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Disruption by Lithium of Phosphatidylinositol-4,5-bisphosphate Supply and Inositol-1,4,5-trisphosphate Generation in Chinese Hamster Ovary Cells Expressing Human Recombinant m_1 Muscarinic Receptors

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SUMMARY

Inhibitory effects of the anti-manic agent lithium on carbachol-stimulated phosphoinositide signaling have been investigated in Chinese hamster ovary (CHO) cells transfected with human m1 muscarinic receptor cDNA (B_{max}, 816 fmol/mg of protein). In the presence of Li^+, a time-dependent inhibition of inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] mass accumulation was observed within 10 min of agonist addition (IC_{50} for lithium inhibition at 20 min after carbachol addition, 0.5 mM). The Li^+-induced decrease in agonist-stimulated Ins(1,4,5)P_3 levels was preceded by a dramatic increase in CMP-phosphatidate accumulation. The idea that Li^+ blockade of inositol monophosphatase caused a rapid depletion of the cellular *myo*-inositol pool in CHO-m1 cells was supported by the reversal of Li^+ effects by exogenous *myo*-inositol. Carbachol (1 mM) alone caused a rapid and dramatic decrease in phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)-P_2]in CHO-m1 cells labeled to equilibrium with [^{3}H]-inositol. Carbachol-evoked decreases in PtdIns(4,5)P_2 were time-dependently accentuated by Li^+ (IC_{50} for Li^+ inhibition at 20 min after carbachol addition, 1.2 mM). Measurements of changes in PtdIns(4,5)P_2 mass demonstrated that the effect of Li^+ was completely and concentration-dependently reversed by addition of *myo*-inositol. Sequential 30-min periods of carbachol stimulation resulted in similar time courses of Ins(1,4,5)P_3 accumulation when an intervening 20-min recovery period was included in the protocol. Inclusion of Li^+ throughout resulted in a more rapid and dramatic attenuation of Ins(1,4,5)P_3 during the agonist rechallenge period, which could be correlated with accentuated changes in PtdIns(4,5)P_2. These data demonstrate that, although mechanisms operate to efficiently resynthesize PtdIns(4,5)P_2, the temporal correlation of carbachol-evoked decreases in PtdIns(4,5)P_2 levels in the presence of Li^+ strongly suggests that phosphoinositide-specific phospholipase C substrate depletion may be causal in the subsequent decrease in Ins(1,4,5)P_3 levels.

Although lithium has been used in the treatment of manic depression for many years, the therapeutic mechanism of action of this simple cation remains unknown. The discovery that Li^+, at concentrations comparable to those used therapeutically, can uncompetitively inhibit inositol monophosphatase (1) led Berridge *et al.* (2) to hypothesize that Li^+ might exert its anti-manic action through disruption of the phosphoinositide cycle in dysfunctional neuronal populations (2).

Subsequently, much evidence has been accumulated to support, refine, and expand this hypothesis (3, 4). Thus, it has been demonstrated that, by inhibiting agonist-stimulated inositol monophosphate hydrolysis, Li^+ also causes other effects on phosphoinositide cycle intermediates. In brain slice preparations the decreased availability of *myo*-inositol, due to inositol monophosphatase inhibition by Li^+, results in a dramatic accumulation of CMP-phosphatidate (5–7). This has been interpreted as evidence that cellular inositol levels decrease rapidly to limit PtdIns synthase activity (with consequent accumulation of the co-substrate CMP-phosphatidate) and therefore potentially to limit provision of substrate for PI-PLC activity (3, 4). Additional indirect evidence that Li^+ affects cellular levels of inositol phospholipids has come from the observation of time-dependent and *myo*-inositol-reversible decreases in agonist-stimulated Ins(1,4,5)P_3 and inositol-1,3,4,5-tetrakisphosphate accumulations (7–13).

Despite much evidence that Li^+ can disrupt inositol phospholipid synthesis and inositol polyphosphate generation in brain preparations, only small effects of Li^+ on levels of PtdIns, PtdIns(4)P, or PtdIns(4,5)P_2 have been reported (14–16), al-

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ABBREVIATIONS: PtdIns, phosphatidylinositol; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; PtdIns(4)P, phosphatidylinositol-4-phosphate; PtdIns-(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

though these can reach significance when high concentrations of Li^+ are used (17). This is perhaps not surprising, considering the heterogeneity of the brain preparations used; however, attempts to demonstrate Li^+ effects on agonist-stimulated changes in inositol phospholipids, and in particular PtdIns-(4,5)P_2, in more homogeneous cell systems have also been disappointing. Thus, although the presence of Li^+ caused marked decreases in PtdIns levels in angiotensin II-stimulated glomerulosa cells (18), decreased PtdIns(4)P synthesis in thrombin-stimulated platelets (19) and muscarinic cholinoceptor-stimulated parotid gland slices (20), and decreases in both PtdIns and PtdIns(4)P levels in thyrotropin-releasing hormone-stimulated GH_3 cells (21), no Li^+-dependent effect on PtdIns(4,5)P_2 was observed in any of these studies. Indeed, a number of studies have concluded that, despite large decreases in PtdIns and/or PtdIns(4)P, there appear to be regulatory mechanisms by which at least some cell types maintain levels of PtdIns(4,5)P_2 at the expense of PtdIns and PtdIns(4)P (20–22).

In the present study, we have attempted to fully characterize the effect of Li^+ on agonist-stimulated phosphoinositide turnover in CHO cells expressing the human recombinant m_1 muscarinic cholinoceptor subtype. In particular, we report a detailed and novel description of lithium effects on PI-PLC substrate-product relationships during m_1 muscarinic receptor stimulation, and we provide evidence that PtdIns(4,5)P_2 depletion leads to the observed decrease in agonist-stimulated Ins(1,4,5)P_3 accumulation.

Materials and Methods

Cell culture. CHO-m1 cultures (originally generously provided by Dr. N. J. Buckley, National Institutes for Medical Research, London, UK) were maintained in α-minimal essential medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 10% (v/v) newborn calf serum. Cultures were seeded into 24-well multiwells containing 0.5 ml of supplemented medium/well and were allowed to approach confluence at 37° in 5% CO_2/95% humidified air. For [^{3}H]inositol labeling experiments, CHO-m1 cells were seeded into multiwells containing 0.5 ml of supplemented medium and 2.5 μCi of [^{3}H]inositol and were maintained at 37° in 5% CO_2/95% humidified air. Cultures were incubated for 48 hr before experimentation, to allow equilibrium labeling to be approached.

Incubation methods. Incubations were conducted essentially as described previously (23). In brief, plated CHO-m1 cells grown in 24-well dishes were washed with Krebs/HEPES buffer (composition identical to that described in Ref. 23 but supplemented with 10 mM HEPES) and allowed to stabilize for 15 min at 37° in Krebs/HEPES buffer. To initiate experiments, the buffer was removed and replaced by Krebs/HEPES buffer containing appropriate drug additions. Incubations were stopped by addition of an equal volume of ice-cold 10% (v/v) perchloric acid.

For experiments assessing [^{3}H]CMP-phosphatidate accumulation, nearly confluent cells in multiwell dishes were washed and preincubated with Krebs/HEPES buffer. Medium was replaced by fresh Krebs/HEPES buffer (300 μl) containing 0.2 μCi of [^{3}H]cytidine, and cells were incubated for 60 min. After this labeling period, cells were washed in Krebs/HEPES buffer and incubations were started by addition of fresh Krebs/HEPES buffer containing appropriate drug additions. Incubations were terminated by addition of an equal volume of ice-cold 1 M trichloroacetic acid. After extraction on ice for 20 min, samples were centrifuged (3000 × g for 10 min at 4°) and the supernatant was discarded. Pellets were sequentially washed and the total lipid fraction was recovered as described below. Preliminary experiments have shown that >95% of the radioactivity associated with this fraction is in CMP-phosphatidate.

