Time-dependent effects of lithium on the agonist-stimulated accumulation of second messenger inositol 1,4,5-trisphosphate in SH-SY5Y human neuroblastoma cells

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In order to approach the molecular mechanism of Li^+'s mood-stabilizing action, the effect of Li^+ (LiCl) on inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] mass was investigated in human neuroblastoma SH-SY5Y cells, which express muscarinic M_3 receptors, coupled to PtdIns hydrolysis. Stimulation of these cells, with the cholinergic agonist acetylcholine, resulted in a rapid and transient increase in Ins(1,4,5)P_3 with a maximum at 10 s. This was followed by a rapid decline in Ins(1,4,5)P_3 within 30 s to a plateau level above baseline, which gradually declined to reach a new steady state, which was significantly higher than resting Ins(1,4,5)P_3 at 30 min. Li^+ had no effect on Ins(1,4,5)P_3 in resting cells, as well as on the acetylcholine-dependent peak of Ins(1,4,5)P_3. However, Li^+ caused a transient reduction (at 45 s), followed by a long lasting increase in the Ins(1,4,5)P_3 (30 min), as compared with controls. The Li^+ effects were dose-dependent and were observed at concentrations used in the treatment of bipolar disorders. Supplementation with inositol had no effect on the level of Ins(1,4,5)P_3, at least over the time periods studied. Stimulation of muscarinic receptors with consequent activation of phospholipase C were necessary for the manifestation of Li^+ effects in SH-SY5Y cells. Li^+ did not interfere with degradation of Ins(1,4,5)P_3 after receptor-blockade with atropine, suggesting that Li^+ has no direct effect on the Ins(1,4,5)P_3-metabolizing enzymes. A direct effect of Li^+ on the phospholipase C also is unlikely. Blockade of Ca^{2+} entry into the cells by Ni^{2+}, or incubation with EGTA, which reduces agonist-stimulated accumulation of Ins(1,4,5)P_3, had no effect on the Li^+-dependent increase in Ins(1,4,5)P_3.

INTRODUCTION

The unique property of Li^+ in the treatment of manic-depressive disorders is well established; however, the molecular mechanism of its mood-stabilizing effects remains unclear. Diverse hypotheses have been proposed to explain lithium's therapeutic action (see recent review [1]), including inhibition of neurotransmitter-stimulated adenylate cyclase [2,3], inhibition of the binding of GTP to G-proteins [4], increases in diacylglycerol [5] and thus activation of protein kinase C [6], effects on gene transcription [7], and effects on Na^+,K^+-ATPase [8].

The most widely accepted is the 'inositol depletion' hypothesis, involving effects of Li^+ on the phosphoinositide signalling system [9,10]. This hypothesis postulates that lithium's inhibition of the inositol monophosphatase [11,12] and, to a lesser extent, inositol polyphosphate 1-phosphatase [13], traps inositol in the form of inositol monophosphates as well as Ins(1,4)P_2 and Ins(1,3,4)P_3 and creates a shortage of inositol as substrate for regeneration of phosphoinositides. This in turn leads to a fall in Ins(1,4,5)P_3 and disruption of the signalling in the over-reactive neurons [9,10].

A majority of the data in support of the 'inositol depletion' hypothesis [14–17], as well as the data arguing against it [18–20], were obtained on cerebral cortex slices from laboratory animals [14–16,18–20] or cultured cells of non-neuronal origin [17]. However, brain slices lose a significant amount of cellular inositol on incubation (up to 80% for rat brain slices [21,22]), and therefore, artifactual inositol 'depletion' may be created. Use of the cells of non-neuronal origin is also limited, since the activity of the PtdIns signalling system, as well as the level of cellular inositol and mechanisms maintaining this level, is tissue specific.

In the present study, we have examined the effects of LiCl on Ins(1,4,5)P_3 levels in SH-SY5Y human neuroblastoma cells. These cells are a pure neuroblastoma subclone [23,24], express muscarinic receptors (predominantly M_3 type, [25]) coupled to PtdIns hydrolysis [26], as well as nerve-growth factor receptors [27], μ and δ opioid receptors [28], G-proteins [29], and protein kinases [30] similar in pattern to those in brain cortex, and a variety of ion channels [31–33].

We report here that, starting with therapeutic concentrations, Li^+ has a biphasic effect on the agonist-dependent accumulation of Ins(1,4,5)P_3 in SH-SY5Y cells. These effects consist of a transient reduction, followed by a long lasting increase in Ins(1,4,5)P_3 compared with controls. Reduction of Ins(1,4,5)P_3 was observed under conditions where Li^+ causes only a moderate increase in the inositol mono- and inositol bis-phosphates. Supplementation with exogenous inositol had no effect on the level of Ins(1,4,5)P_3, indicating that the mechanism of the Li^+-dependent reduction of Ins(1,4,5)P_3 is not due to inositol depletion. Stimulation of muscarinic receptors with consequent activation of phospholipase C (PLC) are necessary for the manifestation of Li^+ effects in SH-SY5Y cells. Li^+ did not interfere with degradation of Ins(1,4,5)P_3 after receptor-blockade with atropine, suggesting that Li^+ has no direct effect on the Ins(1,4,5)P_3-metabolizing enzymes. A direct effect of Li^+ on the PLC also is unlikely. We found that blocking of Ca^{2+} entry into the cells, which reduces agonist-stimulated accumulation of Ins(1,4,5)P_3, had no effect on the Li^+-dependent increase in

Abbreviations used: Ins1:2-cyclic(4,5)P_3, *myo*-inositol 1:2 cyclic,4,5-trisphosphate; DPBSH, Dulbecco's phosphate saline supplemented with Hepes; ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular free calcium concentration; PLC, phospholipase C; PCA, perchloric acid; FBS, fetal bovine serum; DPBS, Dulbecco's phosphate-buffered saline.

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Ins(1,4,5)P_3 level. A preliminary account of part of this study has appeared elsewhere [34].

EXPERIMENTAL

Materials

Dulbecco's modified minimal essential medium, non-essential amino acids, Ham's F12 nutrient mixture, fetal bovine serum (FBS), trypsin, Dulbecco's phosphate-buffered saline (DPBS), acetylcholine (ACh), ezerine, penicillin G, streptomycin sulphate, BSA, EDTA, atropine, Trypan Blue, Hepes, LiCl, and all other salts were supplied by Sigma Chemical Co. [^{3}H]Ins(1,4,5)P_3 (17–25 Ci/mmol) and *myo*-[2-^{3}H]inositol (15–17 Ci/mmol) was purchased from NEN/DuPont. D-Ins(1,4,5)P_3 was from LC Laboratories. Cell culture plasticware was obtained from Fisher Scientific.

Cells, cell culture methods and drug pretreatment

Cells of the human adrenergic neuroblastoma cell line SH-SY5Y, passages 19 and 42 (initial stock kindly provided by Dr. June Biedler, Sloan-Kettering Institute for Cancer Research, Rye, NY, U.S.A.), were cultured in a 1:1 mixture of Ham's F12 nutrients and Dulbecco's modified minimal essential medium supplemented with 10% FBS, 100 units/ml of penicillin G, 100 μg/ml of streptomycin sulphate and 0.3% glutamine at 37 °C in 5% CO_2 in a humidified incubator. When 90–100% confluency was achieved, cells were harvested by preincubation with Ca^{2+}/Mg^{2+}-free DPBS containing 1 mM EDTA for 5 min at 37 °C and subcultured in vented cell culture flasks (25–150 cm^3) at a splitting ratio of 1:6. Usually, cells were allowed to grow for 1 week and the medium was changed once during this time. Because the SH-SY5Y human neuroblastoma cell line can undergo spontaneous transformation [24], subculturing was limited to no more than 10 passages.

