted cells to sustain carbachol-stimulated PI turnover in the presence of Li^+

Inositol-replete (a, c) and -depleted (b, d) cells were prelabelled with $[^3\text{H}]\text{inositol}$ ($1 \mu\text{Ci}/\text{ml}$) for 3 days, and then incubated for the times indicated with 10 mM LiCl in the absence (○) or presence (●) of 1 mM carbachol before extraction and assay of total $[^3\text{H}]\text{InsP}_{1-4}$ (a, b) or $[^3\text{H}]\text{lipids}$ (c, d). Results are expressed as a proportion of agonist-stimulated $[^3\text{H}]\text{InsPs}$ accumulation at 90 min (a, b) or of $[^3\text{H}]\text{lipid}$ labelling at zero time (c, d). Data are means of three paired experiments performed in triplicate. Values representing 100% for $[^3\text{H}]\text{InsPs}$ and $[^3\text{H}]\text{lipid}$ in inositol-replete or -depleted cells respectively are 121985 ± 1651 and 61594 ± 623 or 1245406 ± 54345 and 1425682 ± 74031 (means $\pm$ S.E.M.). Comparison of the rates of stimulated $[^3\text{H}]\text{InsPs}$ accumulation between 60 and 90 min as a fraction of that between 0 and 15 min indicates a significantly slower secondary rate in both replete and depleted cells, but a significantly greater fall in secondary rate in the latter. Similar data were also obtained in further experiments.

(depleted) normal inositol ($40 \mu\text{M}$). In cells cultured under inositol-depleting conditions, labelling of PtdIns , PtdInsP and PtdInsP_2 each achieved steady state within 2 days, whereas in inositol-replete cells, labelling approached steady state only after 3–4 days. The relative level of $[^3\text{H}]\text{inositol}$ incorporation into each phosphoinositide was ~ 20 -fold greater under the former conditions, as reported previously [22], although the distribution of radiolabel between lipids was not significantly different, with PtdInsP and PtdInsP_2 each representing ~ 4 – 6% of total label in both depleted and replete cells by 3–4 days. This suggests that culture of 1321N1 cells in low-inositol medium does not in itself modify phosphoinositide synthesis. This point was confirmed directly by mass assay of total inositol phospholipid, which indicated concentrations of 2.04 ± 0.26 and $3.12 \pm 0.46 \text{ nmol/well}$ of cells ($n = 3$ independent experiments) in inositol-replete and -depleted cells respectively. Thus differences in phosphoinositide labelling must reflect a much higher specific radioactivity (~ 750 – 1000 d.p.m./pmol for $\text{PtdIns} \approx 2.5 \text{ nmol}$) in inositol-depleted cells.

In spite of this, cells cultured in low-inositol medium accumulated ~ 10 – 20 -fold less radiolabel as free $[^3\text{H}]\text{inositol}$ than did those grown in normal DMEM. Thus in inositol-replete and -depleted cells $[^3\text{H}]\text{inositol}$ was $2195 \pm 159\%$ (mean $\pm$ S.E.M. of

6 experiments) and $8 \pm 1\%$ (mean $\pm$ S.E.M. of 49 experiments) $[^3\text{H}]\text{PtdIns}$ respectively, indicating that culture in medium lacking added inositol decreases cellular concentrations by a factor of ~ 250 . Over many experiments the proportionate labelling of $[^3\text{H}]\text{inositol}$ in depleted cells varied from ~ 1 to 50% $[^3\text{H}]\text{PtdIns}$, though most typically was as indicated. Our previous studies [21], supported by the present data, suggest that in normal DMEM 1321N1 cells express intracellular inositol concentrations of $\sim 20 \text{ mM}$. Under depletion conditions these are likely to fall to ~ 0.1 – 0.5 mM .