Ins(1,4,5)P_3 mass determination. Acidified supernatants (200 μl) were transferred to tubes containing 40 μl of 10 mM EDTA, and the mixture was neutralized by addition of Freon/tri-*n*-octylamine (1:1, v/v), centrifugation (14,000 × g for 4 min), and recovery of 200 μl of the upper phase. Each sample was adjusted to pH 7 by addition of 25 μl of 60 mM $NaHCO_3$ and was stored at 4°. Ins(1,4,5)P_3 was measured as described previously (24). The cell pellet was washed with 0.9% NaCl and digested in 1 M NaOH, and the protein concentration was determined to allow Ins(1,4,5)P_3 levels to be expressed as picomoles/milligram of protein.

Inositol phospholipid extraction and separation. Inositol phospholipids were isolated according to the method of Ref. 25. The original acidified supernatant was discarded and the cell pellet was washed consecutively with 1 ml of 5% (v/v) perchloric acid/1 mM EDTA and 1 ml of water. Lipids were extracted by addition of 0.94 ml of chloroform/methanol/12 M HCl (40:80:1) for 15 min at room temperature, followed by addition of 0.31 ml of chloroform and 0.56 ml of 0.1 M HCl to achieve phase partition. A sample of the lower phase (400 μl) was removed, dried, and stored under N_2 for subsequent processing.

For deacylation of [^{3}H]polyphosphoinositides, dried lipid samples were dissolved in 1.2 ml of chloroform/methanol (5:1, v/v), and 0.4 ml of 0.5 M NaOH in methanol/water (19:1, v/v) was added. After regular vortex-mixing for 20 min at room temperature, chloroform (1 ml), methanol (0.6 ml), and water (0.6 ml) were added and samples were thoroughly mixed and centrifuged (3000 × g for 10 min). A sample of the upper phase (1 ml), which contained the [^{3}H]glycerophosphoinositol (phosphates) as deacylation products, was recovered and neutralized by passage through a Dowex-50 (H^+ form) column (1-ml bed volume). The column was washed with 1 ml of water, and the pooled eluate was adjusted to pH 7 by addition of $NaHCO_3$ before addition to a Dowex (AG1-x8, formate form) anion exchange column and elution of [^{3}H]-glycerophosphoinositol, [^{3}H]glycerophosphoinositol-4-phosphate, and [^{3}H]glycerophosphoinositol-4,5-bisphosphate fractions as described in (25).

For determination of PtdIns(4,5)P_2 mass, the method described by Chilvers *et al.* (26) was used; this employs the mass assay described above to quantify the Ins(1,4,5)P_3 released by alkaline hydrolysis of PtdIns(4,5)P_2. In brief, dried lipid extracts were dissolved in 0.25 ml of 1 M KOH and heated to 100° for 15 min in tightly capped tubes. After this period, tubes were immediately transferred to an ice-bath and after 15 min added to Dowex-50 (H^+ form) columns (0.5-ml bed volume). Columns were washed with an additional 1.5 ml of water, and the pooled column eluate was washed with 2 × 2 ml of butan-1-ol/light petroleum ether (5:1, v/v). After lyophilization of a sample of the washed column eluate, the dried sample was reconstituted and the Ins(1,4,5)P_3 content was determined. Preliminary experiments, where lipid extracts were 'spiked' with [^{3}H]PtdIns(4,5)P_2, confirmed the proportions of Ins(1,4,5)P_3, inositol-2,4,5-trisphosphate, and inositol-4,5-bisphosphate generated by the alkaline hydrolysis and the recoveries achieved through each stage of the procedure (26).

Data analysis. Results are expressed as mean ± standard error of at least three determinations. Where appropriate, statistical significance was assessed by Student's t test and was considered significant when p was <0.05. Concentration-response curves were analyzed using the computer program GraphPAD (ISI Software).

Materials. [^{3}H]Ins(1,4,5)P_3 (17–20 Ci/mmol), *myo*-[2-^{3}H]inositol (17–20 Ci/mmol), and [5-^{3}H]cytidine (20–40 Ci/mmol) were purchased from DuPont-NEN. Ptd[3-^{3}H]Ins(4,5)P_2 (1 Ci/mmol) was a kind gift from Amersham International. Cell culture media, newborn calf serum, and antibiotics were from GIBCO. Analytical grade Dowex anion and cation exchange resins were from Bio-Rad. All other biochemicals and reagents were of analytical grade.

Results

Carbachol and Li^+ effects on Ins(1,4,5)P_3 mass levels. Preliminary experiments demonstrated that CHO-m1 cells express M_1 receptors at a density of 816 ± 46 fmol/mg of cell

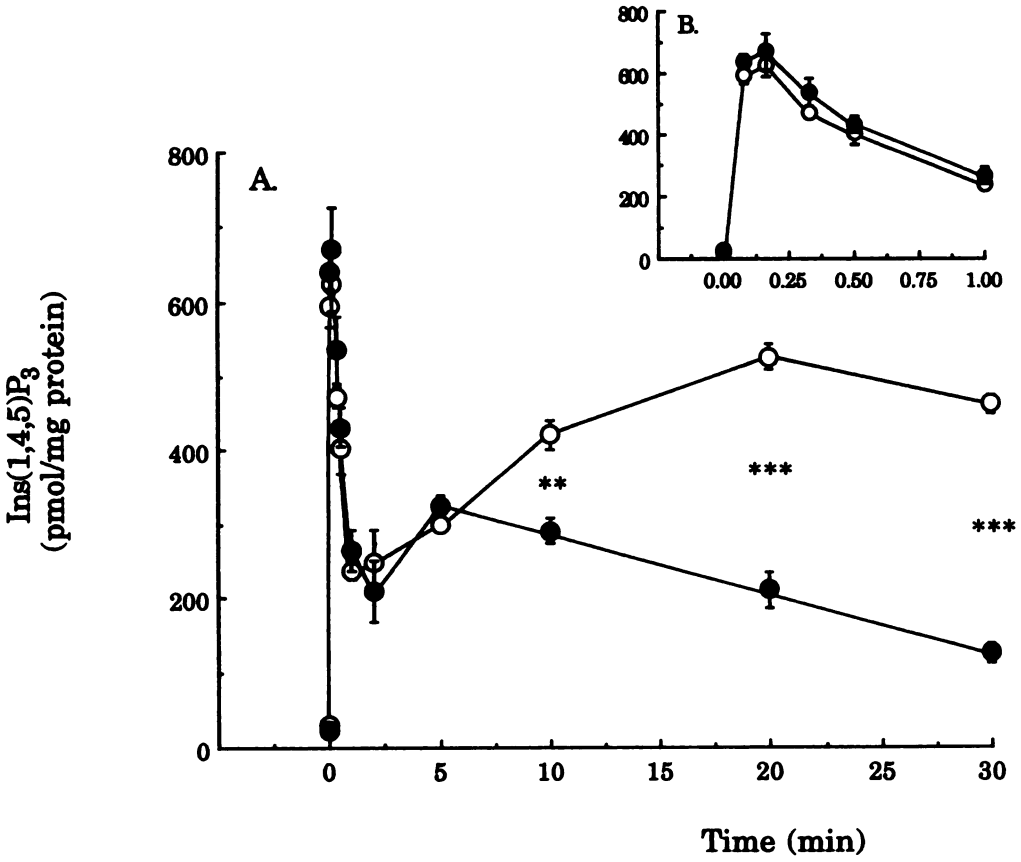

Fig. 1. Effect of lithium on the time course of carbachol-stimulated Ins(1,4,5)P_3 mass accumulation in CHO-m1 cells. Cell monolayers were incubated as described in Materials and Methods. After addition of LiCl (5 mM) (●) or vehicle (○), cells were challenged with carbachol (1 mM) for the indicated times. A, Full 30-min time course investigated; B, changes in Ins(1,4,5)P_3 accumulation occurring during the first 1 min after carbachol addition. Values are shown as means ± standard errors for at least three cell preparations, each assayed in triplicate. Statistically significant differences (Student's *t* test) in the presence, compared with the absence, of lithium are indicated as follows: **, $p < 0.01$; ***, $p < 0.001$.

protein.[1] Stimulation of CHO-m1 cells with a maximally effective concentration of carbachol (1 mM) caused a prompt and dramatic increase (>20-fold) in Ins(1,4,5)P_3 mass, which was maximal at 10 sec [basal, 28 ± 4; plus carbachol (10 sec), 625 ± 37 pmol/mg of protein] (Fig. 1). A subsequent decrease in Ins(1,4,5)P_3 levels to 8-fold over basal by 60 sec was followed by a slowly developing secondary rise to a new plateau level (~18-fold over basal) 10–20 min after carbachol addition (Fig. 1A). In the presence of the muscarinic receptor antagonist atropine (10 μM), no increases in Ins(1,4,5)P_3 level were seen during the 30 min after carbachol challenge (data not shown).