For mass measurement of Ins(1,4,5)P_3 in cell suspensions, 90% confluent SH-SY5Y cells were harvested by treatment with Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffer containing 5 mM EDTA, sedimented by centrifugation at 500 *g* for 3 min, and resuspended in Dulbecco's phosphate–Hepes buffer (DPBSH; 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.4), centrifuged again at 500 *g* for 2 min, and finally resuspended in DPBSH. Cell suspensions were then divided into portions (0.3–0.7 mg of cell protein), and 0.2 vol. of DPBSH or DPBSH-Li (NaCl replaced by LiCl) was added to achieve a final concentration of LiCl of 0–50 mM. Cells were incubated at 37 °C for 30 min, challenged with ACh (supplemented with 100 μM ezerine), and the incubation was continued for various periods of time as specified in the Figure legends.

For the measurement of different [^{3}H]inositol phosphates in a monolayer attached to substrate cells, *myo*-[2-^{3}H]inositol was added directly to the growth medium in 25 cm^2 cell culture flasks (7–10 μCi/ ml), and cells were maintained at standard conditions for 48–72 h. After labelling, the medium was removed, and cells were washed twice with 5 ml of DPBSH buffer and then incubated in 2 ml of DPBSH or DPBSH-Li^+ buffer for 30 min at 37 °C. In some experiments, cells were challenged with 100 μM ACh (an equal volume of DPBSH was added to control cells), and the incubation was continued for another 30 min.

Termination of the reaction and extraction of inositol phosphates

At the end of incubation, the reactions were terminated by addition of an ice-cold solution containing 15% perchloric acid (PCA) and 20 mM EDTA (final concentration: 3% PCA/4 mM EDTA), and Eppendorf tubes or cell culture flasks were placed on ice. After 10 min, the PCA extracts from flasks were transferred to the glass siliconized tubes, cells were rinsed with 1 ml of ice-cold 3% PCA/4 mM EDTA, and extracts were combined. Acid-insoluble material was removed by centrifugation (10000 *g* for 7 min), dissolved in 0.1 M NaOH, and used for cell protein determination. For adherent cells, this material was combined with that remaining in the flask (< 95%), dissolved in 0.1 M NaOH, and used for determination of cell protein and radioactivity in total phosphoinositides.

The acid extracts were neutralized with KOH in the presence of 5 mM EDTA, 10 mM $NaHCO_3$, and 5 μl of pHydrion indicator solution (range 5–11). The neutralized mixtures were centrifuged at 10000 *g* for 7 min to remove $KClO_4$ and subsequently stored at −20 °C. Before Ins(1,4,5)P_3 mass measurement or application of the ^{3}H-labelled samples on the HPLC column, a further accumulation of $KClO_4$was removed by centrifugation (7 min, 10000 *g*).

For the measurements of acid-labile *myo*-inositol 1:2 cyclic, 4,5-trisphosphate [Ins1:2cyclic(4,5)P_3], a modified boiling extraction method was used [35]. ^{3}H-Labelled cells were pretreated as described above, except that at the end of the incubation the volume of the buffer was reduced by 2-fold. Reactions were stopped by addition of 2 ml of hot water (> 90 °C) and immediately transferred to a water bath at > 90 °C for 7 min. Subsequently, samples were supplemented with sodium phytate (final concentration, 50 μg/ml, pH 7.2), cooled on ice for 10 min, and cell debris were removed by centrifugation (at 11000 *g* for 10 min, 4 °C). Supernatants were frozen and stored at −20 °C until analysis.

Mass measurement of Ins(1,4,5)P_3

Ins(1,4,5)P_3 mass was measured by a radioreceptor assay using a liver binding protein prepared as described by Donié and Reiser [36]. Aliquots of neutralized extracts (100 μl; pH 7.0–7.5) were added to tubes containing 100 μl of 0.35 nM D-[^{3}H]Ins(1,4,5)P_3, and the assay was initiated by addition of 200 μl of binding protein (1.0 mg) in buffer [25 mM Tris/HCl, pH 9.0; 1 mM EDTA, 2.5 mg/ml BSA (final concentrations)]. After incubation for 15 min at 4 °C, bound [^{3}H]Ins(1,4,5)P_3 was separated from the reaction mixture by centrifugation (14000 *g* for 3 min) through a 0.5 ml cushion of 5% (w/v) sucrose in 25 mM Tris/HCl, pH 9.0. After aspiration of the supernatant, the pellet (binding protein) was dispersed in 0.35 ml of water, transferred to 8-ml glass vials, and radioactivity was determined by liquid scintillation counting in 6.5 ml of Polyfluor, a biodegradable scintillation mixture with solubilizer. Non-specific binding (usually less then 10% of total binding) was determined with 2.5 μM 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, and only the linear part of this curve was used for determination of Ins(1,4,5)P_3. Buffers and drugs used in our experiments, taken through an extraction procedure, had no effect on [^{3}H]Ins(1,4,5)P_3 binding.

HPLC analysis of ^{3}H-inositol phosphates

Neutralized aqueous extracts (3–4 ml; pH 7.0–7.5) were applied to a Whatman Partisil SAX analytical column (4.6 mm × 25 cm; Clifton, NJ, U.S.A.), and the various inositol phosphate isomers were separated with an elution programme based on the method of Dean and Moyer [37]. This programme consisted of a series of isocratic elutions and linear gradients of $NH_4H_2(PO_4)$, pH 3.8, as follows: 0 min, water; 5 min, 10 mM [in $NH_4H_2(PO_4)$]; 53 min,

10 mM; 58 min, 160 mM; 113 min, 600 mM; 143 min, 800 mM; 143 min, 1.75 M; 188 min, 1.75 M. The column was then washed with water for 60 min before its next use. An automated HPLC system with radioactive peak detection was used in these studies. For quantitative determination of the radioactivity, collected peaks were re-counted in a Packard 2000 liquid scintillation counter. Separation of isoforms has been carefully validated by co-elution with authentic ^{3}H-labelled inositol polyphosphate standards in separate runs.

Miscellaneous

Protein was measured by the microtitre protocol of the Pierce BCA Protein Assay (Pierce Chemical Co.), using BSA as a standard. The samples were incubated at 37 °C for 2 h and read at 590 nm.

Cells were counted with a haemocytometer, and cell viability was determined by the Trypan Blue exclusion test.