Effects of receptor stimulation in inositol-depleted and -replete cells

Figure 2 compares muscarinic-receptor-stimulated activation of PLC in inositol-replete and -depleted cells and the corresponding effects on $[^3\text{H}]\text{PI}$ concentrations. PLC activity was assayed by accumulation of total $[^3\text{H}]\text{InsP}_{1-4}$ ($[^3\text{H}]\text{InsPs}$) in the presence of Li^+ both to trap these at the level of $[^3\text{H}]\text{InsP}_1$ and to inhibit recycling to the free inositol pool. Exposure of inositol-replete cells to a maximally effective concentration of carbachol evoked a large stimulated accumulation of $[^3\text{H}]\text{InsPs}$ which was linear for 45–60 min, implying a continuous stimulated rate of PLC activity, as previously reported in this system [17,18]. Between 60 and 90 min this rate declined to $58.3 \pm 4.7\%$ (mean $\pm$ S.E.M. of 6 experiments) of that observed over the initial 15 min, presumably reflecting a modest receptor desensitization. The latter phenomenon has not previously been reported for this system, and it is probable that the shorter pre-labelling with $[^3\text{H}]\text{inositol}$ previously employed [17,18] allowed increases in $[^3\text{H}]\text{InsPs}$ specific radioactivity which off-set the effects noted here.

Carbachol also promoted a large increase in $[^3\text{H}]\text{InsPs}$ in inositol-depleted cells, with an initial linear rate (60 – 70 pmol/min per well of cells) of accumulation similar to that in inositol-replete cells, implying that these systems do not differ in receptor, G-protein or PLC expression or coupling. However, in inositol-depleted cells this rate was sustained for only 15–30 min and then declined dramatically, falling to $10.5 \pm 1.1\%$ (mean $\pm$ S.E.M. of 43 experiments, with values between 0 and 30%) of the initial rate by 60–90 min. This marked difference in the responses of inositol-replete and -depleted cells is also reflected by the effects of agonist on total $[^3\text{H}]\text{PI}$ concentrations, as shown in Figures 2(c) and (d). Thus, although concentrations of $[^3\text{H}]\text{lipid}$ were largely unaffected by carbachol in inositol-replete cells, those in depleted cells were substantially decreased, falling by $54.8 \pm 2.1\%$ (mean $\pm$ S.E.M. of 49 experiments) after 90 min compared with control (Li^+ alone). Comparison of Figures 2(b) and 2(d) further shows that changes in $[^3\text{H}]\text{PI}$ concentrations mirrored those in $[^3\text{H}]\text{InsPs}$ accumulation in depleted cells, suggesting that the implied diminished capacity of these cells to sustain persistent PLC activation is a consequence of decreased lipid supply. These data also demonstrate that carbachol evokes turnover of the equivalent of the total cellular $[^3\text{H}]\text{inositol}$ lipid pool ~ 1 – 1.5 times/h, and the results from inositol-depleted cells unequivocally show that $> 50\%$ of cellular $[^3\text{H}]\text{lipid}$ participates in this response.

Inhibition of inositol re-cycling by Li^+ and effects on PLC activity

Figure 3 provides a comprehensive comparison of the effects of muscarinic-receptor stimulation on PI metabolism in inositol-depleted cells in the presence and absence of Li^+ . Carbachol alone promoted a several-fold rise in the concentration of $[^3\text{H}]\text{inositol}$ and each $[^3\text{H}]\text{InsP}_{1-4}$, with the most dramatic early changes apparent in $[^3\text{H}]\text{inositol}$ polyphosphates, implying that

Figure 3 Effects of Li⁺ on muscarinic-receptor-mediated PI turnover in inositol-depleted cells

Inositol-depleted cells, pre-labelled with [³H]inositol (2 μ Ci/ml) for 3 days, were incubated for the times indicated in the absence (results not shown) or presence (□) of 10 mM LiCl or with 1 mM carbachol (●) or carbachol + LiCl (○) before extraction and analysis of ³H-labelled metabolites: (a) inositol, (b) InsP₁, (c) InsP₂, (d) InsP₃, (e) InsP₄, (f) PtdIns, (g) PtdInsP, (h) PtdInsP₂. Data are means $\pm$ S.E.M. of triplicate incubations in a single experiment broadly representative of 11 (see text). Where not indicated, S.E.M. is within the symbol shown. Control values in the absence of both LiCl and agonist are omitted for clarity, but paired comparison between these values at 0 and 90 min across all experiments indicated small, significant, increases in [³H]InsP₄, [³H]PtdInsP and [³H]PtdInsP₂ of 13.4 ± 4.9 , 7.2 ± 3.2 and 13.9 ± 4.3 % respectively. Similar comparison of control values with those obtained in the absence and presence of LiCl alone at 90 min indicated small, significant, LiCl-dependent increases in [³H]PtdInsP and [³H]PtdInsP₂ of 8.8 ± 1.7 and 5.9 ± 3.5 % respectively.