Addition of Li^+ (5 mM) for 30 min did not affect basal Ins(1,4,5)P_3 accumulation (without Li^+, 28.1 ± 4.3; plus Li^+, 24.4 ± 4.8 pmol/mg of protein). The initial changes in carbachol-stimulated Ins(1,4,5)P_3 mass in the presence of Li^+ (5 mM) were similar to those observed in its absence (Fig. 1B); however, Li^+ dramatically affected the secondary rise in Ins(1,4,5)P_3 seen 10–30 min after carbachol addition. Thus, in the presence of Li^+ the agonist-stimulated increase in Ins(1,4,5)P_3 was significantly attenuated by 32%, 62%, and 77% at 10, 20, and 30 min, respectively (Fig. 1A).

The concentration dependence of Li^+ inhibition of Ins(1,4,5)P_3 accumulation 20 min after carbachol addition is shown in Fig. 2A. A maximally effective concentration of Li^+ (10 mM) caused an 88% inhibition of the response observed in the absence of Li^+, and a half-maximal inhibitory effect was observed at 0.5 mM. The inhibitory effect of Li^+ on carbachol-stimulated Ins(1,4,5)P_3 accumulation could be completely prevented by prior addition of *myo*-inositol to the incubation medium (Fig. 2B). Thus, addition of 10 mM *myo*-inositol 30 min before carbachol plus Li^+ challenge resulted a Ins(1,4,5)P_3 accumulation at 20 min similar to that observed in the absence of Li^+. Relatively high concentrations of *myo*-inositol were required to overcome the Li^+ effect, with 50% reversal of Li^+ inhibition being observed in the presence of 4 mM *myo*-inositol (Fig. 2B).

Carbachol and Li^+ effects on [^{3}H]CMP-phosphatidate accumulation. Carbachol (1 mM) and Li^+ (5 mM) each caused small (~25%) but significant ($p < 0.05$ in each case) increases in [^{3}H]CMP-phosphatidate accumulation 30 min after addition (control, 1408 ± 88; plus carbachol, 1781 ± 104; plus Li^+, 1784 ± 126 dpm/well). However, simultaneous addition of carbachol plus Li^+ caused a dramatic increase in [^{3}H]CMP-phosphatidate accumulation, which, after an initial 2-min lag, reached 10-fold above basal levels within 10 min (Fig. 3). The initial lag between agonist addition and increased [^{3}H]CMP-phosphatidate accumulation does not appear to relate to any time dependence of Li^+ uptake into CHO cells, because a similar lag phase was observed when Li^+ was added to incubations 30 min before agonist challenge. Additional experiments to explore the con-

[1] S. Jenkinson, R. A. J. Challiss, and S. R. Nahorski, unpublished observations.

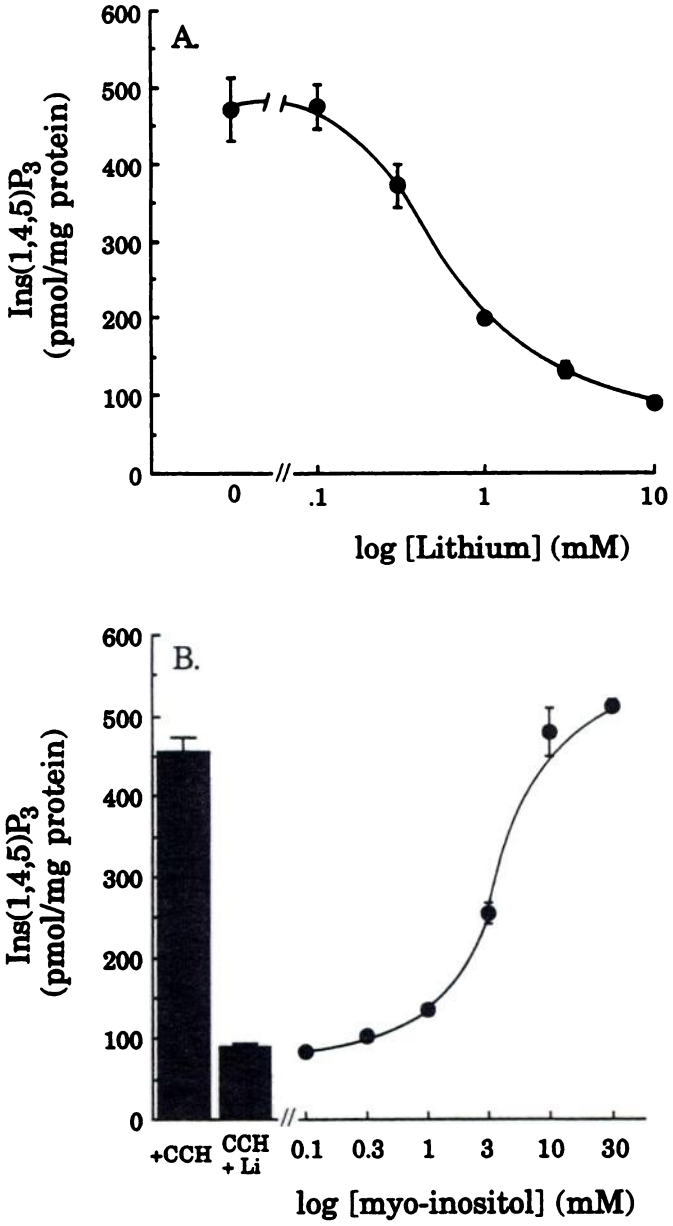

Fig. 2. Concentration dependence of lithium effect on carbachol-stimulated Ins(1,4,5)P_3 accumulation in CHO-m1 cells (A) and reversal by *myo*-inositol addition (B). A, The indicated concentrations of LiCl were added to cell monolayers and then carbachol (1 mM) was added for 20 min. Basal Ins(1,4,5)P_3 accumulation was 37 ± 4 pmol/mg protein, and this was unaffected by 30-min exposure to 10 mM LiCl (35 ± 5 pmol/mg protein). B, Cell monolayers were incubated with the indicated concentrations of *myo*-inositol for 30 min before addition of LiCl (5 mM) and challenge with carbachol (*CCH*) (1 mM) for an additional 20 min. Addition of 30 mM *myo*-inositol did not significantly affect either basal or carbachol-stimulated Ins(1,4,5)P_3 accumulations. In all cases, values are shown as means ± standard errors for at least three cell preparations, each assayed in triplicate.

centration dependence of the Li^+ effect on carbachol-stimulated [^{3}H]CMP-phosphatidate accumulation demonstrated that a half-maximal enhancement was observed at 0.5 mM Li^+, whereas preincubation with 10 mM *myo*-inositol for 30 min before carbachol plus Li^+ addition completely prevented increases in [^{3}H]CMP-phosphatidate accumulation over basal levels (data not shown).

