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Chronic Lithium Treatment Inhibits Basal and Agonist-Stimulated Responses in Rat Cerebral Cortex and GH_3 Pituitary Cells

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SUMMARY

Li^+ is used clinically in the management of bipolar-disordered (manic-depressive) illness, but the mechanism of its clinical efficacy remains unclear. Li^+ inhibits the metabolism of certain inositol phosphates, leading to a decreased cycling of inositol that may be sufficient to reduce phosphoinositide metabolism. We have tested this hypothesis in slices of rat cerebral cortex and in rat pituitary GH_3 cells grown in the presence of low extracellular inositol. We show that basal and stimulated mass levels of inositol-1,4,5-trisphosphate were reduced in rat cerebral cortex and in GH_3 cells after chronic, but not acute, treatment with a therapeutic concentration of Li^+. In GH_3 cells chronic treatment with Li^+ also decreased basal levels of intracellular Ca^{2+} and secretion of prolactin, effects that were prevented by the presence of *myo*-inositol. Agonist-stimulated mobilization of Ca^{2+} and prolactin release were also reduced in Li^+-treated cells. These findings show that chronic perturbation of the phosphoinositide pathway by Li^+ is sufficient to reduce basal and agonist-stimulated cellular responses, an action that may underlie its effectiveness in the alleviation of affective disorders.

Lithium ions are used widely in the treatment of affective disorders, particularly in patients with bipolar disorders, which are characterized by oscillations in mood between mania and depression. In the majority of these patients, a maintained serum level of approximately 1 mM Li^+ alleviates both the manic and depressive phases of this disorder. The mechanism by which Li^+ produces these therapeutic effects is unclear, and it has been suggested that Li^+ may act by altering signaling pathways such as adenylyl cyclase (1), guanine nucleotide-binding proteins (2), or the phosphoinositide pathway (3–5).

Li^+ is an uncompetitive inhibitor of inositol monophosphatase, with a K_i close to its therapeutic plasma concentration. Several studies have shown a large accumulation of inositol monophosphates in brain and a corresponding decrease in inositol (6–8). It has been suggested that Li^+ acts by reducing the supply of inositol for the resynthesis of phosphoinositides and that this impairs receptor-stimulated generation of $Ins(1,4,5)P_3$. In support of this hypothesis, various *in vitro* and *ex vivo* studies have demonstrated a reduction in agonist-stimulated formation of total [^{3}H]inositol phosphates (9–11) and $Ins(1,4,5)P_3$ mass in brain slices in the presence of Li^+ (12).

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In this study, we show that chronic treatment with therapeutic concentrations of Li^+ reduces basal and agonist-stimulated mass levels of $Ins(1,4,5)P_3$ in slices of rat cerebral cortex. These observations were extended to the rat pituitary GH_3 cell line, and these effects were found to be accompanied by a decrease in basal and agonist-stimulated intracellular levels of Ca^{2+} and secretion of prolactin.

Materials and Methods

Experiments with Rat Cerebral Cortex and Rat Ileum

Li^+ treatment. Male Sprague-Dawley rats (150–250 g) were used for all experiments. Rats receiving chronic Li^+ treatment were fed a standard laboratory diet containing 0.1% Li_2CO_3, a treatment regimen that produces therapeutic levels of Li^+ in the plasma of rats (13). Control and acutely Li^+-treated rats received standard laboratory chow. An acute treatment regimen involved a single subcutaneous injection of LiCl or NaCl (10 meq/kg, made up in 0.9% saline) 4 hr before removal of the cerebral cortex. This regimen produces plasma levels of Li^+ of about 2.7 mM (11).

Tissue preparation, incubation, and stimulation. Rat cerebral cortex slices were prepared as described previously (11) and were dispersed in 25 ml of Krebs-Ringer HEPES buffer. The absolute levels of Li^+ in rat cerebral cortex slices are dependent on the treatment regimen, and it was, therefore, decided to incubate the slices in excess Li^+ so that its concentration in cortical slices would be similar despite different treatments. After washing with 100 ml of warmed buffer, the

ABBREVIATIONS: $Ins(1,4,5)P_3$, inositol-1,4,5-trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CNS, central nervous system; TRH, thyrotropin-releasing hormone; EC_{50}, concentration required to produce 50% of the maximal effect.

slices were incubated for 45 min in the absence or presence of 10 mM LiCl (unless otherwise stated), replacing NaCl in the buffer. Tissue slices were then allowed to settle under gravity, and 50-μl aliquots were incubated in a final volume of 250 μl at 37° in a shaking water bath; agonist (10 μl) was added for the appropriate time, and reactions were terminated with 0.31 ml of ice-cold 10% perchloric acid, and samples were extracted and assayed for $Ins(1,4,5)P_3$, as described below.

In some experiments, the ileum was removed from control and chronically Li^+-treated rats and washed with Krebs buffer. The longitudinal muscle was dissected from the ileum, and cross-chopped slices were prepared as described previously (14). Experiments were performed as described above for rat cerebral cortex slices.

Tissue slices were dissolved in 0.5 ml of 1 M NaOH over 24 hr, and protein was measured using the protein assay of Lowry *et al.* (15); with bovine serum albumin as a standard. Levels of $Ins(1,4,5)P_3$ are expressed as pmol/mg of protein.

[^{3}H]$Ins(1,4,5)P_3$ binding to rat cerebral cortex membranes. For each experiment, membranes were prepared from cerebral cortices of two animals, to allow a complete displacement curve to be obtained. Cortical membranes were prepared by homogenization (Polytron, setting 9) in ice-cold buffer A (20 mM NaCl, 100 mM KCl, 50 mM Tris, 1 mM EDTA, pH 8.3). The homogenate was then centrifuged at 1000 × *g* for 15 min at 4°, and the supernatant was centrifuged at 35,000 × *g* for 15 min at 4°. The resulting pellet was resuspended in buffer at a protein concentration of 5 mg/ml. A full displacement curve was performed in parallel for control and Li^+-treated samples, in the presence of 0.01 to 1000 nM $Ins(1,4,5)P_3$, and the data were transformed by Scatchard analysis. Protein was measured using the method of Lowry *et al.* (15), with bovine serum albumin as a standard.

Li^+ Treatment of Rat Pituitary GH_3 Cells

Rat pituitary GH_3 cells were grown in Ham's F-10 medium supplemented with 16% fetal calf serum. For experiments involving low inositol, custom-made Ham's F-10 medium lacking inositol was purchased from GIBCO BRL and supplemented with 5% serum (approximate final *myo*-inositol concentration of 25 μM). Treated cells were grown in this medium supplemented with an additional 1 mM LiCl or 1 mM NaCl (controls). Both control and Li^+-treated cells received 10 mM *myo*-inositol in the relevant experiments. After the appropriate treatment, cells were removed from flasks and incubated in Hanks' modified buffer (composition, in mM: 143 NaCl, 5.6 KCl, 2 $MgCl_2$, 1.3 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.2), gassed with 100% O_2. After 30-min preincubation, 150 μl of cell suspension were aliquoted into plastic tubes (approximately 7.5 × 10^5 cells/tube) and incubated for an additional 10 min. After this, cells were stimulated with TRH (added in 50 μl) for the required period, incubations were terminated by addition of an equal volume of 10% perchloric acid, and $Ins(1,4,5)P_3$ was measured as described below. Cells were counted with the aid of a haemocytometer, and their viability was assessed by trypan blue exclusion. Results are expressed as pmol/10^6 viable cells.