receptor-mediated PI turnover in this system occurs through cleavage of polyphosphoinositides, as reported previously [20]. In the continued presence of agonist, concentrations of [³H]inositol increased further, whereas those of [³H]InsP₁₋₄ achieved new steady-state values by ~ 15 min and then remained elevated. Thus, by 90 min stimulated increases above basal in [³H]InsP₁, [³H]InsP₂, [³H]InsP₃ and [³H]InsP₄ were 87.7 ± 3.9 %, 79.3 ± 5.8 %, 59.4 ± 4.4 % and 72.3 ± 2.7 % (means $\pm$ S.E.M. of 10 experiments) respectively of those measured at 15 min. Addition of the muscarinic-receptor antagonist atropine (10–100 μ M) between 15 and 90 min caused concentrations of each [³H] metabolite to return toward basal values (results not shown). These results suggest that muscarinic-receptor activation in the absence of Li⁺ evokes continuous turnover of inositol lipids and is subject to only limited desensitization.

In the presence of Li⁺, this profile was drastically altered. Stimulated increases in [³H]inositol were prevented, and in some experiments concentrations actually declined, whereas initial stimulated [³H]InsP₁ accumulation was dramatically potentiated, demonstrating inhibition of inositol monophosphatase. Li⁺ also enhanced initial agonist-evoked increases in the concentrations of [³H]InsP₂ and [³H]InsP₃, but did not influence [³H]InsP₄ concentrations. These results are consistent with the inhibition of Ins(1,3,4)P₃/Ins(1,4)P₂ 1-phosphatase by Li⁺ [31]. Control levels of ³H-labelled metabolites were largely unaffected by Li⁺, suggesting a very much slower resting rate of turnover. Consistent with the data in Figure 2, however, after ~ 30 min in the continued presence of agonist and Li⁺, the rate of [³H]InsP₁ accumulation declined markedly, suggesting a diminished rate of PLC activity. This was accompanied by a fall in the stimulated concentrations of [³H]inositol polyphosphates, particularly

[³H]InsP₃ and [³H]InsP₄, although the extent of this effect varied between experiments. A similar secondary attenuation of inositol polyphosphate accumulations by Li⁺ has been reported previously in several systems [32–37], and in Chinese-hamster ovary (CHO) cells parallels a decreased rate of stimulated [³H]InsP₁ accumulation [38].

Figure 3 also correlates these effects of Li⁺ with those on [³H]PI concentrations. In stimulated cells, concentrations of [³H]PtdIns fell dramatically in the presence, but much less so in the absence, of Li⁺ over the first 30–60 min and then approached a new steady state. Importantly, stimulation in the presence of Li⁺ also severely decreased concentrations of [³H]PtdInsP, but influenced those of [³H]PtdInsP₂ to a much lesser extent. Thus, over several paired experiments, concentrations of [³H]PtdIns, [³H]PtdInsP and [³H]PtdInsP₂ were decreased to 81.6 ± 2.2 %, 83.8 ± 3.4 % and 92.9 ± 3.5 % or 39.2 ± 3.1 %, 47.7 ± 4.5 % and 81.0 ± 4.0 % (means $\pm$ S.E.M. of 10–11 experiments) in the presence of carbachol or carbachol + Li⁺ respectively relative to appropriate controls in the absence or presence of Li⁺. Decreases in each [³H]lipid in response to both agonist and agonist + Li⁺ were significant by comparison with the relevant control, and were significantly greater in response to carbachol + Li⁺ than to carbachol alone. These effects are similar to others reported previously [39,40].