Carbachol and Li^+ effects on [^{3}H]polyphosphoinositides. Incubation of CHO-m1 cells with [^{3}H]inositol (5 μCi/ml) for 48 hr allows inositol phospholipid labeling to approach equilibrium. Under these conditions [^{3}H]inositol was incorporated into inositol phospholipids in an approximate ratio of 89:7:4 for PtdIns/PtdIns(4)P/PtdIns(4,5)P_2 [PtdIns, 98,460 ± 6,104; PtdIns(4)P, 8,092 ± 783; PtdIns(4,5)P_2, 4,402 ± 592 dpm/well]. The time courses of agonist-stimulated changes in each inositol phospholipid species are illustrated in Fig. 4.

In the absence of Li^+, carbachol-stimulated inositol phospholipid hydrolysis resulted in an immediate decrease in PtdIns(4,5)P_2 (maximal 78% depletion at 60 sec). PtdIns(4,5)P_2 levels recovered gradually during the time course but were still 40% lower than prestimulation levels 30 min after carbachol addition. Agonist-stimulated changes in PtdIns(4)P showed a similar trend; an immediate decrease to about 40% of initial resting values was seen within the first 1 min after carbachol addition, and PtdIns(4)P remained at this new level throughout the 30 min of carbachol challenge. In contrast, there was an initial small increase in PtdIns labeling upon agonist stimulation (Fig. 4). A significant decrease in PtdIns was observed only after 10 min, and by 30 min after carbachol addition a new pseudo-steady state PtdIns level of about 65% of prestimulation values had been attained.

In all cases, initial carbachol-stimulated changes in inositol phospholipids were similar in the absence and in the presence of 5 mM Li^+ (Fig. 4); however, significant differences emerged as early as 5 min after agonist challenge. Thus, the decrease in the level of PtdIns was significantly greater in the presence, compared with the absence, of Li^+ by 5 min after carbachol addition, and the difference increased over the remainder of the time course. Similar time-dependent effects of Li^+ on the agonist-stimulated decreases in PtdIns(4)P and PtdIns(4,5)P_2 were also observed. Whereas in the absence of Li^+ carbachol caused 36, 48, and 39% reductions in PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2, respectively, in the presence of Li^+ these depletions were significantly greater, decreasing by 73, 77, and 72%, respectively, relative to prestimulation levels (Fig. 4).

The concentration dependences of Li^+ effects on each of the inositol phospholipid species are shown in Fig. 5. It should be noted that basal labeling of PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 was approximately doubled in this experiment (see Fig. 5, legend), principally to increase the 'window' in which the Li^+ concentration-effect curve was constructed (i.e., 20-min carbachol challenge with or without Li^+; see Fig. 4). For each inositol phospholipid, Li^+ caused a concentration-dependent decrease relative to the value obtained in the presence of carbachol only. Half-maximal decreases in PtdIns and PtdIns(4)P were observed at 0.5 and 0.4 mM Li^+, respectively, whereas the concentration of Li^+ required to half-maximally reduce PtdIns(4,5)P_2 was somewhat higher (1.2 mM) (Fig. 5).

Carbachol and Li^+ effects on PtdIns(4,5)P_2 mass levels. Basal levels of PtdIns(4,5)P_2 were unaffected by incubation of CHO-ml monolayers in the presence of Li^+ (5 mM) or *myo*-inositol (30 mM) for 30 min (basal, 399 ± 24; plus Li^+, 394 ± 24; plus inositol, 456 ± 37 pmol/mg of protein). The time courses of carbachol-stimulated changes in PtdIns(4,5)P_2 mass (with or without Li^+) mirrored those previously observed for [^{3}H]PtdIns(4,5)P_2 (data not shown); thus, 20 min after carbachol addition PtdIns(4,5)P_2 levels were decreased by 40%

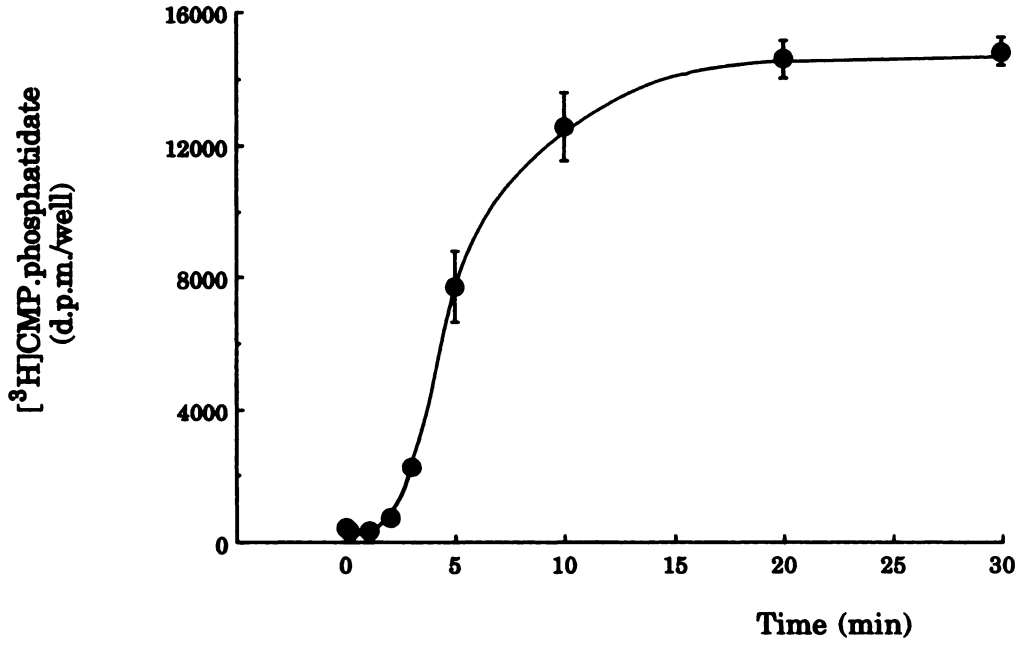

Fig. 3. Time course of carbachol-stimulated [^{3}H]CMP-phosphatidate accumulation in CHO-m1 cells in the presence of lithium. CHO-m1 cell monolayers were labeled with [^{3}H]cytidine as described in Materials and Methods. After addition of LiCl (5 mM), carbachol (1 mM) was added for the times indicated. Values are shown as means ± standard errors for at least three cell preparations, each assayed in triplicate.

and 64% in the absence and presence of Li^+, respectively (Fig. 6). Incubation with *myo*-inositol (added 30 min before carbachol challenge) concentration-dependently reversed the effect of Li^+ on PtdIns(4,5)P_2 levels but had no effect on the decrease in PtdIns(4,5)P_2 levels caused by carbachol alone (Fig. 6).

Effects of rechallenge with carbachol on Ins(1,4,5)P_3 mass and [^{3}H]polyphosphoinositide levels. Using cells in monolayer it is possible to completely change the incubation medium many times, allowing the effects of re-exposure to one or more agents to be studied. Here we have investigated how both Ins(1,4,5)P_3 mass and [^{3}H]polyphosphoinositide labeling are affected by two sequential periods of carbachol challenge when Li^+ is either absent, present only during the second stimulation, or present throughout the experimental period. The experimental paradigm and the time courses of changes in Ins(1,4,5)P_3 mass are illustrated in Fig. 7. In all cases, an initial 30-min period of carbachol (1 mM) challenge was followed by a 20-min period during which multiple changes of medium were used to wash out the agonist; this was followed by a 30-min period of rechallenge with carbachol.

In the absence of lithium, the reproducibility of the time courses of changes in Ins(1,4,5)P_3 levels between the first and second periods of carbachol exposure was clearly seen. The 20-min wash period at the end of the first stimulation caused a 96–98% return towards basal Ins(1,4,5)P_3 levels, suggesting that this protocol is effective in washing out the agonist. The effect of Li^+ added after the first period of stimulation and subsequent wash period (i.e., at t = 50 min) (Fig. 7) was somewhat greater than that seen when Li^+ was added at the start of the experiment. Although in both cases the initial responses were identical, Li^+ added during the second period of carbachol exposure more profoundly affected the time course of changes in Ins(1,4,5)P_3 during the 10–30-min period of agonist challenge. For example, whereas Li^+ caused a 62% decrease in Ins(1,4,5)P_3 20 min after carbachol addition, Li^+ added during the second stimulation period caused a 91% decrease in the concentration of this second messenger at the comparable time point.