Data analysis

Data were expressed as means ± S.E.M. values from an experiment performed in triplicate, representative of at least three independent experiments with similar results. Statistical significance was assessed by Student's t test and considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Effects of Li^+ on Ins(1,4,5)P_3 accumulation in SH-SY5Y cells in the presence and absence of ACh

Our preliminary experiments established that incubation of SH-SY5Y cells in Dulbecco's phosphate/Hepes-buffered saline supplemented with 10 mM glucose and containing different concentrations of LiCl [0.5–25 mM (NaCl was reduced to maintain isotonicity)] for 2.5 h at 37 °C maintained viability of the cells, as determined by the Trypan Blue exclusion test (results not shown). After 3 h of incubation, there was about a 5% reduction in viable cells in LiCl-treated cells as well as in control cells. The total incubation time in most of our subsequent experiments was reduced to 1 h (2.5 h in a few experiments). We found that the level of Ins(1,4,5)P_3 in control resting SH-SY5Y cells remained constant for at least 2.0 h (Figure 1).

The application of a maximal dose of ACh (100 μM) to intact SH-SY5Y cells resulted in a rapid increase in the Ins(1,4,5)P_3 mass, peaking at 172 ± 5 pmol/mg of cell protein at approximately 10 s. The peak level was 17 times the basal level (Figure 1). This was followed by a rapid decline in Ins(1,4,5)P_3 within 30 s to a plateau level above baseline, which gradually declined to reach a new steady state, which was significantly higher than resting Ins(1,4,5)P_3 at 30 min. Thereafter, the concentration of Ins(1,4,5)P_3 was maintained virtually at the same level throughout the remainder of the experiment (Figure 1). Dose–response curves conducted at 10 s and 30 min of ACh stimulation revealed that the peak and plateau Ins(1,4,5)P_3 levels had EC_{50} values of 12.7 ± 1.2 μM and 8.9 ± 1.0 μM respectively. Pretreatment with atropine sulphate (1 μM final concentration) completely blocked both peak and plateau responses, whereas treatment during the plateau phase caused a rapid reduction in Ins(1,4,5)P_3, indicating that both peak and plateau responses require continuous ACh stimulation. The accumulation of Ins(1,4,5)P_3 resulting from muscarinic receptor activation in our experiments is similar to that previously reported in SK-N-SH [38] and SH-SY5Y human neuroblastoma cells [25,39,40].

Incubation with 0.5–25 mM Li^+ had no effect on the Ins(1,4,5)P_3 level in the resting cells (Figure 1). The presence of Li^+ (0.5–25 mM, 30 min, 37 °C) had no statistically significant effect on the initial Ins(1,4,5)P_3 peak upon agonist application. However, initially the decay of the Ins(1,4,5)P_3-peak was faster in the presence of Li^+, leading to a lower level of Ins(1,4,5)P_3 than in control cells at 45 s (Figure 1). However, this was followed by a gradual fall in Ins(1,4,5)P_3, and after 30 min of agonist stimulation the Ins(1,4,5)P_3 level reached a new steady state which was significantly higher in Li^+-treated cells than in control cells, and this persisted for at least 2 h (Figure 1).

Effect of Li^+ on the level of [^{3}H]inositol phosphates

In order to determine the effect of Li^+ on different inositol phosphate isomers, we used the [^{3}H]inositol prelabelling technique and separation of individual isomers by HPLC (Table 1). In these experiments we have examined monolayers of cells attached to the surface of cell culture flasks, which minimized

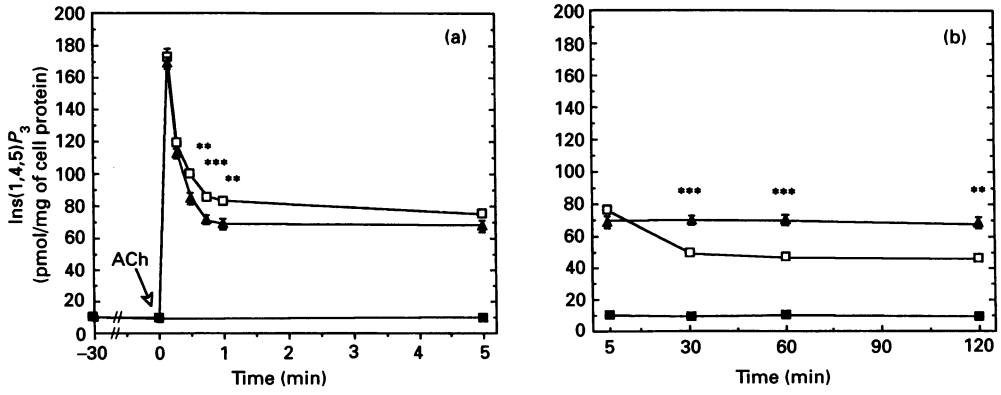

Figure 1 Effect of LiCl on the time-dependent accumulation of Ins(1,4,5)P_3 in control and ACh-stimulated human neuroblastoma SH-SY5Y cells

Cells (0.3–0.4 mg of protein) were preincubated in DPBSH buffer at 37 °C for 30 min with (■, ▲) or without (□) LiCl (10 mM) and were challenged with ACh (100 μM; □, ■). Samples were quenched at various periods of time by addition of ice-cold PCA, and Ins(1,4,5)P_3 was extracted and measured as described under the Experimental section. Data are means ± S.E.M. values from an experiment performed in triplicate, representative of at least three independent experiments with similar results. The P values of the difference between Li^+-treated and control samples were *** $P < 0.001$; ** $P < 0.005$.

Table 1 Effect of ACh and/or LiCl on the accumulation of [^{3}H]inositol phosphates in SH-SY5Y cells

Monolayers of cells, prelabelled with *myo*-[^{3}H]inositol (48–72 h, 10 μCi/ml), were incubated in DPBSH buffer with or without Li^+ (10 mM) for 30 min at 37 °C and challenged with ACh (100 μM, 45 s or 30 min, 37 °C). Samples were quenched at various times by PCA or boiling, as described in the Experimental section. Inositol phosphate isomers were separated by HPLC. Background radioactivity was subtracted from each fraction. Data are means ± S.E.M. values of three experiments performed in quadruplicate.

		Radioactivity (d.p.m.)					
		(−) ACh		ACh (45 s)		ACh (30 min)	
Inositol phosphate	Extraction	Na^+	Li^+	Na^+	Li^+	Na^+	Li^+
Monophosphates	PCA	19938 ± 1306	43242 ± 1212	54072 ± 1921	60276 ± 1872	74907 ± 2903	639936 ± 14521
Bisphosphates	PCA	12177 ± 977	14409 ± 1052	47160 ± 2151	60009 ± 2466	41562 ± 3142	173391 ± 5922
Ins(1,3,4)P_3	PCA	411 ± 63	519 ± 75	5937 ± 521	6210 ± 407	2259 ± 306	4056 ± 292
Ins(1,4,5)P_3	PCA	1392 ± 171	1453 ± 242	6686 ± 238	5374 ± 277	5171 ± 211	6818 ± 254
Ins(1,3,4,5)P_4	PCA	1644 ± 260	1710 ± 243	7257 ± 421	6798 ± 372	8609 ± 460	11064 ± 443
Ins(1,4,5)P_3	Boiling	1042 ± 101	1105 ± 128	6082 ± 265	4726 ± 251	3352 ± 274	4339 ± 193
Ins(c1-2,4,5)P_3	Boiling	390 ± 74	432 ± 59	477 ± 81	562 ± 64	1867 ± 108	2322 ± 132