Measurement of $Ins(1,4,5)P_3$ using a Radioreceptor Assay

Experiments were terminated at the appropriate time by addition of an equal volume of 10% (v/v) ice-cold perchloric acid, and tubes were left for at least 10 min at 4°. Tubes were centrifuged (1000 × *g* for 10 min at 4°), and the supernatant was removed and added to 50 μl of 20 mM EDTA (pH 7.5). After this, 500 μl of freshly made 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine (1:1; v/v) were added to each tube and vortex-mixed for at least 30 sec, to extract perchloric acid. Phase separation was added by centrifugation (2 min; 13,000 × *g*); and the top, aqueous, phase was removed and neutralized by addition of 60 mM $NaHCO_3$.

Either 25-μl aliquots (rat cerebral cortex slices) or 50-μl aliquots (rat ileal slices and GH_3 cells) of this neutralized extract (now diluted 10–20-fold) were added to tubes containing approximately 3 nM [^{3}H] $Ins(1,4,5)P_3$, and the assay was initiated by addition of 50 μl of binding protein (~400 μg); this binding protein was prepared from bovine adrenal cortex, as described previously (16). After 15 min, the assay was terminated by centrifugation (6 min, 13,000 × *g*), the supernatants were discarded, and the pellets were washed in distilled water and dried by inversion. The pellets were dissolved in tissue solubilizer (Soluene, Packard), scintillant was added to the tubes, and the radioactivity was determined by liquid scintillation counting. Nonspecific binding (typically around 10% of total binding) was determined in the presence of 10 μM DL-$Ins(1,4,5)P_3$. The displacement of [^{3}H]$Ins(1,4,5)P_3$ binding was measured by comparison against a curve obtained with authentic $Ins(1,4,5)P_3$ standards.

Measurement of Prolactin Secretion

The secretion of prolactin was measured in GH_3 cells grown on sterilized microcarrier Cytodex beads (Pharmacia). After 21 days, these were transferred to a water-jacketed column held at 37° and were constantly perfused (0.4 ml/min) with modified Hanks' buffer. Fractions were collected in tubes containing 25 μl of 4% (w/v) bovine serum albumin. Prolactin was measured by radioimmunoassay, the reagents for which were kindly supplied by Dr Salvatore Raiti of the NIDDK, National Hormone and Pituitary Program, University of Maryland. The reference prolactin standard used was RP-3. Investigation of the dose-response relationship for prolactin secretion by TRH was carried out on cells that had been grown in flasks, preincubated for 30 min in modified Hanks' buffer, centrifuged (250 × *g*, 5 min), and resuspended in fresh buffer. After a 10-min stimulation with TRH, incubations were terminated by addition of 4 volumes of ice-cold modified Hanks' buffer. Samples were centrifuged (250 × *g*, 5 min), and prolactin was assayed in the supernatant fraction.

Measurement of cAMP and cGMP

The procedure for obtaining samples for cyclic nucleotide measurements was exactly as described above for the preparation of samples for measurement of $Ins(1,4,5)P_3$. cAMP was measured in the aqueous extracts by using a competitive protein binding assay (17). cGMP was measured by radioimmunoassay, using antisera obtained from Amersham International (Amersham, UK). Authentic standards were assayed with each experiment, and samples were measured from the standard curve.

Measurement of Ca^{2+} in GH_3 Cells

On the day of the experiment, cells were resuspended at a density of approximately 3 × 10^6 cells/ml, in Hanks' modified buffer, and incubated with 3 μM fura-2/acetoxymethyl ester at room temperature. After 45 min, cells were centrifuged (200 × *g*, 5 min), resuspended in Hanks' buffer, and left at room temperature for up to 1 hr. An aliquot of the cell suspension (0.5 ml) was taken, centrifuged briefly (5 sec; microcentrifuge), and resuspended in 1 ml of fresh Hanks' buffer at 37°. The cell suspension was placed in a quartz cuvette and left for 2 min to equilibrate in the water-jacketed cuvette holder, heated to 37°, of a Perkin-Elmer LS-3 spectrofluorimeter. Basal Ca^{2+} was calculated from the ratio of emissions obtained at 510 nm after excitation at 340 nm and 380 nm; a recording was also performed in the presence of 1 mM $MnCl_2$, used to quench dye that had leaked from the cells. Autofluorescence values were subtracted by measuring the excitation spectra from cells suspended at the same density but not loaded with fura-2. F_{max} was determined using 2 mM sodium deoxycholate in the presence of at least 1 mM Ca^{2+}, and F_{min} was determined by addition of excess Tris-buffered EGTA (10 mM EGTA, 100 mM Tris-HCl, pH 7.2), calculated to give values of free Ca^{2+} of ≤1 nM. The values of intracellular Ca^{2+} were calculated using the equation derived in Ref. 18. For agonist-induced responses, only excitation at 340 nm was monitored, because the spectrofluorimeter was unable to measure two wavelengths simultaneously.

Data Analysis

Results are expressed as the mean ± standard error. Statistical significance was determined for data points obtained from three or

more experiments, using either Student's paired t test (for GH_3 cell work, in which treated cells were directly compared with control cells) or unpaired t test (for studies using rats). A statistically significant difference was taken to be where $p < 0.05$. Where appropriate, concentration-response curves were analyzed using the computer program GraphPAD (ISI Software), implemented on an IBM Nimbus computer.

Materials

[^{3}H]Ins(1,4,5)P_3, [8-^{3}H] cAMP, Na^{125}I, and guanosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl 3-[^{125}I]iodotyrosine methyl ester were purchased from New England Nuclear. Fura-2/acetoxymethyl ester was obtained from Calbiochem. TRH, carbachol, Ins(1,4,5)P_3, cAMP, and cGMP were all purchased from Sigma. GH_3 cells were obtained from Flow Laboratories. Growth media, fetal calf serum, and antibiotics were from GIBCO, and sterile plastic tissue culture equipment was from Griffiths & Neilson. All other standard laboratory reagents were of analytical grade.

Results

Studies on rats after acute or chronic treatment with Li⁺. Carbachol (1 mM) stimulated a rapid increase in Ins(1,4,5)P_3 mass in rat cerebral cortical slices, which reached a maximum by 10 sec and remained elevated for at least 30 min (Fig. 1). However, in the presence of 10 mM Li⁺, carbachol-stimulated levels of Ins(1,4,5)P_3 returned towards basal values after 20 min of stimulation (Fig. 1). The presence of Li⁺ did not affect carbachol-stimulated levels of Ins(1,4,5)P_3 over the first 10 min of stimulation and, as a consequence, incubations of up to 5 min were used in subsequent experiments.