More exaggerated effects of Li^+ were observed during the second stimulation period if Li^+ was present throughout the experiment. In this case, profound effects of Li^+ on Ins(1,4,5)P_3 levels could be observed as early as 1 min, with levels falling to values not significantly above basal values by 20 min after carbachol readdition (Fig. 7).

Measurement of [^{3}H]polyphosphoinositide labeling in parallel experiments allowed the effects of Li^+ on carbachol-stimulated changes in Ins(1,4,5)P_3 to be correlated with inositol phospholipid levels during the two stimulation periods (Fig. 8). After 30-min exposure to carbachol, the 20-min washing phase allowed full recovery of PtdIns(4)P and PtdIns(4,5)P_2 levels but only a partial 50% recovery of PtdIns (t = 0, 98,460 ± 6,104; t = 50 min, 80,212 ± 1,768 dpm/well; $P < 0.05$). Comparison of the effects of Li^+ addition during the first period or second period of carbachol challenge did not reveal any clear-cut differences in the time courses of changes for any of the [^{3}H]inositol phospholipids. The presence of Li^+ throughout the protocol had a number of effects. Most notably, the presence of Li^+ during the wash period between stimulations had a differential effect on the recoveries seen for each of the [^{3}H]-inositol phospholipids; thus, only a 17% recovery of PtdIns occurred during the 20-min wash phase, whereas PtdIns(4)P and PtdIns(4,5)P_2 recovered to 65% and 98% of respective prestimulation levels. The initial decreases in PtdIns(4)P and PtdIns(4,5)P_2 upon readdition of carbachol were similar in all groups; however, significantly lower levels of PtdIns(4,5)P_2, and to a lesser extent PtdIns(4)P, were observed 5–30 min after carbachol readdition for cells constantly exposed to Li^+ (Fig.

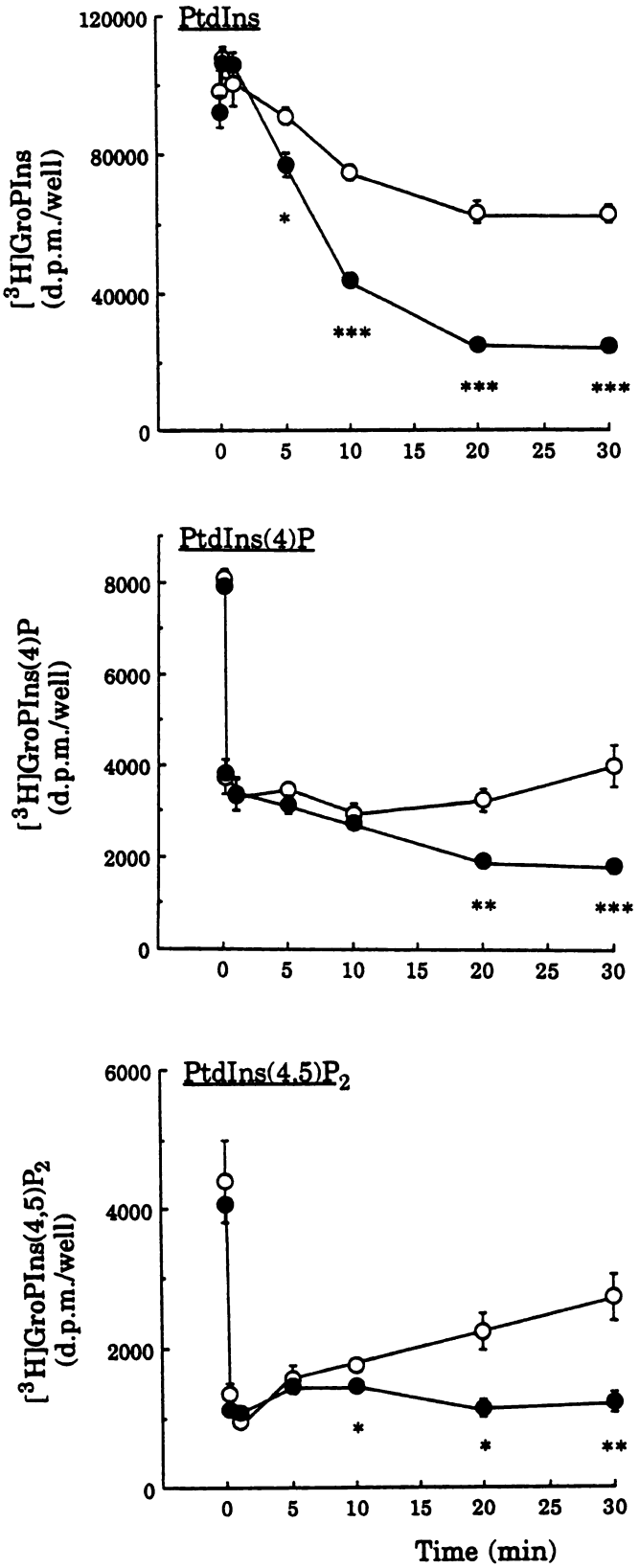

Fig. 4. Effect of lithium on the time courses of carbachol-stimulated changes in inositol phospholipid levels in CHO-m1 cells. Cell monolayers were labeled with [^{3}H]inositol as described in Materials and Methods. After addition of LiCl (5 mM) (●) or vehicle (○), cells were challenged with carbachol (1 mM) for the indicated times. After termination and extraction, the inositol phospholipid fraction was deacylated and glycerophosphoinositol (phosphates) [*GroPIns*, glycerophosphoinositol; *GroPIns(4)P*, glycerophosphoinositol-4-phosphate; *GroPIns(4,5)P_2*, glycerophosphoinositol-4,5-bisphosphate] were separated. Values are shown as means ± standard errors for at least three cell preparations, each assayed in triplicate. Statistically significant differences (Student's *t* test) in the presence, compared with the absence, of lithium are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

8). PtdIns also rapidly decreased (from already depressed values) upon carbachol readdition and remained at <20% of initial levels throughout the stimulation period.

Discussion

In the present study we have investigated the actions of Li^+ in CHO cells transfected to express M_1 muscarinic cholinoceptors, the predominant muscarinic receptor subtype preferentially coupled to phosphoinositide turnover in the central nervous system (27). In agreement with a recent study (28), we have demonstrated that Li^+ causes disruption of phosphoinositide turnover in CHO-m1 cells similar to that previously observed in brain slice preparations (5–11). Thus, only in the presence of Li^+ does carbachol stimulate a substantial accumulation of CMP-phosphatidate, followed by a significant decrease in Ins(1,4,5)P_3 mass accumulation. In both cases the effects of Li^+ are not immediate. CMP-phosphatidate accumulates after a brief 1–2-min lag (which can be equated with the time required to deplete free cellular *myo*-inositol), whereas significant decreases in Ins(1,4,5)P_3 are evident only 10 min after carbachol addition. We have also demonstrated for the first time that the inhibition by Li^+ of carbachol-stimulated Ins(1,4,5)P_3 mass accumulation temporally correlates with the occurrence of a significantly greater decrease in PtdIns(4,5)P_2, suggesting that PI-PLC substrate depletion may be causal in the subsequent decrease in Ins(1,4,5)P_3 levels.