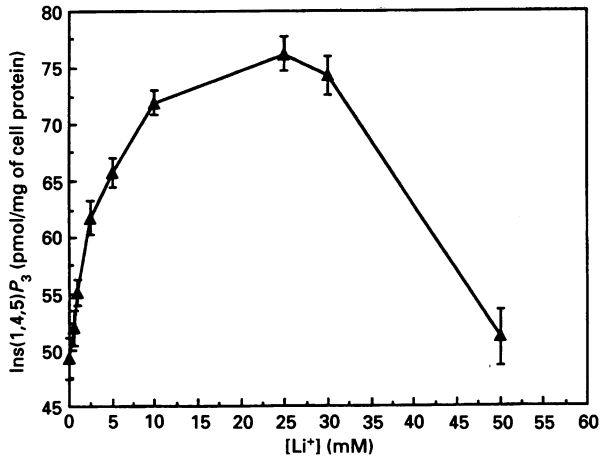

Figure 2 Concentration dependence of Li^+ effects on the level of Ins(1,4,5)P_3 in SH-SY5Y cells

Cells (0.3–0.4 mg of protein) were preincubated at 37 °C for 30 min in DPBSH buffer with or without LiCl at indicated concentrations and challenged with ACh (100 μM, 30 min at 37 °C). Samples were quenched by addition of ice-cold PCA, and Ins(1,4,5)P_3 was extracted and measured as described under the Experimental section. Data are means ± S.E.M. values from an experiment performed in triplicate, representative of at least three independent experiments with similar results. The *P* values of the difference between the control samples and the Li^+-treated samples at all Li^+ concentrations (except 0.5 mM and 50 mM) were < 0.005; for [Li^+] at 0.5 mM and 50 mM, *P* 0.137.

any possible side effects caused by cell damage during harvesting. (Similar results were obtained on suspension of [^{3}H]inositol-prelabelled cells). For such analysis, we chose two incubation times (45 s and 30 min), at which Li^+-dependent reduction and steady-state increase of Ins(1,4,5)P_3, respectively, were observed. In confirmation of the results of others [26,38,39], radioactivity eluting with characteristics of [^{3}H]Ins1*P*/[^{3}H]Ins3*P*/[^{3}H]Ins4*P*, [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,3,4)P_3, and [^{3}H]Ins(1,3,4,5)P_4 were all significantly increased by muscarinic receptor stimulation in a time-dependent manner (Table 1). The level of [^{3}H]Ins(1,4,5)P_3 was also increased; however, the level of this inositol phosphate at 45 s was higher than at 30 min of agonist stimulation, which confirms the results of Ins(1,4,5)P_3 mass measurements. Pre-treatment with 10 mM Li^+ increased levels of [^{3}H]Ins1*P*/[^{3}H]Ins3*P*/[^{3}H]Ins4*P*, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,3,4)P_3 in the cells challenged with ACh, and to a lesser extent in control cells as reported previously [39,41]. The effects of Li^+ on the agonist-dependent accumulation of the second messenger Ins(1,4,5)P_3 has not been reported before. In agreement with our mass measurements, Li^+ significantly decreased the [^{3}H]Ins(1,4,5)P_3 level at 45 s, and increased accumulation of [^{3}H]Ins(1,4,5)P_3 at 30 min of agonist-stimulation (Table 1). In addition, we observed that Li^+ significantly increased levels of putative second messenger [^{3}H]Ins(1,3,4,5)P_4, which may be involved in the regulation of cellular Ca^{2+} [42]. Under these conditions, the three isomers of InsP_4 [Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, and Ins(3,4,5,6)P_4] co-elute [43]. However, other experiments (G. V. Los, I. P. Artemenko and L. E. Hokin, unpublished work) showed that extracts from Li^+-treated cells had a much greater ability to displace [^{3}H]Ins(1,3,4,5)P_4 from a highly specific binding site. The affinity of this binding site for Ins(1,3,4,6)P_4 and Ins(3,4,5,6)P_4 is two orders of magnitude lower than that of Ins(1,3,4,5)P_4, and thus unrealistic amounts of these isomers would be required to produce the observed results.

Hydrolysis of PtdIns(4,5)P_2 by PLC(s) releases a mixture of Ins(1,4,5)P_3 and Ins1:2cyclic(4,5)P_3 [44,45]. Ins1:2cyclic(4,5)P_3 is formed at a much lower rate than Ins(1,4,5)P_3 and is slowly metabolized by the 5′-phosphatase [46]; however, it can accumulate in cells during persistent stimulation [35,47]. Ins1:2cyclic(4,5)P_3 is acid-labile, and treatment of cells with PCA, a highly reproducible and widely used method for inositol phosphate extraction [48], hydrolyses Ins1:2cyclic(4,5)P_3 and releases a mixture of Ins(1,4,5)P_3 and Ins(2,4,5)P_3 [49]. One would anticipate that part of the Li^+-dependent increase in Ins(1,4,5)P_3 observed in ACh-stimulated cells could be due to the accumulation of Ins1:2cyclic(4,5)P_3. In order to determine the contribution of Ins1:2cyclic(4,5)P_3 to Ins(1,4,5)P_3 in PCA extracts, we analysed accumulation of [^{3}H]InsP_3 in the cells using extraction by boiling (at neutral pH), which allows recovery of intact inositol 1:2 cyclic diester phosphates, and Ins1:2cyclic(4,5)P_3 in particular [35]. [Control experiments showed similar efficiency in extraction of different InsP_3 isomers from SH-SY5Y cells by boiling- or PCA-extraction (G. V. Los, I. P. Artemenko, and L. E. Hokin, unpublished work).]

After 30 min of ACh stimulation, [^{3}H]Ins1:2cyclic(4,5)P_3 accounted for about 39% of the [^{3}H]Ins1:2cyclic(4,5)P_3 + [^{3}H]Ins(1,4,5)P_3 isomer mixture. Therefore, [^{3}H]Ins1:2cyclic-(4,5)P_3 indeed makes a contribution to the [^{3}H]Ins(1,4,5)P_3

Table 2 Effect of 1 mM LiCl on the accumulation of Ins(1,4,5)P_3 in ACh-stimulated human neuroblastoma SH-SY5Y cells

Cells (0.3–0.4 mg of protein) were preincubated at 37 °C for 30 min in DPBSH buffer with or without LiCl at indicated concentrations and challenged with ACh (100 μM, 30 min at 37 °C). Samples were quenched by addition of ice-cold PCA, and Ins(1,4,5)P_3 was extracted and measured as described in the Experimental section. Data are means ± S.E.M. values from an experiment performed in triplicate. [Percentage of lithium-dependent Ins(1,4,5)P_3 increase was calculated after subtraction of Ins(1,4,5)P_3 value in non-stimulated cells.]

	Ins(1,4,5)P_3 content (pmol/mg of cell protein)			
	Control	Li^+ (1 mM)	Increase (%)	*P*
1	49.1 ± 0.7	55.2 ± 1.1	15.5	0.001
2	54.7 ± 1.2	62.4 ± 1.2	17.1	0.002
3	46.4 ± 1.5	51.8 ± 2.1	14.7	0.005

observed in acidic extracts. {[^{3}H]Ins(2,4,5)P_3 is a minor product of [^{3}H]Ins1 : 2cyclic(4,5)P_3 hydrolysis and could be neglected (results not shown).} The level of [^{3}H]Ins(1,4,5)P_3 in neutral extracts was 40% higher in Li^+-treated cells (10 mM) than in control cells. This is in good agreement with the Li^+ effects observed with acidic extraction of [^{3}H]Ins(1,4,5)P_3, as well as Ins(1,4,5)P_3 mass. Li^+ also increased the level of [^{3}H]Ins1 : 2cyclic(4,5)P_3 by 28 ± 3%, which is somewhat smaller than that of [^{3}H]Ins(1,4,5)P_3 (Table 1).