Acute treatment (4 hr) of rats with 10 meq/kg LiCl (subcutaneously), a dose that is approximately 10-fold greater than that used clinically, did not alter carbachol-stimulated Ins(1,4,5)P_3 levels in cerebral cortical slices, compared with NaCl-treated control animals (Fig. 2A). However, basal levels of Ins(1,4,5)P_3 were reduced in cerebral cortical slices obtained from rats treated chronically with Li⁺ for 21 days, relative to controls, from 15.2 ± 2.9 to 7.98 ± 1.4 pmol/mg of protein (Fig. 2B). In addition, maximal levels of Ins(1,4,5)P_3 in response to 1 mM carbachol were reduced by a similar extent in cortical slices, from 30.8 ± 4.0 to 15.2 ± 2.8 pmol/mg of protein (mean ± standard error; six experiments).

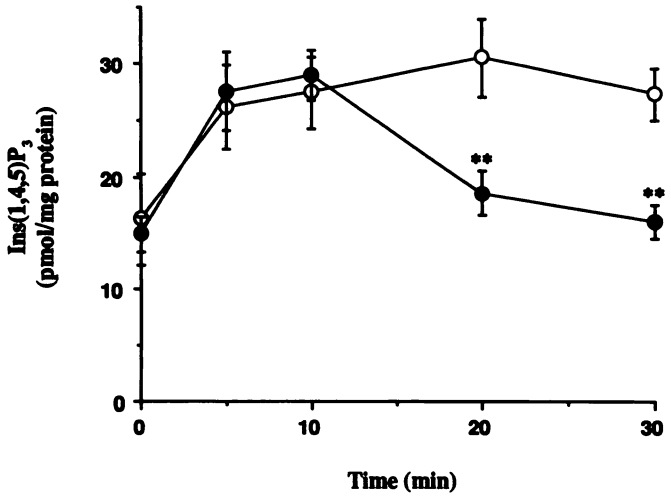

Fig. 1. Effect of Li⁺ on carbachol-stimulated Ins(1,4,5)P_3 mass in slices of rat cerebral cortex. Rat cerebral cortical slices were stimulated with 1 mM carbachol in the absence (○) or presence (●) of 10 mM LiCl, replacing NaCl in the buffer. Ins(1,4,5)P_3 was measured using a mass assay, and results are presented as mean ± standard error from at least four experiments, with each *point* determined in triplicate. **, Significantly different from controls ($p < 0.05$), as determined using Student's t test.

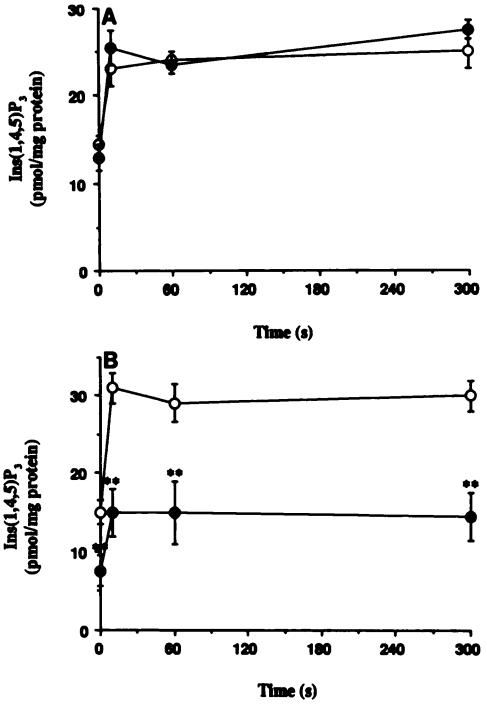

Fig. 2. Effect of acute and chronic Li⁺ treatment on carbachol-stimulated Ins(1,4,5)P_3 mass in slices of rat cerebral cortex. A, Control rats (○) received an injection of 10 meq/kg NaCl (subcutaneously), and Li⁺-treated rats (●) received 10 meq/kg LiCl subcutaneously, 4 hr before tissue preparation. B, Chronically Li⁺-treated rats (●) were fed a diet containing 0.1% Li_2CO_3 for at least 21 days; control animals (○) were fed a standard laboratory diet. Tissue slices were prepared from the cerebral cortex, incubated in buffer containing 10 mM LiCl, and stimulated with 1 mM carbachol. Ins(1,4,5)P_3 levels are expressed as pmol/mg of protein, and data points represent mean ± standard error from five to eight experiments, each performed in triplicate. **, Significantly different from controls ($p < 0.05$), as determined using Student's t test.

The density and affinity of Ins(1,4,5)P_3 receptors in the cerebral cortex of control and chronically Li⁺-treated rats were investigated from the specific binding of [^{3}H]Ins(1,4,5)P_3 to cortical membranes. Scatchard transformation of [^{3}H]Ins(1,4,5)P_3 binding revealed both a similar affinity of Ins(1,4,5)P_3 for its receptor, as determined by the slope of the curve, and a similar density of Ins(1,4,5)P_3 receptors (Fig. 3).

The effect of chronic Li⁺ treatment was investigated in a peripheral tissue, to assess the selectivity of the action of Li⁺. In contrast to the results obtained from cerebral cortex, basal and carbachol-stimulated levels of Ins(1,4,5)P_3 in slices of ileal longitudinal muscle were similar in control and chronically Li⁺-treated rats (Fig. 4).

Studies on the rat pituitary GH_3 cell line. Experiments were extended to rat pituitary GH_3 cells grown in low inositol-containing medium (approximately 25 μM). This strategy was used so that cells would rely heavily on recycling of inositol, as is thought to occur in rat cerebral cortex (see below). In GH_3 cells, TRH (1 μM) stimulated a rapid formation of Ins(1,4,5)P_3, producing maximal levels after 10 sec. A 1-hr acute treatment with Li⁺ did not alter basal or peak TRH-induced formation of Ins(1,4,5)P_3 (Fig. 5A). However, cells grown under the same conditions in the presence of 1 mM Li⁺ for 4–7 days showed

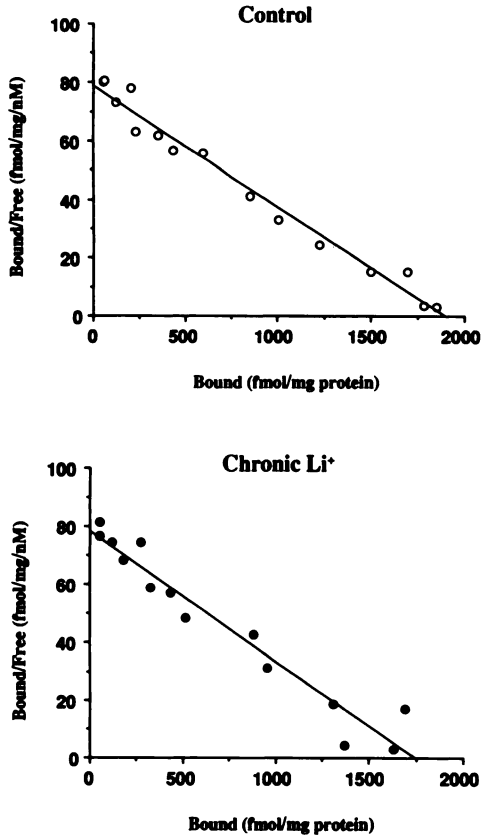

Fig. 3. Scatchard analysis of [^{3}H]Ins(1,4,5)P_3 binding to membranes prepared from the cerebral cortex of control and chronically Li^+-treated rats. Membranes were prepared from the cerebral cortex of control rats (○) or rats treated chronically for at least 21 days with Li^+ (●). Membranes (~250 μg) were incubated with ~0.7 nM [^{3}H]Ins(1,4,5)P_3 and increasing concentrations of Ins(1,4,5)P_3 (0.01–1000 nM). Each *point* represents triplicate determinations from a single representative experiment, repeated in two additional experiments.