Similarly, in CHO-m1 cells the concentrations of Li^+ necessary to evoke half-maximal effects on carbachol-stimulated Ins(1,4,5)P_3 mass, [^{3}H]CMP-phosphatidate accumulation, and [^{3}H]PtdIns, [^{3}H]PtdIns(4)P, and [^{3}H]PtdIns(4,5)P_2 levels approximate those previously reported in brain slice preparations. Furthermore, preincubation of cells with *myo*-inositol can concentration dependently and fully reverse the effects of Li^+ on agonist-stimulated Ins(1,4,5)P_3 and PtdIns(4,5)P_2 mass and [^{3}H]CMP-phosphatidate accumulation, strongly supporting the contention that Li^+ exerts its action by disrupting the ability of the cell to efficiently recycle *myo*-inositol. However, preincubation of CHO-m1 cells with relatively high concentrations of *myo*-inositol (5–10 mM) is necessary to completely prevent the effects of Li^+ on Ins(1,4,5)P_3 and PtdIns(4,5)P_2 mass levels. Although a high affinity (K_m, 12 μM), Na^+-dependent inositol transport system has been reported in CHO cells (29), it is likely that the maximal rate at which inositol can be carried by this system is insufficient to overcome phosphoinositide cycle disruption by Li^+ and additional influx of inositol via a low affinity uptake mechanism is also necessary (29).

It should be noted that even in the absence of Li^+ a maximally effective concentration of carbachol caused immediate and profound decreases in PtdIns(4)P (50–55%) and PtdIns(4,5)P_2 (70–75%) levels. These changes exceed those previously reported in thrombin-stimulated human platelets (19), thyrotropin-releasing hormone-stimulated GH_3 cells (21), and carbachol-stimulated 1321N1 astrocytoma cells (22), whereas

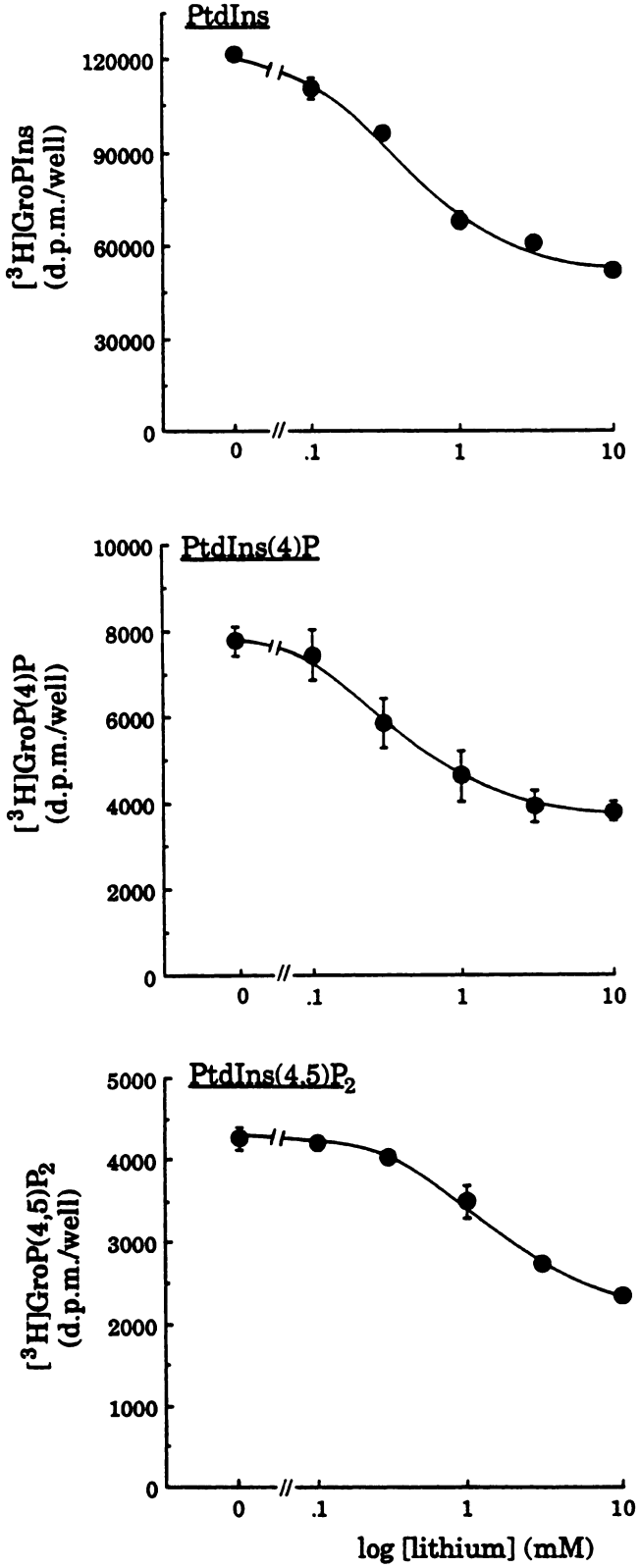

Fig. 5. Concentration dependence of lithium effect on carbachol-stimulated changes in inositol phospholipid levels in CHO-m1 cells. Cell monolayers were labeled with [^{3}H]inositol as described in Materials and Methods. The indicated concentrations of LiCl were added to cells and then carbachol (1 mM) was added for 20 min. After termination and extraction, the inositol phospholipid fraction was deacylated and glycerophosphoinositol(phosphates) [*GroPIns*, glycerophosphoinositol; *GroP(4)P*, glycerophosphoinositol-4-phosphate; *GroP(4,5)P$_2$*, glycerophosphoinositol-4,5-bisphosphate] were separated. Addition of LiCl (10 mM) had no effect on inositol phospholipid labeling in the absence of carbachol. Values are shown as means ± standard errors for at least three cell preparations, each assayed in triplicate.

rapid PtdIns(4,5)P_2 depletions of similar magnitude have been reported in angiotensin II-stimulated adrenal glomerulosa cells (18) and carbachol-stimulated tracheal smooth muscle (26). It is presently unclear whether the initial decrease in PtdIns(4,5)P_2 contributes to the rapid decrease from the initial peak Ins(1,4,5)P_3 mass accumulation observed from 15 sec after agonist addition or whether, like CHO cells expressing M_3 muscarinic receptors (23, 30), rapid receptor phosphorylation can contribute to a partial desensitization of the M_1 muscarinic receptor (31).

The extent to which PtdIns(4,5)P_2 is depleted is dependent not only on the extent of PI-PLC activation but also on the ability of PtdIns-4-kinase and PtdIns(4)P-5-kinase to coordinately resynthesize PtdIns(4,5)P_2 from PtdIns. Little is known about the latter process, although it is likely that the increase in flux necessary to maintain the new steady state PtdIns(4,5)P_2 level requires external regulation; a number of potential regulators of PtdIns-4-kinase (32) and PtdIns(4)P-5-kinase (33), including protein kinase C and G proteins, have been proposed. Although in the presence of Li^+ the additional agonist-stimulated depletion of PtdIns(4,5)P_2 that develops with time appears to be modest, it is likely to be crucial with respect to substrate provision for agonist-stimulated PI-PLC activity.

Subfractionation studies on CHO cells by Helms *et al.* (34) have demonstrated that, although the majority of PtdIns(4,5)P_2 is localized to the plasma membrane, a significant proportion (~20%) is found in the microsomal fraction and is therefore unlikely to be available to agonist-stimulated PI-PLC. Thus, the depletion of plasma membrane PtdIns(4,5)P_2 may be greater than indicated by the present experimental protocols, which assess changes in total cell phosphoinositides. Furthermore, although studies have demonstrated the potential for phosphoinositide resynthesis in the plasma membrane of GH_3 cells (35) and avian erythrocytes (36), the majority of studies have provided evidence that PtdIns synthesis occurs in a subcellular fraction consistent with PtdIns synthase activity being predominantly or exclusively localized to the endoplasmic reticulum (37–39). Indeed, PtdIns synthase activity in CHO cells appears to be absent from the plasma membrane (34). These data suggest that PtdIns is synthesized in the endoplasmic reticulum of CHO cells and PtdIns [or possibly PtdIns(4)P and PtdIns(4,5)P_2] needs to be translocated to the agonist-sensitive plasmalemmal phosphoinositide pool. Such a conclusion is also supported by the recent demonstration that a PtdIns transfer protein is essential for sustained phosphoinositide signaling (40).