Lithium and ACh dose–response curves

As reported above the transient reduction in Ins(1,4,5)P_3 due to Li^+ was followed by its long lasting increase above control values. This increase is more likely to be related to lithium's therapeutic action, which takes a week or longer to be established. We therefore concentrated our efforts on this latter phase of Li^+ action.

The data in Figure 2 show that the effect of Li^+ on the Ins(1,4,5)P_3 plateau phase in ACh-stimulated human neuroblastoma cells was dose-dependent. Starting at 1.0 mM, Li^+ progressively increased Ins(1,4,5)P_3 accumulation and was maximally effective at 25 mM. The Li^+-dependent increase in Ins(1,4,5)P_3 level is not a result of the reduced Na^+ content (up to 25 mM), because similar effects of Li^+ were observed when Li^+ was added to the buffer with normal Na^+ concentrations (results not shown). In contrast, replacement of 50 mM of Na^+ with 50 mM Li^+ caused a reduction in agonist-dependent Ins(1,4,5)P_3 formation, probably due to lithium's toxicity or the decrease in Na^+ content. The mean EC_{50} (± S.E.M.) for the Li^+ effect was 2.4 ± 0.3 mM. The therapeutic serum concentration of Li^+ in the treatment of bipolar disorders ranges from 0.5 to 1.5 mM. In our experiment, 1 mM Li^+ raised the ACh-dependent increment of Ins(1,4,5)P_3 level only by 15% (Table 2). However, the elevations in brain under therapeutic conditions may not be large; and in fact, larger elevations may indeed be toxic.

We next conducted ACh dose–response curves in control and Li^+-pretreated cells at the plateau phase of Ins(1,4,5)P_3 accumulation (at 30 min). Li^+-dependent increments in Ins(1,4,5)P_3 could be seen at all tested ACh concentrations (Figure 3). The absolute values of these increments increased progressively with increasing ACh concentrations; however, if presented as a percentage of the ACh-dependent accumulation, the increment due to Li^+ remained virtually unchanged at all ACh concentrations. Dose–response curves in the presence and absence of Li^+ show typical sigmoidicity. Li^+ had no effect on the major

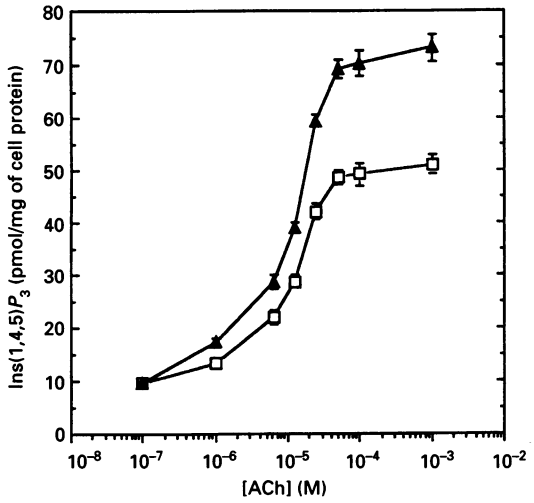

Figure 3 Acetylcholine dose dependence of Ins(1,4,5)P_3 accumulation in control and Li^+-treated SH-SY5Y cells

Cells (0.3–0.4 mg of protein) were preincubated in DPBSH buffer at 37 °C for 30 min with (▲) or without (□) LiCl (10 mM) and challenged with ACh at indicated final concentrations (10^{-6}–10^{-3} M). After 30 min, samples were quenched by addition of ice-cold PCA, and Ins(1,4,5)P_3 was extracted and measured as described in the Experimental section. Data are means ± S.E.M. values from an experiment performed in triplicate, representative of at least three independent experiments with similar results. The *P* values of the difference between Li^+-treated and control samples at all (except 10^{-6} M) ACh concentrations were < 0.005; at 10^{-6} M ACh, $P = 0.05$.

characteristics of the dose–response curve for ACh: the EC_{50} values were 8.9 ± 1.3 μM and 8.7 ± 2.1 μM in control and Li^+-treated cells respectively.

Effects of atropine

The mechanism for the Li^+-dependent increase in Ins(1,4,5)P_3 in agonist-stimulated SH-SY5Y cells is not known. However, we analysed several possibilities to account for the Li^+-dependent long lasting elevation of Ins(1,4,5)P_3. The level of inositol phosphates in cells is a result of the dynamic balance between formation and degradation of these compounds. Increased Ins(1,4,5)P_3 levels could result from either activation of PtdIns(4,5)P_2 hydrolysis by PLC or inhibition of dephosphorylation, most probably by the 5-phosphatase, or phosphorylation to Ins(1,3,4,5)P_4, followed by dephosphorylation [50].

We approached the mechanism of the Li^+-dependent elevation of Ins(1,4,5)P_3 in human neuroblastoma cells by analysis of the kinetics of decay on atropine quenching. Briefly, inositol phosphate accumulation was stimulated to an elevated steady state by ACh in the absence or presence of Li^+, and the muscarinic antagonist atropine was then added to stop accumulation. This provides a measure of Ins(1,4,5)P_3 metabolism (degradation).

If Li^+ causes elevation of Ins(1,4,5)P_3 by inhibition of one (or more) of the phosphatases, as has been shown for accumulation of inositol monophosphates, the rate of Ins(1,4,5)P_3 decay in the presence of Li^+ should be lower than in control cells. Figure 4 shows the time-dependent disappearance of Ins(1,4,5)P_3 in the absence and presence of Li^+ (10 mM) in ACh-stimulated (100 μM) cells. Atropine sulphate (1–10 μM) was added to the cell suspensions after 30 min of agonist stimulation, and the decay of Ins(1,4,5)P_3 was examined. The Ins(1,4,5)P_3 level rapidly decreased within 10 s. This was followed by a slower decay of Ins(1,4,5)P_3 to the basal level within 2 min. Similar dynamics

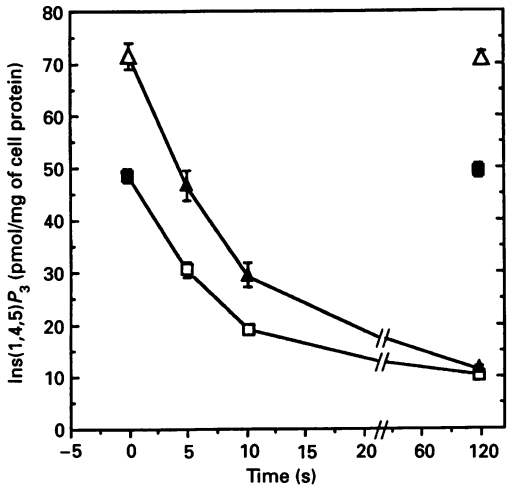

Figure 4 Effect of Li^+ on the rate of disappearance of Ins(1,4,5)P_3 after atropine blockade of ACh-stimulated SH-SY5Y cells