The relationship between Li⁺ inhibition of inositol monophosphatase and the secondary consequences of this on stimulated PLC activity in inositol-depleted cells is further examined in Figure 4. This shows that the accumulation of total [³H]InsP₁₋₄ in response to carbachol and Li⁺ approached a new steady state after ~ 60 min, but reveals that, when a large excess of atropine was then added to prevent continued muscarinic-receptor ac-

Figure 4 Decrease in muscarinic-receptor-mediated PLC activity by Li^+ in inositol-depleted cells

Inositol-depleted cells pre-labelled with $[^3\text{H}]\text{inositol}$ ($2 \mu\text{Ci}/\text{ml}$) were incubated in buffer containing 10 mM LiCl and in the absence ($\square$) or presence ($\bullet$) of 1 mM carbachol. After 60 min , incubations were either stopped or continued after addition of buffer ($\bullet$) or $100 \mu\text{M}$ atropine ($\blacktriangledown$) before extraction and analysis of a fraction containing $[^3\text{H}]\text{InsP}_3$. Data represent the means of duplicate incubations within a single experiment representative of 9. The rate shown as $\blacktriangledown$ defines residual PLC activity (see the text).

tivation, the concentration of pre-accumulated $[^3\text{H}]\text{InsPs}$ declined at a slow, continuous, rate for up to 60 min . Since at steady state product formation and degradation are equal, the rate of decline immediately after antagonist addition gives a measure of both the rate of $[^3\text{H}]\text{InsPs}$ removal through phosphatase activity and the equal rate of continued $[^3\text{H}]\text{InsPs}$ release by PLC activity immediately before receptor blockade [41–43]. Thus the rate of $[^3\text{H}]\text{InsPs}$ removal after atropine treatment approximates to the residual rate of PLC after 60 min previous exposure to carbachol + Li^+ . The initial stimulated activity of this enzyme is given by the increased accumulation of $[^3\text{H}]\text{InsPs}$ over the first 15 min of exposure to agonist. Comparison of these two rates shows that after 60 min in the combined presence of carbachol and Li^+ the stimulated activity of PLC is decreased to $23.6 \pm 2.2\%$ (mean $\pm$ S.E.M. of 9 experiments) of that immediately after receptor activation. Although it should be recalled that a component of this decreased activity may reflect receptor desensitization, the data in Figures 2(a) and 2(b) clearly emphasize the extent to which decreased $[^3\text{H}]\text{InsPs}$ formation is directly attributable to a limiting inositol supply. The present data are thus the first in any system to demonstrate directly a diminished rate of receptor-stimulated PLC by Li^+ as a secondary consequence of inositol monophosphatase inhibition.

Effects of Li^+ on InsP_3 concentrations in inositol-depleted cells

An expected consequence of diminished PLC activity is decreased $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol concentrations. Figure 5 therefore compares the effects of Li^+ on carbachol-stimulated accumulation of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ and $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ in inositol-depleted cells. Agonist alone evoked a rapid increase in the concentrations of both $[^3\text{H}]\text{InsP}_3(\text{s})$, as previously reported [20], and these early changes were then largely sustained. However, it will be noted that, although stimulated concentrations of both $[^3\text{H}]\text{InsP}_3(\text{s})$ were similar in the absence of Li^+ , its very much lower basal level makes $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ a more sensitive indicator of receptor activation. In the presence of Li^+ , stimulated concentrations of

Figure 5 Effects of Li^+ on carbachol-stimulated $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ and $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ accumulation

Inositol-depleted cells labelled with $[^3\text{H}]\text{inositol}$ ($2 \mu\text{Ci}/\text{ml}$) were incubated in the absence and presence ($\square$) of LiCl (10 mM) or with 1 mM carbachol ($\bullet$) or 1 mM carbachol + LiCl ($\circ$) before extraction and analysis of $[^3\text{H}]\text{InsP}_3(\text{s})$ by h.p.l.c. Data are broadly representative of values obtained in 3 experiments, and derive from the same experiment illustrated in Figure 3, although extracts from triplicate incubations were pooled before h.p.l.c. analysis. Values for control incubations run in the absence of LiCl and carbachol are omitted for clarity, but see the legend to Figure 3.