The fact that only a proportion of total cellular PtdIns is available within the plasma membrane to 'buffer' agonist-stimulated decreases in PtdIns(4)P and PtdIns(4,5)P_2 strongly suggests that, during prolonged agonist challenge, the presence of Li^+ would so deplete the PtdIns concentration in the plasma membrane that PtdIns-4-kinase activity might become substrate limited. Indeed, considering the universal distribution of PtdIns in cellular membranes, it is remarkable that >80% of the cellular PtdIns can be utilized in the presence of agonist and Li^+.

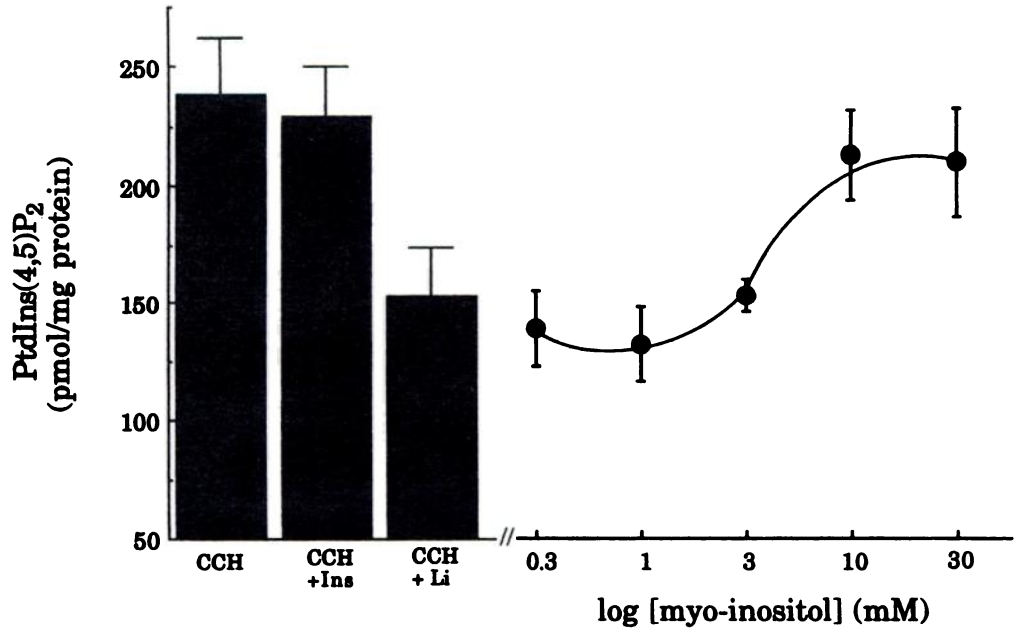

Fig. 6. Reversal of the lithium effect on carbachol-stimulated changes in PtdIns(4,5)P_2 mass levels in CHO-m1 cells by *myo*-inositol addition. Cell monolayers were incubated with the indicated concentrations of *myo*-inositol for 30 min before addition of LiCl (5 mM) and carbachol (1 mM) for 20 min. In the absence of carbachol, PtdIns(4,5)P_2 mass levels were 399 ± 24 pmol/mg of protein in the absence of *myo*-inositol supplementation and 456 ± 37 pmol/mg of protein in the presence of 30 mM *myo*-inositol. *CCH* and *CCH + Ins columns*, effects of carbachol on these values. Values are shown as means ± standard errors for four cell preparations, each assayed in triplicate.

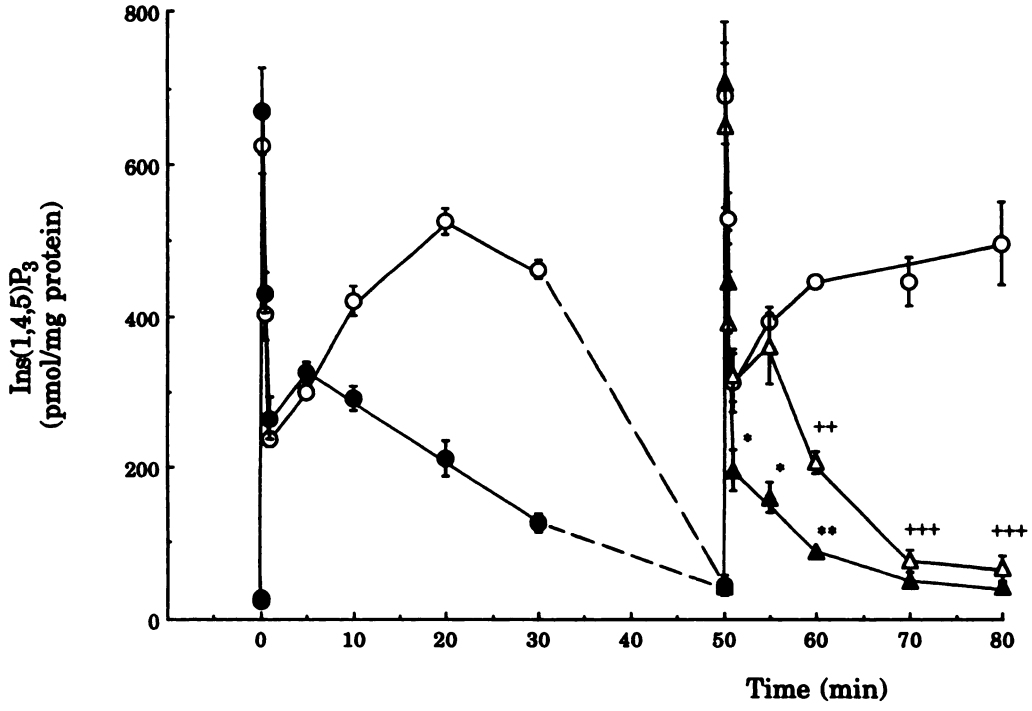

Fig. 7. Effect of rechallenge with agonist on the time course of lithium inhibition of carbachol-stimulated Ins(1,4,5)P_3 mass accumulation in CHO-m1 cells. Carbachol (1 mM) was added to cell monolayers for 0–30 min, in the absence (○) or presence (●) of 5 mM LiCl. After an initial exposure period of 30 min, monolayers were washed with repeated changes of either lithium-free Krebs/HEPES buffer, (○, △) or Krebs/HEPES buffer containing 5 mM LiCl (▲), over a period of 20 min. After this period, carbachol (1 mM) was again added for the indicated periods, in either the absence (○) or the presence (△, ▲) of 5 mM LiCl. Values are shown as means ± standard errors for at least three cell preparations, each assayed in triplicate. Statistically significant differences are indicated as follows: $^{++}$, $p < 0.01$; $^{+++}$, $p < 0.001$, for comparisons between monolayers washed in the absence of LiCl and rechallenged with carbachol in the presence and absence of LiCl; *, $p < 0.05$; **, $p < 0.01$, for monolayers washed in the absence or presence of LiCl and rechallenged with carbachol plus LiCl.