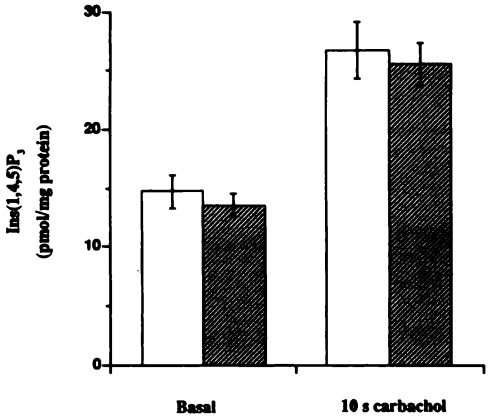

Fig. 4. Effect of chronic Li^+ treatment on basal and carbachol-stimulated Ins(1,4,5)P_3 mass in rat ileum longitudinal muscle slices. Mass levels of Ins(1,4,5)P_3 were determined in slices of rat ileum longitudinal muscle. Basal and 1 mM carbachol-stimulated (10-sec stimulation) mass levels of Ins(1,4,5)P_3 are shown for tissue slices obtained from control animals (□) or from rats treated chronically with Li^+ for at least 21 days (▨). Data represent the mean ± standard error for five to eight experiments, with each *point* determined in quadruplicate.

reduced basal and TRH-stimulated levels of Ins(1,4,5)P_3 (Fig. 5B), an effect that was maintained for at least 21 days (Fig. 5C). The reduction in cellular Ins(1,4,5)P_3 could be prevented by supplementing the medium with 10 mM *myo*-inositol throughout the time of incubation with Li^+ (Fig. 5D). Mass levels of Ins(1,4,5)P_3 were concentration-dependently increased by TRH (Table 1). After chronic Li^+ treatment, TRH-stimulated levels of Ins(1,4,5)P_3 showed a reduced maximal response, compared with controls (Table 1), but the EC_{50} values were similar between control and chronically Li^+-treated cells, with values of 26.3 nM (−5.9, +9.5 nM) and 21.5 nM (−5.8, +10.5 nM), respectively (geometric mean, −standard error, +standard error; three experiments). These results suggest that the decrease in Ins(1,4,5)P_3 is the consequence of a reduced inositol bioavailability within the cell.

Effect of Li^+ on intracellular Ca^{2+} levels. Ins(1,4,5)P_3 releases Ca^{2+} from the intracellular stores in GH_3 cells. In control cells the mean basal level of Ca^{2+} was 182 ± 6.2 nM, and this was reduced to 146 ± 6.0 nM (15 experiments; $p < 0.01$) in cells treated chronically with Li^+. Furthermore, this decrease in basal Ca^{2+} could be prevented by growing cells in 10 mM *myo*-inositol (control cells, 184 ± 8.2 nM; Li^+ plus *myo*-inositol, 186 ± 6.5; 12 experiments). TRH-induced mobilization of Ca^{2+} was also decreased in cells treated chronically with Li^+, both in the presence and in the absence of extracellular Ca^{2+} (Fig. 6). However, intracellular Ca^{2+} levels in response to a depolarizing concentration of K^+ were unaffected by chronic Li^+ treatment (Fig. 6).

Effect of Li^+ on prolactin secretion. The functional consequence of decreased levels of Ins(1,4,5)P_3 and Ca^{2+} in GH_3 cells was investigated by measuring the rate of prolactin secretion. GH_3 cells were grown on microcarriers and placed in a constantly perfused water-jacketed column. Under these conditions, control cells secreted prolactin at a rate of 4.42 ± 0.53 ng/min/10^7 cells. However, after Li^+ treatment for 21 days, basal secretory activity was significantly reduced, to 2.66 ± 0.30 ng/min/10^7 cells (35 observations, five experiments; $p < 0.01$). This decrease in basal secretion by Li^+ was prevented in medium supplemented with inositol; control cells grown in the presence of 10 mM *myo*-inositol secreted 4.95 ± 0.87 ng/min/10^7 cells, compared with 4.88 ± 0.99 ng/min/10^7 cells in the presence of Li^+ and 10 mM *myo*-inositol.

The rate of secretion of prolactin from GH_3 cells was greatly increased by the presence of TRH. The peak secretion (burst phase) after 1 μM TRH stimulation in control cells was 82.3 ± 7.8 ng/min/10^7 cells, and this was reduced in Li^+-treated cells to 49.0 ± 8.0 ng/min/10^7 cells (three experiments; $p < 0.01$). A representative perfusion experiment is shown in Fig. 7. Prolactin release was also measured under 'static' conditions, in which cells were incubated in the presence of an agonist for 30 min. Using this method, a dose-response curve was determined (Table 1) and revealed that secretion of prolactin was reduced at all concentrations of TRH after chronic Li^+ treatment, but the EC_{50} was unchanged; in control cells the mean EC_{50} was 3.45 nM (−1.85, +4.15 nM), compared with 3.14 nM (−1.94, +4.26 nM) for Li^+-treated cells (geometric mean, −standard error, +standard error; three experiments).

In the presence of 50 mM K^+, which depolarizes GH_3 cells and leads to entry of extracellular Ca^{2+} through voltage-sensitive Ca^{2+} channels, prolactin secretion was increased. Under static conditions, K^+-stimulated secretion of prolactin in con-