Cells (0.3–0.4 mg of protein; 0.5 ml suspension) were preincubated at 37 °C for 30 min in DPBSH buffer with (△, ▲) or without (□, ■) LiCl (10 mM), challenged with ACh (100 μM), and the incubation continued. After 30 min (0 s on the graph) aliquots of atropine sulphate (50 μl, final concentration 1–10 μM; ▲, □) or equal aliquots of Dulbecco's buffer were added (△, ■). The samples were then quickly mixed and quenched by addition of ice-cold PCA at indicated times. Ins(1,4,5)P_3 was extracted and measured as described in the Experimental section. Data are means ± S.E.M. values of quadruplicate determinations, representative of at least three independent experiments with similar results. The P values of the difference between the control samples and the Li^+-treated samples at all time points (except 120 s of atropine treatment) were < 0.001. The P values of the difference between time points of atropine addition: 0, 5, 10, and 120 s in the control groups, as well as in the Li^+-pretreated cells, were < 0.001. The level of Ins(1,4,5)P_3 in the cells incubated without atropine remained constant.

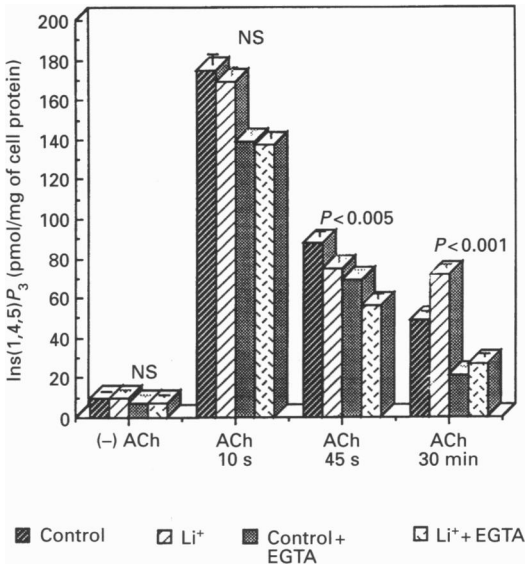

Figure 5 Effect of EGTA on the time-dependent accumulation of Ins(1,4,5)P_3 in ACh-stimulated human neuroblastoma SH-SY5Y cells

Cells (0.3–0.4 mg of protein) were preincubated in DPBSH buffer at 37 °C for 30 min with or without LiCl (10 mM) and challenged with ACh (100 μM). In some experiments, 3 mM EGTA (pH 7.4) was added to the cells 45 s before agonist stimulation. Samples were quenched at indicated periods of time by addition of ice-cold PCA, and Ins(1,4,5)P_3 was extracted and measured as described in the Experimental section. Data are means ± S.E.M. values from an experiment performed in triplicate, representative of at least three independent experiments with similar results. The P values of the difference between control samples and samples incubated with EGTA (with or without Li^+) were $P < 0.005$. The P values of the difference between control samples and samples incubated with Li^+ are indicated on the graph.

were observed in Li^+-treated cells: the steady-state Ins(1,4,5)P_3 level fell within 10 s and was completely abolished by 2 min. Attempts were made to slow the rate of decay by incubation at room temperature in the hope that a Li^+ effect, if it exists, could more easily be seen, but similar results were obtained as at 37 °C (results not shown). The results underline the complexity of Ins(1,4,5)P_3 metabolism and argue against the possibility that Li^+ affects the rate of Ins(1,4,5)P_3 degradation in neuroblastoma cells. Connolly et al. [51] and Shears et al. (for a review, see [50]) found no direct inhibition by Li^+ of at least one of the key enzymes of Ins(1,4,5)P_3 metabolism, Ins(1,4,5)P_3-5-phosphatase. It is also unlikely that Li^+ significantly inhibited the Ins(1,4,5)P_3-3-kinase since we found Li^+-stimulated accumulation of Ins(1,3,4,5)P_4 of the same magnitude as that of Ins(1,4,5)P_3. It should be noted that the initial absolute rate of Ins(1,4,5)P_3 degradation was higher in Li^+-treated than in control cells (24 ± 1.7 pmol/5 s per mg versus 17 ± 1.3 pmol/5 s per mg; Figure 4). An estimation based on Ins(1,4,5)P_3 mass measurements and cell volume (10 μl per mg of cell protein) suggested agonist-dependent concentrations of Ins(1,4,5)P_3 in SH-SY5Y cells in the 'plateau phase' to be about 4 μM in control cells and about 6 μM in Li^+-treated cells. The K_m of Ins(1,4,5)P_3-metabolizing enzymes is in the same micromolar range [52], so the concentration of substrate is probably the rate-limiting factor in its degradation.

Another possible enzymic mechanism is suggested by the observation that low concentrations of Li^+ stimulate PLC in PC 12 cells pretreated with nerve growth factor [53]. In preliminary experiments with guinea-pig and rhesus monkey cerebral cortex preparations (G. V. Los and L. E. Hokin, unpublished work), Li^+ had no effect on membrane or cytosolic PLC activity *in vitro*, arguing against a direct effect of Li^+ on these enzymes.

Effect of extracellular Ca^{2+} on the level of Ins(1,4,5)P_3 in Li^+-treated neuroblastoma cells

One factor which could impact on the Li^+ effect is intracellular free Ca^{2+}, which plays an important role in the regulation of PtdIns signalling [54]. Stimulation of muscarinic receptors expressed in SH-SY5Y cells leads to increased turnover of membrane phosphoinositides (this report; [25,39]) and increased intracellular free Ca^{2+} [25] as a result of the release of Ca^{2+} from intracellular Ins(1,4,5)P_3-sensitive stores as well as increased Ca^{2+} entry across the plasma membrane [25,32]. It has also been suggested [55] that Li^+, by blocking the delayed rectifier potassium conductance (I_k), may prolong the duration of action potentials, causing depolarization [56], which activates voltage-sensitive Ca^{2+} channels, thereby raising agonist-dependent levels of ionized Ca^{2+} in the cells.

In order to probe the role of Ca^{2+} in Li^+-enhanced Ins(1,4,5)P_3 levels the cells were incubated in DPBSH buffer supplemented with the 3 mM Ca^{2+} chelator, EGTA. Under these conditions, the Ca^{2+} concentration is reduced from 1.3 mM to ~ 200 nM. In agreement with previous reports [39], we found that omission of Ca^{2+} had only marginal effects on basal Ins(1,4,5)P_3. At the same time, the initial ACh-dependent Ins(1,4,5)P_3 peak, as well as the level of Ins(1,4,5)P_3 in the plateau phase (Figure 5), was significantly reduced, indicating the involvement of extracellular Ca^{2+}