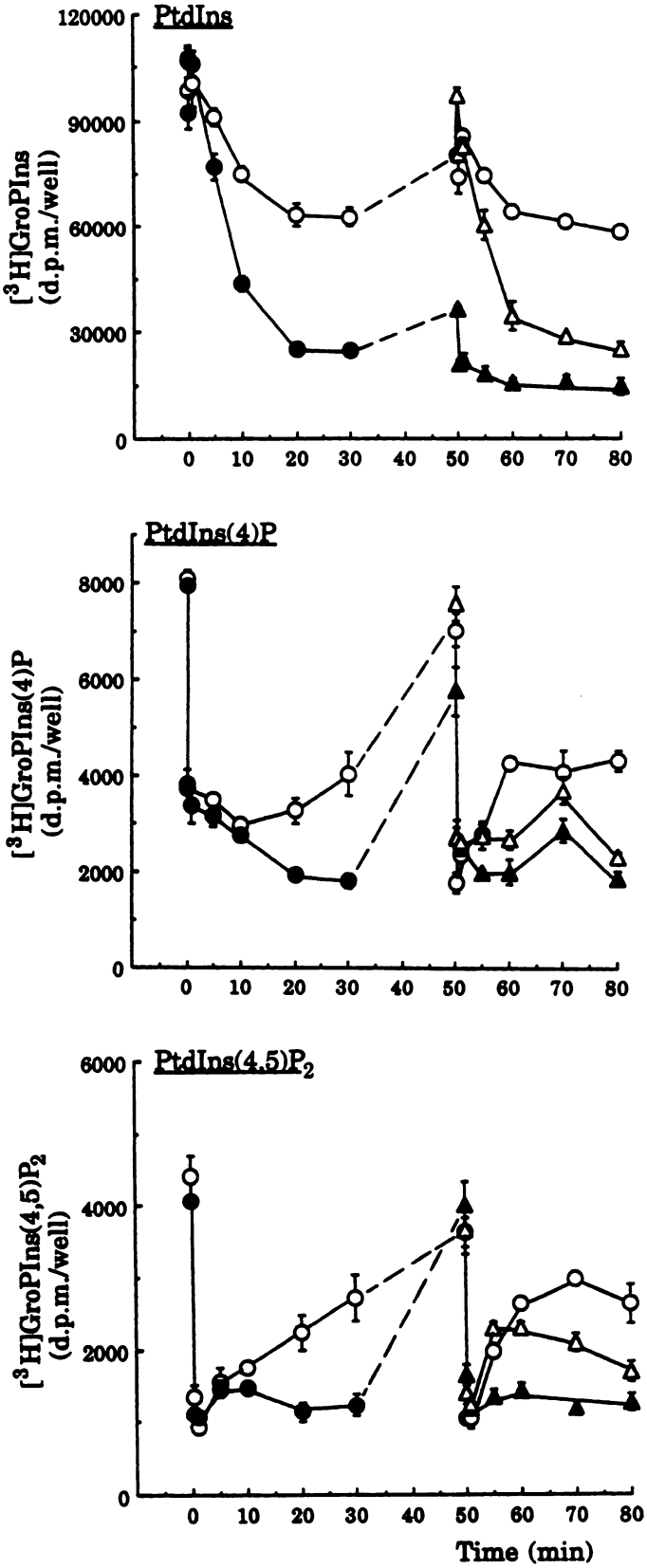

Fig. 8. Effect of rechallenge with agonist on the time course of lithium-induced effects on carbachol-stimulated changes in inositol phospholipid levels in CHO-m1 cells. Cell monolayers were labeled with [^{3}H]inositol as described in Materials and Methods. Carbachol (1 mM) was added to cells for 0–30 min in the absence (○) or presence (●) of 5 mM LiCl. After an initial exposure period of 30 min, monolayers were washed with repeated changes of either lithium-free Krebs/HEPES buffer (○, △) or Krebs/HEPES buffer containing 5 mM LiCl (▲), over a period of 20 min. After this period, carbachol (1 mM) was again added for the indicated periods, in either the absence (○) or the presence (△, ▲) of 5 mM LiCl. Values are shown as means ± standard errors for at least three cell preparations, each assayed in triplicate. *GroPIns*, glycerophosphoinositol; *GroPIns(4)P*, glycerophosphoinositol-4-phosphate; *GroPIns(4,5)P$_2$*, glycerophosphoinositol-4,5-bisphosphate.

Further insight into how the individual inositol phospholipids are regulated was gained from experiments that investigated the effects of sequential periods of agonist stimulation in the absence or presence of Li^+. In the absence of Li^+, carbachol readdition after an initial 30-min carbachol exposure and 20-min recovery period, during which CHO-ml cell monolayers were repeatedly washed to remove the agonist (see Fig. 7), resulted in two identical time-course profiles for changes in agonist-stimulated Ins(1,4,5)P_3 accumulation. In parallel experiments where CHO-ml cells were equilibrium-labeled with [^{3}H]inositol, it was found that the 20-min wash period between the two periods of carbachol challenge was sufficient to allow complete resynthesis of all phosphoinositides to prestimulation levels. However, when Li^+ was present throughout the protocol, significant differences were seen in Ins(1,4,5)P_3 accumulation between the first and second periods of agonist challenge. Although the initial peak increase in Ins(1,4,5)P_3 upon carbachol readdition was unaffected, the Ins(1,4,5)P_3 level subsequently decreased rapidly, was significantly lower than in the two time-matched control groups by 1 min, and had declined to basal values by 20 min after agonist readdition. Again, parallel labeling experiments provided a basis for the exaggerated effect of Li^+ during the second period of carbachol exposure. After the initial 30-min carbachol exposure, marked differences were observed in the extent to which PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 recovered if Li^+ was included in the wash buffer. Little recovery of PtdIns occurred, consistent with the continued, nearly complete, blockade of inositol monophosphatase. However, PtdIns(4)P and PtdIns(4,5)P_2 labeling recovered to 65 and 98%, respectively, of prestimulation values.

Additional support for the effectiveness of the blockade of inositol monophosphatase by Li^+ was provided by the fact that levels of CMP-phosphatidate, previously elevated by incubation in the presence of carbachol plus Li^+ for 30 min, remained constant during the 20-min wash period if Li^+ was continually present.[2]

How substantial resynthesis of PtdIns(4,5)P_2 can be brought about despite the continued presence of Li^+ is unclear. Activation of the 4- and/or 5-kinases by modulators that remain active during the wash period and redistribution of PtdIns [or PtdIns(4)P/PtdIns(4,5)P_2] to the plasma membrane to locally increase substrate availability are obvious mechanistic possibilities. The consequence of PtdIns(4,5)P_2 resynthesis is that the ability of the cell to generate Ins(1,4,5)P_3 upon carbachol readdition is preserved despite the presence of Li^+. However, the low initial levels of PtdIns quickly fall further to levels that limit the capacity of the cell to resynthesize PtdIns(4,5)P_2, and consequently Ins(1,4,5)P_3 generation by PI-PLC is rapidly and dramatically attenuated.

Since the original proposal by Berridge *et al.* (2) that Li^+ exerts its highly selective therapeutic action through inositol depletion and consequent disruption of phosphoinositide turn-

[2] S. Jenkinson, R. A. J. Challiss, and S. R. Nahorski, unpublished observations.

over stimulated by specific, but as yet undefined, neurotransmitters, questions have been raised intermittently regarding the hypothesis. For example, the demonstration that Li^+ can affect G protein function (41) and more recently the observation that Li^+ can increase, rather than attenuate, agonist-stimulated phosphoinositide turnover and second messenger generation under certain experimental conditions (42) led those authors and others (43) to question or dismiss the inositol depletion hypothesis. However, the latter remains the only comprehensive explanation that adequately accounts for the vast majority of experimental findings, including the *myo*-inositol-reversible actions of Li^+ on functional synaptic responses *in vitro* (44, 45) and *in vivo* (46, 47), as well as affording a clear mechanistic basis for the selectivity of Li^+ action (3, 4).

The current study provides unequivocal support for the inositol depletion hypothesis for Li^+ action, although data establishing that this model provides the definitive explanation for the clinical actions of lithium *in vivo* remain elusive. Our understanding of the selective vulnerability of neurons to lithium *in vivo* will require further investigation of the handling of *myo*-inositol, the critical regulatory features of key phosphoinositide cycle enzymes, notably PtdIns synthase, PtdIns-4-kinase, and PtdIns(4)P-5-kinase, and the dependence of receptor stimulus strength on the uncompetitive inhibition of inositol monophosphatase (4).

In summary, the detailed analysis of lithium effects on agonist-stimulated phosphoinositide turnover in CHO-m1 cells reported in this study has provided a number of significant novel findings. In particular, the manner in which lithium affects PtdIns(4,5)P_2 and Ins(1,4,5)P_3 mass provides important insight into PI-PLC substrate-product relationships, whereas the simple reversibility of lithium effects on agonist-stimulated changes in PtdIns(4,5)P_2 and Ins(1,4,5)P_3 mass by *myo*-inositol supplementation is entirely consistent with the inositol depletion hypothesis proposed to explain lithium action.

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