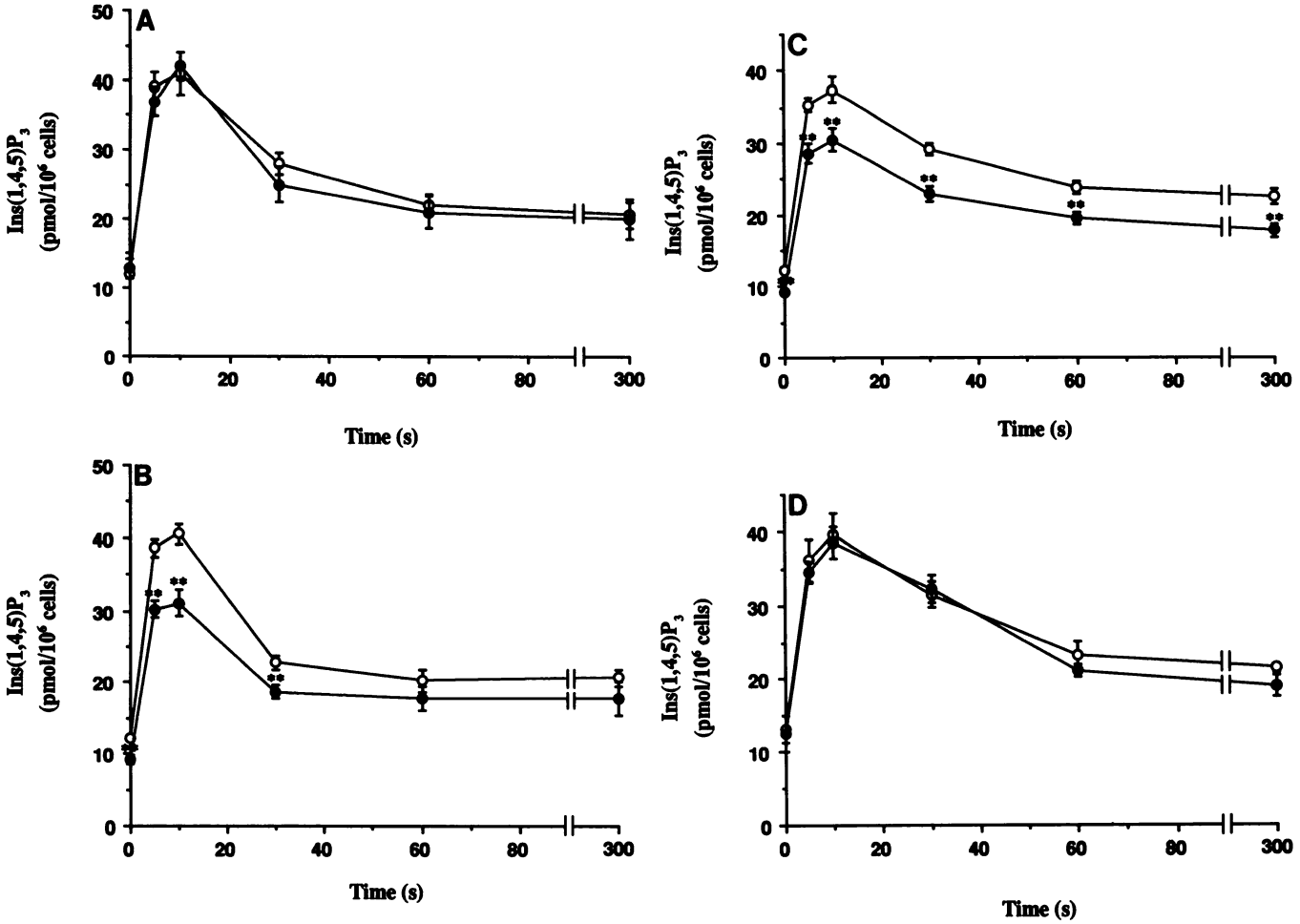

Fig. 5. Effect of Li^+ on basal and TRH-stimulated Ins(1,4,5)P_3 mass levels in rat pituitary GH_3 cells. Mass levels of Ins(1,4,5)P_3 were measured in control cells (○) and Li^+-treated GH_3 cells (●) under basal conditions and after stimulation with 1 μM TRH. A, Control cells were incubated in Hanks' buffer or buffer supplemented with 1 mM Li^+ for 1 hr. B and C, Cells were grown in low inositol-containing medium (~25 μM), in the absence or presence of 1 mM Li^+, for 4–7 days (B) or for at least 21 days (C). D, Cells were grown under the same conditions as C, but the growth medium was supplemented with 10 mM *myo*-inositol. **, Different from control values ($p < 0.05$), as determined using Student's paired *t* test.

TABLE 1

Increases in Ins(1,4,5)P_3 and prolactin secretion

GH_3 cells were grown in low inositol-containing medium for 21 days, in the absence or presence of 1 mM Li^+. Values show the increase in TRH-stimulated Ins(1,4,5)P_3 (measured after 10 sec) or prolactin secretion (measured after 10 min), expressed as percentages of control values. Values are mean ± standard error obtained from 3 to 16 experiments, each performed in at least triplicate.

[TRH]	Ins(1,4,5)P_3		Prolactin	
	Control	Lithium	Control	Lithium
nM	*% of control*		*% of control*	
Basal	100	76 ± 5	100	49 ± 5
0.1	107 ± 2	84 ± 4	144 ± 12	77 ± 11
1	117 ± 6	105 ± 3	224 ± 42	122 ± 19
10	173 ± 10	156 ± 5	336 ± 17	210 ± 33
100	298 ± 29	209 ± 26	491 ± 29	288 ± 47
1000	342 ± 12	257 ± 15	513 ± 36	293 ± 40

trol cells was 176 ± 23 ng/10^7 over a 10-min incubation period, compared with 144 ± 24 ng/10^7 cells from cells treated with Li^+ for 21 days (three experiments; $p > 0.05$).

Effect of Li^+ on other second messenger pathways. Although there was no evident difference in the viability of GH_3 cells treated with Li^+, we considered whether the decreased response to TRH may have been a result of nonspecific Li^+ toxicity. To test this, we measured basal and stimulated levels

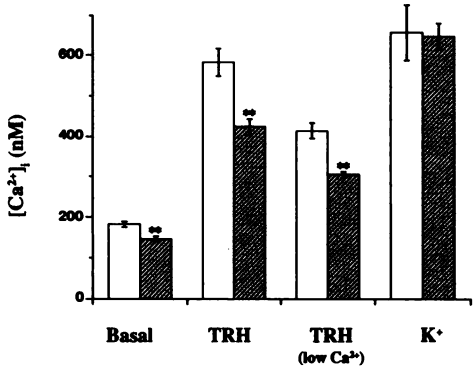

Fig. 6. Effect of chronic Li^+ treatment on basal and stimulated intracellular Ca^{2+} responses in GH_3 cells. Control (□) and chronically Li^+-treated (▨) GH_3 cells were loaded with fura-2 and stimulated with 1 μM TRH (both in the presence and in the absence of extracellular Ca^{2+}) or with 50 mM K^+. Data represent the mean ± standard error from at least six experiments. **, Different from control values ($p < 0.05$), as determined using Student's paired *t* test.

of cyclic nucleotides in GH_3 cells treated chronically for up to 21 days with Li^+ (summarized in Table 2). In these experiments, basal, vasoactive-intestinal polypeptide-stimulated and NaF-stimulated levels of formation of cAMP in control and Li^+-

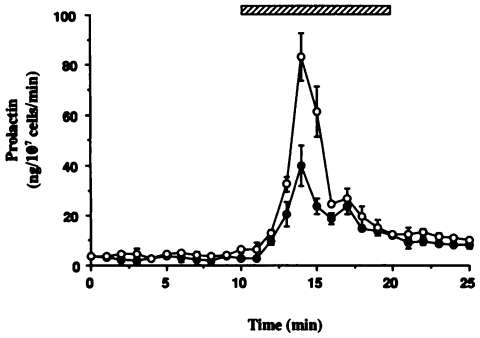

Fig. 7. Effect of chronic Li^+ treatment on prolactin secretion from GH_3 cells. GH_3 cells were grown on Cytodex beads for 21 days in low inositol-containing medium, in the absence (○) or presence (●) of 1 mM Li^+. Beads were transferred to a column held at 37° and constantly perfused with Hanks' buffer. *Hatched bar*, introduction of TRH (1 μM) into the column. Prolactin was measured by radioimmunoassay and is expressed as pmol/10^7 cells/min. Data points represent the mean ± standard error from a single representative experiment.