$[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ were initially enhanced, indicating inhibition of $\text{Ins}(1,3,4)\text{P}_3/\text{Ins}(1,4)\text{P}_2$ 1-phosphatase [31], but after 30 min declined sharply. This secondary effect is similar to that previously reported in other systems [33,35] and shows close temporal correlation with attenuated PLC activity and decreased $[^3\text{H}]\text{phosphoinositide}$ concentrations. Surprisingly, however, stimulated concentrations of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ were not notably affected by Li^+ .

A probable explanation of this last result is revealed by the data presented in Table 1 and Figure 6. These illustrate the results of experiments in which the extraction of $[^3\text{H}]\text{InsPs}$ was modified to facilitate intact recovery of metabolites containing an inositol 1:2cyclic diester phosphate. These are released as minor side-products during hydrolysis of phosphoinositides by various PLC(s) [44–46]. Cleavage of $\text{PtdIns}(4,5)\text{P}_2$ releases a mixture of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}1:2\text{cyclic}(4,5)\text{P}_3$ [45,46]. The latter is only slowly metabolized by $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase [47] and can accumulate in cells during persistent stimulation [24,48], but is acid-labile, releasing a mixture of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(2,4,5)\text{P}_3$ [24,49].

Figure 6 shows the separation of each of these $[^3\text{H}]\text{InsP}_3(\text{s})$ by h.p.l.c. $[^3\text{H}]\text{Ins}1:2\text{cyclic}(4,5)\text{P}_3$ is identified both by its retention relative to $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ [24] and by virtue of its presence in neutral extracts from stimulated cells, but not in acid extracts, which show proportionately greater $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ and

Figure 6 H.p.l.c. separation of carbachol-sensitive [³H]InsP₃

Cells pre-labelled with [³H]inositol as described in the legend to Table 1 were incubated for 30 min with or without carbachol (1 mM), and [³H]-labelled metabolites were then extracted under neutral or acidic conditions as described in the Materials and methods section and analysed by h.p.l.c. The traces shown are for stimulated cells after subtraction of corresponding controls (see Table 1). All samples also contained a further unidentified [³H]InsP₃, which was eluted between Ins1:2cyclic(4,5)P₃ [c(1,4,5)] and Ins(1,3,4)P₃, but whose concentration was unaffected by agonist and/or LiCl. Data are from one experiment representative of three.

[³H]Ins(2,4,5)P₃. Table 1 presents a quantitative comparison of the relative concentrations of these products extracted under neutral and acidic conditions from control and stimulated cells incubated with and without Li⁺. Both methods gave similar

amounts of total [³H]InsP₃ for each incubation, indicating that each achieves similar extraction. The data for neutrally extracted samples show that, although concentrations of [³H]Ins(1,3,4)P₃ and [³H]Ins(1,4,5)P₃ increased rapidly on stimulation, with changes in the former potentiated by Li⁺, concentrations of [³H]Ins1:2cyclic(4,5)P₃ also increased several-fold. Although apparently unaffected by Li⁺, concentrations of the latter also continued to rise between 1 and 30 min, implying a relatively slow rate of turnover, as expected from previous studies [24]. It is also notable that basal concentrations of [³H]Ins(1,4,5)P₃ far exceeded those of its cyclic counterpart. Table 1 further shows that after 30 min exposure to carbachol with or without Li⁺, ~50% of the total stimulated increment in [³H]Ins(1,4,5)P₃ + [³H]Ins1:2cyclic(4,5)P₃ is present in the latter form. This may account for the apparent failure of Li⁺ to diminish stimulated [³H]Ins(1,4,5)P₃ concentrations, as the co-accumulation of slowly metabolized [³H]Ins1:2cyclic(4,5)P₃ may obscure such effects. Moreover, the high basal levels revealed by Table 1, together with the rapid stimulated turnover of Ins(1,4,5)P₃ in 1321N1 cells [20], which limit accumulation of this second messenger, suggest that it may be difficult to correlate directly Li⁺-dependent decreases in PLC activity and Ins(1,4,5)P₃ concentration in this system.

DISCUSSION

Studies in numerous systems have demonstrated the ability of Li⁺ to influence various aspects of stimulated inositol phospholipid metabolism in a manner compatible with the inhibition of inositol monophosphatase as the primary action of this ion [13–15]. Among these effects of Li⁺ are decreased cellular inositol concentrations [50,51], impaired PI synthesis [39,40], decreased release of inositol polyphosphates [32–37] and inositol-reversible inhibition of functions controlled through PI-coupled receptors [52].