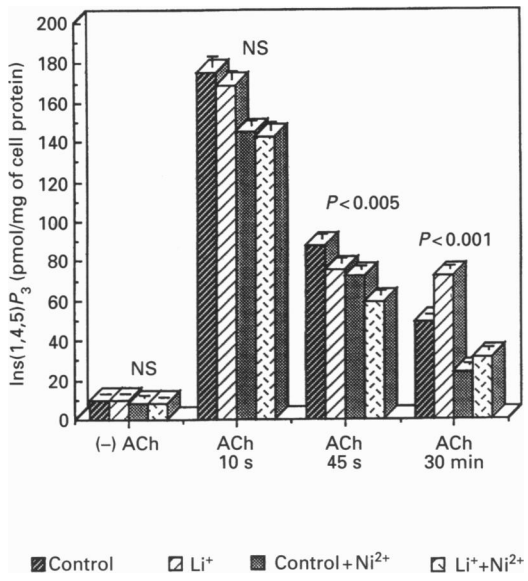

Figure 6 Effect of $NiCl_2$ on the time-dependent accumulation of Ins(1,4,5)P_3 in ACh-stimulated human neuroblastoma SH-SY5Y cells

Cells (0.3–0.4 mg of protein) were preincubated in DPBSH buffer at 37 °C for 30 min with or without LiCl (10 mM) and challenged with ACh (100 μM, 30 min at 37 °C). In some experiments, 2.5 mM $NiCl_2$ was added to the cells 45 s before agonist stimulation. Samples were quenched by addition of ice-cold PCA, and Ins(1,4,5)P_3 was extracted and measured as described in the Experimental section. Data are means ± S.E.M. from an experiment performed in triplicate, representative of at least three independent experiments with similar results. The P values of the difference between control samples and samples incubated with $NiCl_2$ (with or without Li^+) were $P < 0.005$. The P values of the difference between control samples and samples incubated with Li^+ are indicated on the graph.

in the cell response to ACh. Removal of Ca^{2+} had a similar effect on the level of Ins(1,4,5)P_3 in Li^+-pretreated cells (Figure 5). However, Li^+-dependent reduction of Ins(1,4,5)P_3 level at 45 s, as well as amplification of Ins(1,4,5)P_3 at 30 min of agonist stimulation remained statistically significant.

Similar results were obtained when neuroblastoma cells were co-incubated with 2.5 mM Ni^{2+}, which prevents entry of Ca^{2+} into neuroblastoma cells by blocking Ca^{2+} channels [25]; Ni^{2+} reduced the initial ACh-dependent Ins(1,4,5)P_3 peak, as well as the level of Ins(1,4,5)P_3 in the plateau phase (Figure 6). However, the Li^+-dependent reduction of Ins(1,4,5)P_3 level at 45 s, as well as amplification of Ins(1,4,5)P_3 level at 30 min of agonist stimulation, remained statistically significant.

Therefore, our data so far suggest that in spite of the importance of Ca^{2+} influx in agonist-dependent Ins(1,4,5)P_3 accumulation in neuronal cells, the resting $[Ca^{2+}]_i$ level or Ca^{2+} released from intracellular stores is probably sufficient for manifestation of the Li^+ effects.

The long-lasting Li^+-dependent increase of Ins(1,4,5)P_3 in agonist-stimulated human neuroblastoma cells is in apparent contrast with the data supporting the 'inositol depletion' hypothesis. The lithium-dependent transient reduction in Ins(1,4,5)P_3 levels in our experiments also does not appear to be due to inositol depletion. This reduction was observed at early times of agonist stimulation, when Li^+ had trapped only a moderate fraction of the inositol phosphates (Table 1), and supplementation of the cells with inositol over a broad range of concentrations (0.01–10 mM) had no effect on this reduction (results not shown). [Supplementation of the cells with inositol also had no effect on the Ins(1,4,5)P_3 level in the resting or ACh-stimulated SH-SY5Y cells treated or not with LiCl over the entire duration of our experiments (results not shown)]. This is in good agreement with the fact that human neuroblastoma cells SK-N-SH (parental to SH-SY5Y cells line) concentrate inositol from an extracellular medium, achieving comparably high inositol levels (60 ± 4 nmol/mg of protein), and, in contrast to rodent brain slices, these cells retain their inositol even after extensive washing in inositol-free buffer [22].

Our results indicate that Li^+ has significant effects on the PtdIns signalling system, even when the level of the cellular inositol is not a rate-limiting factor for resynthesis of PtdIns. This is supported by several observations. First, the results of human brain biopsy studies, as well as recent results of localized proton magnetic resonance spectroscopy *in vivo*, show that concentration of free *myo*-inositol in grey matter of human brain is 6.2 ± 1.1 mM [57,58]. Secondly, a K_m value for inositol for PtdIns synthase in a crude particulate fraction from rat cerebral cortex is 0.91 ± 0.04 mM [59]. The PtdIns synthase from placenta (the only human enzyme described so far) displayed a K_m of 0.28 mM [60]. The concentration of the brain inositol is thus significantly higher then the K_ms for PtdIns synthase. Therefore, in order to affect activity of PtdIns synthase the level of cellular inositol has to be dramatically reduced. However, chronic or even high acute doses of Li^+ produce relatively small decreases in inositol levels in the brain of laboratory animals *in vivo* [61].

In conclusion, the results presented here demonstrate that in SH-SY5Y human neuroblastoma cells Li^+ has biphasic effects on Ins(1,4,5)P_3 levels. These effects consist of a transient reduction, followed by a long lasting increase in the Ins(1,4,5)P_3 level as compared with controls. The Li^+ effects are dose dependent, and were observed at concentrations used in the treatment of bipolar disorders, which may have therapeutic implications. The mechanism of the Li^+ effect on Ins(1,4,5)P_3 accumulation requires further investigation. Our data do not support an inhibition of Ins(1,4,5)P_3 degradation by Li^+. It appears that a role for agonist-dependent Ca^{2+} entry into the cells through the Ni^{2+}-sensitive channels can be also ruled out. A direct effect of Li^+ on PLC activity could not be demonstrated (at least in brain cortex *in vitro*). However, our results show that manifestation of Li^+ effects in SH-SY5Y cells requires stimulation of muscarinic receptors and activation of PLCs. These enzymes are under complex control by G-proteins [62], as well as protein kinase C [63], and an indirect effect of Li^+ on PLCs has to be considered.

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REFERENCES

1 Jope, R. S. and Williams, M. B. (1994) Biochem. Pharmacol. **47**, 429–441
2 Newman, M. E. and Belmaker, R. H. (1987) Neuropharmacology **26**, 211–217
3 Mork, A., Geisler, A. and Hollund, P. (1992) Pharmacol. Toxicol. **71**, (Suppl. 1) 4–17
4 Avissar, S. and Schreber, G. (1989) Biol. Psychiatry **26**, 113–130
5 Brami, B. A., Leli, U. and Hauser, G. (1991) Biochem. Biophys. Res. Commun. **174**, 606–612
6 Manji, K. H. and Lenox, R. H. (1994) Synapse **16**, 11–28
7 Weiner, E. D., Kalasapudi, V. D., Papolos, D. F. and Lachman, H. M. (1991) Brain Res. **553**, 117–122
8 Hokin-Neverson, M. and Jefferson, J. W. (1989) Neuropsychobiology **22**, 18–25
9 Berridge, M. J., Downes, C. P. and Hanley, M. R. (1989) Cell **59**, 411–419
10 Nahorski, S. R., Ragan, I. C. and Challiss, J. R. A. (1991) Trends Pharmacol. Sci. **12**, 297–303
11 Hallcher, L. M. and Sherman, W. R. (1980) J. Biol. Chem. **255**, 10896–10901