TABLE 2

cAMP and cGMP formation

GH_3 cells were grown in low inositol-containing medium for 21 days, in the absence or presence of 1 mM Li^+. cAMP was measured by radioreceptor assay and cGMP by radioimmunoassay. The reactions were terminated at the times that produced the maximum stimulated levels of cyclic nucleotides. Data points represent mean ± standard error from three to six experiments (*n*), each performed in triplicate. No values obtained from lithium-treated samples are significantly different from their corresponding control values (Student's paired *t* test).

Treatment	cAMP		*n*
	Control	Lithium	
	pmol/10⁶ cells		
Basal	2.32 ± 0.19	2.51 ± 0.14	6
$AlCl_3$/NaF, 10 sec	4.34 ± 0.15	4.39 ± 0.16	3
0.1 μM VIP,[a] 60 sec (100 μM/10 mM)	8.36 ± 1.50	7.67 ± 0.82	3
Treatment	**cGMP**		***n***
	Control	Lithium	
	fmol/10⁶ cells		
Basal	349 ± 35	359 ± 7	4
50 mM K^+, 30 sec	451 ± 24	436 ± 22	3

[a] VIP, vasoactive intestinal peptide.

treated cells were similar. Furthermore, basal and K^+-stimulated cGMP levels were also similar between control and chronically Li^+-treated cells (Table 2).

Discussion

The activity of the phosphoinositide pathway was determined by measurement of $Ins(1,4,5)P_3$ mass, using a radioreceptor assay, rather than measurement of [^{3}H]inositol phosphates, so as to avoid the potential problem of changes in specific radioactivity of inositol phosphates and the complex separation of $Ins(1,4,5)P_3$ from other naturally produced isomers of $InsP_3$.

Experiments on rat cerebral cortex. In the absence of Li^+, the maintained production of $Ins(1,4,5)P_3$ over a 30-min stimulation period with carbachol in rat cerebral cortex indicates that this tissue is able to maintain sufficient levels of receptor-sensitive phosphatidylinositol 4,5-bisphosphate, even under conditions of extreme stimulation. However, in the presence of 10 mM Li^+, stimulated levels of $Ins(1,4,5)P_3$ were not maintained, indicating that recycling of inositol is essential in maintaining the activity of the pathway. A similar time-dependent decline in $Ins(1,4,5)P_3$ mass has been observed by others (12), and this decline is reduced in the presence of high concentrations of inositol.

Chronic treatment of rats with Li^+ reduced both basal and agonist-stimulated levels of $Ins(1,4,5)P_3$. The former observation has not been reported previously, because it would be unlikely to be detected in nonisotropic labeling experiments. We believe that this is an important observation, because the reduction in $Ins(1,4,5)P_3$ is seen under basal conditions and does not require prior stimulation with a high concentration of agonist. The attenuated $Ins(1,4,5)P_3$ levels measured in cerebral cortex of chronically Li^+-treated rats suggest a decreased rate of phosphoinositide metabolism; this is unlikely to be through a direct interaction of Li^+ with guanine nucleotide-binding protein regulation of phospholipase C, because this inhibition was not seen after acute treatment.

It is possible that the method by which these experiments have been performed *in vitro* exaggerates the action of Li^+. Preparation of cortical slices routinely involves a loss of up to 80% inositol (19); because brain levels of inositol are ~10 mM *in vivo* (20), the concentration in the slices is probably <2 mM, a concentration of inositol similar to the K_m of the enzyme phophatidylinositol synthase, which catalyzes the incorporation of inositol into phosphatidylinositol (21, 22). It, therefore, seems likely that, under the conditions of the present study, a further reduction of inositol in the cell through the inhibition of inositol phosphate recycling is a limiting factor for resynthesis of phosphatidylinositol.

The reduction in $Ins(1,4,5)P_3$ levels after chronic Li^+ treatment did not result in a compensatory change in the $Ins(1,4,5)P_3$ receptor affinity or density. Because there was no alteration in the density or affinity of $Ins(1,4,5)P_3$ for its receptor, it is likely that the attenuated levels of $Ins(1,4,5)P_3$ result in changes in the pattern of Ca^{2+} mobilization. This effect would be more prominent in those neurons that have the greatest depletion of $Ins(1,4,5)P_3$.

The inhibition of inositol monophosphatase by Li^+ in preparations obtained from central and peripheral tissues occurs with a similar K_i (23), and inositol 1-monophosphate levels increase in both central and peripheral tissues after the treatment of rats with Li^+ (8). Li^+ is, therefore, nonselective in its inhibition of these enzymes in peripheral and brain tissues. However, in the present study there was no difference between basal and carbachol-stimulated levels of $Ins(1,4,5)P_3$ in rat ileal slices prepared from control and chronically Li^+-treated rats. Although the effects of Li^+ have been examined in only one peripheral tissue, it is tempting to speculate that the selectivity of the reduction of $Ins(1,4,5)P_3$ is limited to the CNS. The interpretation of these results is, therefore, consistent with the inositol depletion hypothesis, suggesting that peripheral tissues are able to maintain levels of phosphatidylinositol 4,5-bisphosphate through uptake of inositol from the serum. However, inositol crosses the blood-brain barrier only poorly (20), and, therefore, cells of the CNS rely on recycling of inositol from inositol phosphates and *de novo* synthesis from glucose to maintain an adequate supply of inositol. The inhibition by Li^+ of both these pathways confers the selectivity of the action of Li^+ to the CNS. Superimposed on this, the rate of phosphoinositide cycling is higher in neuronal cells and, as a consequence, these cells are far more susceptible to the uncompetitive inhibition by Li^+.

GH_3 cell line studies. The lack of effect of chronic Li^+ treatment on levels of cyclic nucleotides demonstrates that Li^+ does not have a toxic effect on GH_3 cells and indicates that neither cAMP nor cGMP levels are responsible for the attenuated secretion of prolactin seen in GH_3 cells after chronic Li^+ treatment.

When GH_3 cells were grown in a low inositol-containing medium, a reduction in levels of basal and TRH-stimulated Ins(1,4,5)P_3 occurred. This effect of Li^+ was seen, at the earliest, after 4 days of treatment with Li^+, and levels remained attenuated for at least 21 days. The decrease in Ins(1,4,5)P_3 can be attributed to a reduced bioavailability of inositol to the cell, because growing the cells in medium containing Li^+ supplemented with 10 mM *myo*-inositol revealed no difference in basal or agonist-stimulated Ins(1,4,5)P_3 levels, compared with controls.

Because Li^+ inhibits the inositol monophosphatase enzyme uncompetitively, the degree of inhibition is strongly stimulus dependent (24). The addition of 10 mM Li^+ to cells labeled with [^{3}H]inositol results in a 2–4-fold increase in the accumulation of [^{3}H]inositol phosphates after 30 min in the absence of an agonist (25),[1] demonstrating that the cycle is active under basal conditions. Furthermore, fetal calf serum, added to the growth medium to maintain the viability of GH_3 cells, induces a small increase in phosphoinositide turnover.[1] These observations may, therefore, explain why Li^+ reduces not only TRH-stimulated Ins(1,4,5)P_3 production but also basal levels of this messenger.