The present study has correlated many of these and additional features of Li⁺ action in a homogeneous cell line cultured under conditions specifically developed to this end and to address the influence of cellular inositol supply. The data show that culture of 1321N1 cells in medium lacking added inositol lowers cellular

Table 1 Effects of carbachol and/or LiCl on the accumulation of [³H]inositol cyclic and non-cyclic trisphosphates

Inositol-depleted cells were pre-labelled (2–3 days) with [³H]inositol (10 μCi/ml) as described in the Materials and methods section and then incubated for either 1 or 30 min in the absence or presence of LiCl (10 mM) and/or carbachol (1 mM) before extraction of [³H]InsPs under neutral (N) or acidic (A) conditions and analysis by h.p.l.c. Ins1:2cyclic(4,5)P₃ is c(1,4,5); other isomers are as indicated. The far right column indicates the proportion of the stimulated increase in the total of Ins1:2cyclic(4,5)P₃ + Ins(1,4,5)P₃ contributed by the former. Data are means ± S.E.M. of 3 experiments, with single incubations for each condition: ND, not detected.

Condition	Extraction	InsP ₃				% c(1,4,5)/increase c(1,4,5) + (1,4,5)
		c(1,4,5)	(1,3,4)	(1,4,5)	(2,4,5)	
1 min control	N	1457 ± 275	1965 ± 297	53188 ± 4201	723 ± 152	—
1 min Li ⁺	N	1504 ± 176	2591 ± 548	52899 ± 5231	966 ± 142	—
1 min carbachol	N	6517 ± 535	132404 ± 7408	97061 ± 17746	953 ± 133	15.5 ± 5.9%
1 min carbachol/Li ⁺	N	6619 ± 587	144231 ± 4117	101584 ± 6168	269 ± 269	9.4 ± 1.0%
30 min control	N	865 ± 447	1000 ± 502	47383 ± 4184	164 ± 164	—
30 min Li ⁺	N	1410 ± 55	1302 ± 73	54683 ± 6016	254 ± 184	—
30 min carbachol	N	32010 ± 1401	94681 ± 1903	83955 ± 11135	520 ± 265	48.0 ± 6.4%
30 min carbachol/Li ⁺	N	34985 ± 2457	188369 ± 14462	80096 ± 8380	1291 ± 110	57.1 ± 0.8%
30 min control	A	400 ± 190	667 ± 189	46264 ± 4322	4220 ± 406	—
30 min Li ⁺	A	ND	964 ± 75	46188 ± 4791	4674 ± 859	—
30 min carbachol	A	40 ± 40	90939 ± 3597	96207 ± 10618	10557 ± 88	—
30 min carbachol/Li ⁺	A	ND	182588 ± 4483	110484 ± 4315	12294 ± 1365	—

inositol concentrations from ~ 20 mM to < 0.5 mM, revealing the profound dependence of these cells on accumulation of normal cellular reserves by uptake from the medium via the active transporter characterized previously [21]. Under the same conditions, PI concentrations are unaffected, implying that the low residual inositol concentrations remain sufficient to support activity of PtdIns synthase at levels appropriate to resting rates of PtdIns turnover. [N.B. Continuous culture of 1321N1 cells through ~ 5 – 10 cell doublings in medium with inositol at < 20 nM caused inositol-preventable cell death.] It follows that the high concentrations of inositol (~ 120 nmol/mg of protein $\equiv 20$ mM [21]) normally expressed by these cells provide considerable spare capacity to fuel the accelerated activity of PtdIns synthase necessary to maintain PtdIns concentrations during maximal muscarinic-receptor-stimulated PI turnover (~ 10 nmol/h per mg of protein), particularly as estimates of the K_m of PtdIns synthase for inositol are in the range 0.05 – 5.00 mM (see [15]). The present data also show that, when these cellular inositol reserves are drained by prior depletion, 1321N1 cells are still able to maintain PtdIns concentrations at levels which are only modestly ($\sim 20\%$) decreased, even during receptor activation of PLC, clearly demonstrating the close coupling between stimulated lipid hydrolysis and re-synthesis permitted by efficient inositol re-cycling. When the latter is prevented by Li^+ , however, both PI concentrations and stimulated PLC activity are markedly attenuated in inositol-depleted, but not replete, cells. This selective effect strongly supports the views that Li^+ influences PI signalling via a mechanism which is secondary to a decreased substrate supply, and consequently that variation in cellular capacity for access to extracellular inositol may be a major factor directing the action of Li^+ against therapeutically beneficial targets [16].