12 Gee, N. S., Ragan, C. I., Watling, K. J., Aspley, S., Jackson, R. G., Reid G. G., Gani, D. and Shute, J. K. (1988) Biochem. J. **249**, 883–889
13 Gee, N. S., Reid, G. G., Jackson, R. G., Barnaby, R. J. and Ragan, C. I. (1989) Biochem. J. **253**, 777–782
14 Kennedy, E. D., Challiss, J. R. A. and Nahorski, S. R. (1989) J. Neurochem. **53**, 1652–1655
15 Kennedy, E. D., Challiss, R. A., Ragan, C. I. and Nahorski, S. R. (1990) Biochem. J. **267**, 781–786
16 Jenkinson, S., Patel, N., Nahorski, S. R. and Challiss, R. A. J. (1993) J. Neurochem. **61**, 1082–1090
17 Atack, J. R., Prior, A. M., Griffith, D. and Ragan, I. (1993) Br. J. Pharmacol. **110**, 809–815
18 Lee, C. H., Dixon, J. F., Reichman, M., Moummi, C., Los, G. V. and Hokin, L. E. (1992) Biochem. J. **282**, 377–385
19 Dixon, J. F., Lee, C. H., Los, G. V. and Hokin, L. E. (1992) J. Neurochem. **59**, 2332–2335
20 Dixon, J. F., Los, G. V. and Hokin, L. E. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8358–8362
21 Sherman, W. R., Gish, B. G., Honchar, M. P. and Munsell, L. Y. (1986) Fed. Proc. **46**, 2639–2646
22 Stubbs, E. B., Jr. and Agranoff, B. W. (1993) J. Neurochem. **60**, 1292–1299
23 Biedler, J. L., Helson, L. and Spengler, B. A. (1973) Cancer Res. **33**, 2643–2652
24 Ross, R. A., Spengler, B. A. and Biedler, J. L. J. (1983) Natl. Cancer Inst. **71**, 741–749
25 Lambert, D. G. and Nahorski, S. R. (1990) Biochem. J. **265**, 555–562
26 Serra, M., Mei, L., Roeske, W. R., Lui, G. K., Watson, M. and Yamamura, H. I. (1988) J. Neurochem. **50**, 1513–1521
27 Martin, D. M., Yee, D., Carlson, R. O. and Feldman, E. L. (1992) Mol. Brain Res. **15**, 241–246
28 Kazmi, S. M. I. and Mishra, R. K. (1986) Biochem. Biophys. Res. Commun. **137**, 813–820
29 Ammer, H. and Schulz, R. (1993) Biochem. J. **295**, 263–271
30 Leli, U., Shea, T. B., Cataldo, A., Hauser, G., Grynspan, F., Beermann, M. L., Liepkalns, V. A., Nixon, R. A. and Parker, P. J. (1993) J. Neurochem. **60**, 289–298
31 Akermann, K. E. O. (1989) FEBS Lett. **242**, 337–340
32 Lambert, D. G., Whithman, E. M., Baird, J. G. and Nahorski, S. R. (1990) Mol. Brain Res. **8**, 263–266
33 Reeve, H. L., Vaughan, P. F. T. and Peers, C. (1992) Neurosci. Lett. **135**, 37–40
34 Los, G. V., Artemenko, I. P. and Hokin, L. E. (1994) J. Neurochem. **62**, S18
35 Hughes, A. R., Takemura, H. and Putney, J. W., Jr. (1988) J. Biol. Chem. **263**, 10314–10319
36 Donié, F. and Reiser, G. (1989) FEBS Lett. **254**, 155–158
37 Dean, N. M. and Moyer, J. D. (1987) Biochem. J. **242**, 361–366
38 Fisher, S. K., Heacock, A. M., Seguin, E. B. and Agranoff, B. W. (1989) Mol. Pharmacol. **38**, 54–63
39 Lambert, D. G., Challis, R. A. J. and Nahorski, S. R. (1991) Biochem. J. **273**, 791–794
40 Larsson, C. and Simonsson, P. (1993) Neurosci. Lett. **150**, 141–144
41 Kanba, S., Yagi, G., Nakaki, T., Kato, R. and Richelson, E. (1991) Neuropharmacology **30**, 497–500
42 Hughes, A. R. and Michell, R. H. (1993) Curr. Opin. Neurobiol. **3**, 383–400
43 Delvaux, A., Erneux, C., Moreau, C. and Dumont, J. E. (1987) Biochem. J. **242**, 193–198
44 Wilson, D. B., Bros, T. E., Sherman, W. R., Berger, R. A. and Majerus, P. W. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4013–4017
45 Kim, J. W., Ryu, S. H. and Rhee, S. G. (1989) Biochem. Biophys. Res. Commun. **163**, 177–182
46 Connolly, T. M., Wilson, D. B., Bross, T. E. and Majerus, P. W. (1986) J. Biol. Chem. **261**, 122–126
47 Dixon, J. F. and Hokin, L. E. (1987) J. Biol. Chem. **262**, 13892–13895
48 Kendall, D. A. and Hill, S. J. (1990) in Methods in Neurotransmitter Receptor Analysis (Yamamamura, H. I., Enna, S. J., Kuhar, M. J., eds.), pp. 69–87, Raven Press, New York
49 Hawkins, P. T., Berrie, C. P., Morris, A. J. and Downes, C. P. (1987) Biochem. J. **243**, 211–218
50 Shears, S. B. (1992) in Advances in Second Messenger and Phosphoprotein Research (Putney, J. W., Jr., ed.), vol. 26, pp. 63–92, Raven Press, New York
51 Connolly, T. M., Bross, T. E. and Majerus, P. W. (1985) J. Biol. Chem. **260**, 7868–7874
52 Ruitz-Larrea, F. and Drummond, A. H. (1993) Biochim. Biophys. Acta **1178**, 63–72
53 Volonte', C. and Racker, E. (1988) J. Neurochem. **51**, 1163–1168
54 Berridge, M. J. (1993) Nature (London) **361**, 315–325
55 Kato, M., Lledo, P.-M. and Vincent, J.-D. (1991) Am. J. Physiol. **261**, C218–C223
56 Adam-Vizi, V., Banay-Schwartz, M., Wajda, I. and Lajtha, A. (1987) Brain Res. **410**, 257–263
57 Michaelis, T., Merboldt, K.-D., Bruhn, H., Hanicke, W. and Frahm, J. (1993) Radiology **187**, 219–227
58 Petroff, O. A. C., Spencer, D. D., Alger, J. R. and Prichard, J. W. (1989) Neurology **39**, 1197–1202
59 Heacock, A. M., Seguin, E. B. and Agranoff, B. W. (1993) J. Neurochem. **60**, 1087–1092
60 Antonsson, B. E. (1994) Biochem. J. **297**, 517–522
61 Sherman, W. R. (1989) in Inositol Lipids in Cell Signalling (Michell, R. H., ed.), pp. 39–79, Academic Press, New York
62 Rhee, S. G. and Choi, K. D. (1992) in Advances in Second Messenger and Phosphoprotein Research (Putney, J. W. Jr., ed.), vol. 26, pp. 35–61, Raven Press, New York
63 Nishizuka, Y. (1992) Science **258**, 607–614

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