The reduction in basal and agonist-stimulated Ins(1,4,5)P_3 after Li^+ treatment resulted in an attenuation of basal and TRH-induced Ca^{2+} levels. Because this TRH-stimulated increase in Ca^{2+} was also reduced in the absence of extracellular Ca^{2+}, this implies that Li^+ does not simply inhibit Ca^{2+} entry. Furthermore, this is supported by the similar increase in Ca^{2+} between control and Li^+-treated cells after addition of a depolarizing concentration of K^+. These results suggest that attenuated levels of Ins(1,4,5)P_3 after treatment with Li^+ result in a decreased mobilization of intracellular Ca^{2+}.

In GH_3 cells we estimate basal levels of Ins(1,4,5)P_3 to be ~6 μM, and they rise to ~25 μM after maximal TRH stimulation. These values are consistent with those reported for other cell lines, such as AR4–2J pancreotoma cells (26) and SH-SY5Y neuroblastoma cells (27). The results obtained by Mollard *et al.* (28), using tight-seal whole-cell recording from GH_3 cells, suggest that this basal concentration of Ins(1,4,5)P_3 would be sufficient to stimulate Ca^{2+} mobilization, leading to Ca^{2+}-activated K^+ currents, and, therefore, it is conceivable that (in GH_3 cells at least) basal Ca^{2+} may be regulated by Ins(1,4,5)P_3.

In this study, we have shown that Li^+ reduces basal levels of intracellular Ca^{2+}, as measured in a whole population of GH_3 cells. However, because the intracellular Ca^{2+} levels vary between individual cells, the reduced 'basal' Ca^{2+} levels may reflect an altered frequency or amplitude in oscillations. For example, under basal conditions Ca^{2+} oscillations occur in some GH_3 cells due to spontaneous action potentials, sufficient to cause a transient entry of Ca^{2+} through voltage-sensitive Ca^{2+} channels (29–31).

There is some evidence to suggest that the reduction in cellular levels of Ins(1,4,5)P_3 could result in attenuated Ca^{2+} oscillations and, hence, a decrease in the population basal Ca^{2+}. For example, a single intracellular injection of Ins(1,4,5)P_3 is sufficient to cause oscillations of intracellular Ca^{2+} in some cells (32, 33), brought about by the periodic increase in Ca^{2+}, primarily from intracellular stores (for review, see Ref. 34). Although there is still much controversy about the mechanism for the generation and maintenance of Ca^{2+} oscillations, Ins(1,4,5)P_3 appears to play a crucial role in their generation. The release of Ca^{2+} from Ins(1,4,5)P_3-sensitive stores by Ins(1,4,5)P_3 has been suggested to be quantal (35, 36), and this quantal release may depend on the sensitivity of the Ins(1,4,5)P_3 receptor being regulated by the Ca^{2+} concentration in the lumen of the endoplasmic reticulum. It has been reported (37) that the Ins(1,4,5)P_3-sensitive Ca^{2+} stores may become overloaded with Ca^{2+} and, because of an increased sensitivity of the Ins(1,4,5)P_3 receptor to endogenous (i.e., basal) Ins(1,4,5)P_3, can spontaneously discharge their accumulated Ca^{2+}. This mechanism could, therefore, have a role in generating Ca^{2+} oscillations and may explain how reduced basal levels of Ins(1,4,5)P_3 after Li^+ treatment could result in reduced Ca^{2+} signaling.

After chronic treatment of GH_3 cells with Li^+, both the basal rate and the TRH-stimulated burst phase of prolactin secretion were reduced. Because this burst secretory activity is stimulated by an increase in intracellular Ca^{2+} (38), it seems likely that the decreased intracellular Ca^{2+} concentration after Li^+ treatment is responsible for the attenuated rate of TRH-stimulated prolactin secretion. Indeed, a decrease in basal Ca^{2+} may also explain the reduced basal secretory activity after Li^+ treatment, because it has been suggested that spontaneous action potentials under nonstimulated conditions (which produce Ca^{2+} oscillations) are responsible for basal secretion from pituitary cells (29). This has been confirmed by using the extremely sensitive reverse hemolytic plaque assay to measure hormone secretion from single pituitary cells; hormone release correlated directly with both the frequency and the amplitude of Ca^{2+} oscillations (39). The secretion of prolactin after K^+ stimulation, which results in an increase in intracellular Ca^{2+} and a subsequent increase in the burst phase of prolactin secretion, was unaffected by Li^+ treatment. This result suggests that the secretory mechanism itself was unaffected by Li^+.

Somatostatin, a hypothalamic peptide, lowers basal prolactin secretion from GH_3 cells (40) and the population basal Ca^{2+} (41). Single-cell studies reveal that somatostatin gradually reduces spontaneous spiking activity and has no effect on intracellular Ca^{2+} in cells that are not displaying spontaneous oscillations (29). Thus, somatostatin reduces basal secretion not by reducing basal Ca^{2+} *per se* but by reducing spontaneous Ca^{2+} oscillations responsible for prolactin secretion. It is, therefore, conceivable that Li^+ reduces spontaneous Ca^{2+} oscillations in those cells that are more active through a reduction in basal Ins(1,4,5)P_3 levels, which ultimately decreases prolactin secretion. It is essential to test these hypotheses at the level of the single cell by using imaging techniques, and these studies are in progress.

Conclusions. These results show that chronic treatment with therapeutic concentrations of Li^+ reduce basal and agonist-stimulated activities of cells in low inositol-containing medium through a decreased level of Ins(1,4,5)P_3. This change, in turn, leads to a reduction in Ca^{2+} mobilization and a decrease in secretory activity. Similar adaptive changes may occur in

[1] M. Varney, unpublished observations.

CNS neurons and could underlie the clinical efficacy of Li^+ in the treatment of manic-depressive disease.