The secondary dependence of decreased PLC activity and phosphoinositide re-synthesis in stimulated cells on a primary inhibition of inositol monophosphatase by Li^+ is evidently demonstrated by several aspects of the data presented and supporting observations. Firstly, the selectivity of these actions in inositol-depleted cells identifies a limiting inositol supply as a causal factor. Secondly, Li^+ dramatically potentiates stimulated $[^3\text{H}]\text{InsP}_1$ accumulation, but prevents the corresponding increase in free $[^3\text{H}]\text{inositol}$ concentrations observed in its absence in depleted cells, demonstrating profound monophosphatase inhibition. Thirdly, the time course for potentiation of $[^3\text{H}]\text{InsP}_1$ accumulation by Li^+ correlates closely with each of those for decreased $[^3\text{H}]\text{PI}$ and $[^3\text{H}]\text{inositol}$ polyphosphate concentrations, enhanced CMP-PA accumulation (results not shown) and impaired PLC activity. Finally, the potentiation of stimulated $[^3\text{H}]\text{InsP}_1$ and CMP-PA levels, the attenuation of $[^3\text{H}]\text{lipid}$ concentrations and the reversal of stimulated increases in $[^3\text{H}]\text{inositol}$ concentrations observed in 1321N1 cells all show a Li^+ dose-dependency (results not shown) characteristic of that established for inhibition of inositol monophosphatase [53,54]. It is thus clear that, in depleted cells, inhibition of monophosphatase by Li^+ prevents a stimulated rise in $[^3\text{H}]\text{inositol}$ concentration and so limits re-synthesis of PI.

These results imply that a stimulated increase in cellular inositol concentration is essential to allow accelerated PI re-synthesis, but other observations suggest that this is a permissive factor rather than the trigger for increased PtdIns synthase activity. In both replete and depleted cells the initial stimulated rate of PLC is similar, necessitating matched enhancement of lipid re-synthesis to maintain PI concentrations essentially unchanged as observed in the absence of Li^+ . As cellular inositol concentrations in replete cells are many times those in depleted cells and are unaffected by stimulation (results not shown),

increased PtdIns synthase activity must be regulated by other factors. Although the present data may be compatible with the regulation of this enzyme by product (PtdIns) inhibition in resting cells, they do not support the recent proposal [55] that relief of such inhibition accounts for enhanced PtdIns synthase activity during stimulation, as PI concentrations are unaffected by agonist in inositol-replete cells. Similarly, acceleration of PtdIns re-synthesis is not readily explained by changes in CMP-PA concentrations, which in both 1321N1 cells (results not shown) and other systems [15] are only markedly elevated when inositol concentrations are limiting, although greater localized changes cannot be excluded. Consequently, regulation of this enzyme by means other than substrate availability should not be discounted.

It is nevertheless clear that stimulation of inositol-depleted cells in the presence of Li^+ results in a marked fall in the concentrations of PtdIns and PtdInsP and that this occurs as a consequence of inadequate inositol supply. The modest contemporary effects of Li^+ on PtdIns P_2 concentrations ($\sim 10\%$ specific decrease) are reminiscent of similar effects in thyrotropin-releasing-hormone-stimulated and carbachol-stimulated GH $_3$ cells and parotid-gland slices respectively [39,40], but are unexpected, as they appear disproportionate to a much more marked attenuation (~ 70 – 80%) of receptor-stimulated PLC activity. As other studies currently in progress suggest that $\sim 50\%$ of each inositol lipid is accessible to muscarinic-receptor stimulation (I. H. Batty and C. P. Downes, unpublished work), these data may indicate that other lipids (e.g. PtdIns, PtdInsP, CMP-PA), whose concentrations are more dramatically altered in the presence of Li^+ , also influence PLC activity. Thus, although the observed effects of carbachol + Li^+ on concentrations of $[^3\text{H}]\text{Ins}(1,3,4)P_3$ are only readily compatible with a continued but secondarily decreased hydrolysis of PtdIns P_2 , it remains possible that hydrolysis of PtdIns and/or PtdInsP contributes to PLC activity. Alternatively, we propose that these lipids and/or other components of this system whose concentrations are selectively modified as a consequence of cellular inositol depletion during stimulation exert allosteric or other regulatory effects on agonist-stimulated PLC activity.