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References

1. Newman, M. E., and R. H. Belmaker. Effects of lithium *in vitro* and *in vivo* on components of the adenylate cyclase from rat brain. *Neuropharmacology* **26**:211–217, (1987).
2. Avissar, S., G. Schreiber, A. Danson, and R. H. Belmaker. Lithium inhibits adrenergic and cholinergic increases in GTP binding in rat cortex. *Nature (Lond.)* **331**:440–442 (1988).
3. Berridge, M. J., C. P. Downes, and M. R. Hanley. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.* **206**:587–595 (1982).
4. Sherman, W. R. Lithium and the phosphoinositide signalling system, in *Lithium and the Cell* (N. J. Birch, ed.). Academic Press, New York, 121–157 (1991).
5. Nahorski, S. R., C. I. Ragan, and R. A. J. Challiss. Lithium and the phosphoinositide cycle: an example of uncompetitive inhibition and its pharmacological consequences. *Trends Pharmacol. Sci.* **12**:297–303 (1991).
6. Allison, J. H., and M. A. Stewart. Reduced brain inositol in lithium-treated rats. *Nature (Lond.)* **233**:267–268 (1971).
7. Allison, J. H., M. E. Blisner, W. H. Holland, P. P. Hipps, and W. R. Sherman. Increased brain *myo*-inositol 1-phosphate in lithium-treated rats. *Biochem. Biophys. Res. Commun.* **71**:664–670 (1976).
8. Sherman, W. R., L. Y. Munsell, B. G. Gish, and M. P. Honchar. Effects of systemically administered lithium on phosphoinositide metabolism in rat brain, kidney and testis. *J. Neurochem.* **44**:798–807 (1985).
9. Casebolt, T. L., and R. S. Jope. Long-term lithium treatment selectively reduces receptor-coupled inositol phospholipid hydrolysis in rat brain. *Biol. Psychiatry* **25**:329–340 (1989).
10. Kendall, D. A., and S. R. Nahorski. Acute and chronic treatments influence agonist and depolarization-stimulated inositol phospholipid hydrolysis in rat cerebral cortex. *J. Pharmacol. Exp. Ther.* **241**:1023–1027 (1987).
11. Godfrey, P. P., S. J. McClue, A. M. White, A. J. Wood, and D. G. Grahame-Smith. Subacute and chronic *in vivo* lithium treatment inhibits agonist- and sodium fluoride-stimulated inositol phosphate production in rat cortex. *J. Neurochem.* **52**:498–506 (1989).
12. Kennedy, E. D., R. A. J. Challiss, C. I. Ragan, and S. R. Nahorski. Reduced inositol polyphosphate accumulation and inositol supply induced by lithium in stimulated cerebral cortex slices. *Biochem. J.* **267**:781–786 (1990).
13. Bond, P. A., B. A. Brooks, and A. Judd. The distribution of lithium, sodium, and magnesium in rat brain and plasma after various periods of lithium in the diet. *Br. J. Pharmacol.* **53**:235–239 (1975).
14. Sasaguri, T., and S. P. Watson. Lowering of the extracellular Na^+ concentration enhances high-K^+-induced formation of inositol phosphates in the guinea-pig ileum. *Biochem. J.* **252**:883–888 (1988).
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
16. Varney, M. A., J. Rivera, A. Lopez Bernal, and S. P. Watson. Are there subtypes of inositol 1,4,5-trisphosphate receptor? *Biochem. J.* **269**:211–216 (1990).
17. Brown, B. L., J. D. M. Albano, R. P. Ekins, and A. M. Sgherzi. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* **121**:561–562 (1971).
18. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
19. Sherman, W. R., B. G. Gish, M. P. Honchar, and L. Y. Munsell. Effects of lithium on phosphoinositide metabolism *in vivo*. *Fed. Proc.* **45**:2639–2646 (1986).
20. Spector, R., and A. V. Lorenzo. *myo*-Inositol transport in the central nervous system. *Am. J. Physiol.* **228**:1510–1518 (1975).
21. Ghalayini, A., and J. Eichberg. Purification of phosphatidylinositol synthetase from rat brain by CDP-diacyglycerol affinity chromatography and properties of the purified enzyme. *J. Neurochem.* **44**:175–182 (1985).
22. Takenawa, T., and K. Egawa. CDP-diglyceride:inositol transferase from rat liver: purification and properties. *J. Biol. Chem.* **252**:5419–5423 (1977).
23. Hallcher, L. M., and W. R. Sherman. The effects of lithium ion and other agents on the activity of *myo*-inositol-1-phosphatase from bovine brain. *J. Biol. Chem.* **255**:10896–10901 (1980).
24. Cornish-Bowden, A. Why is uncompetitive inhibition so rare? A possible explanation, with implications for the design of drugs and pesticides. *FEBS Lett.* **203**:3–6 (1986).
25. Drummond, A. H., and C. A. Raeburn. The interaction of lithium with thyrotropin-releasing hormone-stimulated lipid metabolism in GH_3 pituitary tumour cells. *Biochem. J.* **224**:129–136 (1984).
26. Horstman, D. A., H. Takemura, and J. W. Putney, Jr. Formation and metabolism of [^{3}H]inositol phosphates in AR4–2J pancreatoma cells: substance P-induced Ca^{2+} mobilization in the apparent absence of inositol 1,4,5-trisphosphate 3-kinase activity. *J. Biol. Chem.* **262**:15297–15303 (1988).
27. Lambert, D. G., and S. R. Nahorski. Muscarinic-receptor-mediated changes in intracellular Ca^{2+} and inositol 1,4,5-trisphosphate mass in human neuroblastoma cell line, SH-SY5Y. *Biochem. J.* **265**:555–562 (1990).
28. Mollard, P., B. Dufy, P. Vacher, J. L. Barker, and W. Schlegel. Thyrotropin-releasing hormone activates a $[Ca^{2+}]_i$-dependent K^+ current in GH_3 pituitary cells via Ins(1,4,5)P_3-sensitive and Ins(1,4,5)P_3-insensitive mechanisms. *Biochem. J.* **268**:345–352 (1990).
29. Schlegel, W., B. P. Winiger, P. Mollard, P. Vacher, F. Wuarin, G. R. Zahnd, C. Wollheim, and B. Dufy. Oscillations of cytosolic Ca^{2+} in pituitary cells due to action potentials. *Nature (Lond.)* **329**:719–721 (1987).
30. Winiger, B. P., and W. Schlegel. Rapid transient elevations of cytosolic calcium triggered by thyrotropin releasing hormone in individual cells of the pituitary line GH_3B_6. *Biochem. J.* **255**:161–167 (1988).
31. Benham, C. D. Voltage-gated and agonist-mediated rises in intracellular Ca^{2+} in rat clonal pituitary cells (GH_3) held under voltage clamp. *J. Physiol. (Lond.)* **415**:143–158 (1989).
32. Berridge, M. J. Inositol trisphosphate-induced membrane potential oscillations in *Xenopus* oocytes. *J. Physiol. (Lond.)* **403**:589–599 (1988).
33. Wakui, M., B. V. L. Potter, and O. H. Peterson. Pulsative intracellular calcium release does not depend on fluctuations in inositol trisphosphate concentration. *Nature (Lond.)* **339**:317–320 (1989).
34. Berridge, M. J., and A. Galione. Cytosolic calcium oscillators. *FASEB J.* **2**:3074–3082 (1988).
35. Muallem, S., S. J. Pandol, and T. G. Beeker. Hormone evoked calcium release from intracellular stores is a quantal process. *J. Biol. Chem.* **264**:205–212 (1989).
36. Taylor, C. W., and B. V. L. Potter. The size of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores depends on inositol 1,4,5-trisphosphate concentration. *Biochem. J.* **266**:189–194 (1990).
37. Missiaen, L., C. T. Taylor, and M. J. Berridge. Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores. *Nature (Lond.)* **352**:241–244 (1991).
38. Gershengorn, M. C. Mechanism of thyrotropin releasing hormone stimulation of pituitary hormone secretion. *Annu. Rev. Physiol.* **48**:515–526 (1986).
39. Holl, R. W., M. O. Thorner, G. L. Mandell, J. A. Sullivan, Y. N. Sinha, and D. A. Leong. Spontaneous oscillations of intracellular calcium and growth hormone secretion. *J. Biol. Chem.* **263**:9682–9685 (1988).
40. Schonbrunn, A., and A. H. Tashjian. Characterisation of functional receptors for somatostatin in rat pituitary cells in culture. *J. Biol. Chem.* **253**:6473–6483 (1978).
41. Schlegel, W., F. Wuarin, C. B. Wollheim, and G. R. Zahnd. Somatostatin lowers the cytosolic free Ca^{2+} concentration in clonal rat pituitary cells (GH_3 cells). *Cell Calcium* **5**:223–226 (1984).

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