Despite the marked capacity of Li^+ to impair PLC activity, corresponding changes in the stimulated release of $\text{Ins}(1,4,5)P_3$, though clearly implied by effects on specific metabolites [e.g. $\text{Ins}(1,3,4)P_3$], were difficult to establish directly owing to the apparently restricted stimulated increase in its concentration and the simultaneous release of $\text{Ins}1:2\text{cyclic}(4,5)P_3$. Similar accumulation of this InsP_3 has also been reported previously [24,48]. As the catabolism of $\text{Ins}1:2\text{cyclic}(4,5)P_3$ after stimulation in parotid acinar cells is ~ 50 – 100 times slower than that of $\text{Ins}(1,4,5)P_3$ [24], the relative basal levels of these in 1321N1 cells may indicate that the $\text{Ins}(1,4,5)P_3$ present in resting and stimulated cells arises through distinct routes. We estimate the concentrations of $[^3\text{H}]\text{Ins}1:2\text{cyclic}(4,5)P_3$ and $[^3\text{H}]\text{Ins}(1,4,5)P_3$ in resting cells or those exposed to carbachol (with or without Li^+) for 30 min are ~ 50 nM and ~ 2 μM or ~ 1 μM and ~ 3 μM respectively. Since maximal release of Ca^{2+} from intracellular stores occurs at ~ 1 μM $\text{Ins}(1,4,5)P_3$ [56], the stimulated increment in $\text{Ins}(1,4,5)P_3$ in 1321N1 cells is clearly consistent with this function, and the present data may support the view discussed previously [57,58] that resting levels of $\text{Ins}(1,4,5)P_3$ are confined to a separate compartment such that the dynamic changes in the concentration of this second messenger on stimulation are more comparable with those of its co-product, $\text{Ins}1:2\text{cyclic}(4,5)P_3$.

The present results also allow the concentrations of $[^3\text{H}]\text{InsP}_1$ in inositol-depleted cells incubated as control or with agonist in the absence and presence of Li^+ to be estimated at ~ 30 – 40 μM ,

~ 100 μ M and ~ 1 mM respectively. Similar values have been reported in rat brain after Li⁺ treatment *in vivo* (see [14,53]), whereas previous studies using cell-free systems have shown that, at substrate concentrations ~ 0.1 mM, inositol monophosphatase is profoundly (> 90%) inhibited by 10 mM LiCl. The present data are clearly compatible with these observations and demonstrate the capacity of Li⁺ to impair markedly stimulated PLC activity under conditions in which InsP₁ concentrations are raised to levels similar to those which can be promoted by treatment *in vivo* with this ion.

In summary, the data presented describe a cellular system in which crucial, but previously poorly established, elements of the inositol-depletion hypothesis of Li⁺ action have been examined and correlated directly. This has been used to demonstrate that Li⁺ ions selectively decrease PI re-synthesis and receptor-mediated PLC activity in cells which are highly dependent on efficient inositol re-cycling and do so in a manner consistent with their primary inhibition of inositol monophosphatase. Contrary to expectation, however, impaired PLC activity is not readily attributed to Li⁺-dependent changes in PtdInsP₂ concentrations, and the present data may imply that activity both of this enzyme and of the PtdIns synthase responsible for compensatory PI re-synthesis are subject to complex regulation. In view of these observations, it will be of considerable future interest to examine the potential secondary consequences of Li⁺ action on signalling regulated through stimulation of PI 3-kinase.

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