Investigation of the role of inositol and the phosphatidylinositol signal transduction system in mouse embryonic stem cells

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The aims of this study were to investigate in mouse embryonic stem cells (1) the requirement for *myo*-inositol for cell proliferation, (2) the incorporation of inositol into the phosphoinositides and inositol phosphates of the phosphatidylinositol (PtdIns) signal transduction system and (3) the effect of serum growth factors on PtdIns turnover. Exogenous *myo*-inositol was not essential for embryonic stem cell proliferation. Lithium, an inhibitor of endogenous inositol recycling, inhibited embryonic stem cell proliferation but this effect was not reversible by the addition of high concentrations of exogenous inositol. [^3H]inositol was incorporated into the phosphoinositides, PtdIns, PtdIns4P and PtdIns(4,5) P_2 in similar proportions as reported for other cells. [^3H]inositol was also incorporated into a fourth lipid, tentatively identified as an inositolglycan. [^3H]inositol was also incorporated into a number of inositol phosphates, with the greatest amount of incorporation after 24 h into an inositol pentakisphosphate. After serum starvation for 24 h, the addition of 10% whole or dialysed serum for 2 or 20 min increased ($P < 0.05$) incorporation into inositol tris- and tetrakisphosphates. These results demonstrate the presence of PtdIns system components in embryonic stem cells and increased PtdIns turnover in response to serum growth factors.

Introduction

There is enormous research interest in the effects of peptide growth factors on the control of growth in preimplantation embryos (for reviews see Kane *et al.*, 1992a; Heyner *et al.*, 1993). The role of phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5) P_2) breakdown in transmembrane signalling by growth factors and other hormones is also a focus of major scientific investigation (Berridge, 1993). Activation of the PtdIns signalling system by growth factors or other agonists involves the phospholipase C-catalysed cleavage of PtdIns(4,5) P_2 to yield two second messengers, inositol(1,4,5)trisphosphate (Ins(1,4,5) P_3), which acts to release Ca^{2+} from internal Ca^{2+} stores, and diacylglycerol (DAG), which activates protein kinase C.

While there is considerable information available on the role of Ins(1,4,5) P_3 in oocyte maturation and fertilization (Whitaker, 1989; Swann and Whitaker, 1990; Whitaker and Swann, 1993) and some information on its role in cleavage in sea urchin embryos (Ciapa *et al.*, 1994), there is very little information on its role or that of phosphoinositides generally in early mammalian embryos. We have shown that inositol is essential for rabbit blastocyst expansion and cell proliferation (Kane, 1989; Fahy and Kane, 1992) and that it also stimulates hatching of hamster blastocysts (Kane and Bavister, 1988). We have also

shown the presence of components of the PtdIns system in both mouse (Kane *et al.*, 1992b) and rabbit blastocysts (Fahy and Kane, 1993).

The establishment of embryonic stem (ES) cells, stable cell lines derived from the inner cell mass (ICM) of preimplantation embryos which closely resemble their *in vivo* counterparts, has provided an *in vitro* model in which to investigate early embryo development. Because of their origin from the ICM and their ability to re-integrate with it, they offer a particular advantage as a model system to study the control of cell proliferation and differentiation in ICM cells.

In the work presented here we set out to investigate evidence for PtdIns system activity in mouse ES cells.

Materials and Methods

Embryonic stem cell culture

Mouse ES cells of the cell line, CCE (Robertson *et al.*, 1986), were cultured in the standard culture medium for ES cells (Robertson, 1987a), Dulbecco's modified Eagle's medium (DMEM from GibcoBRL, Life Technologies Ltd, Paisley) supplemented with penicillin G (150 U ml^{-1}), streptomycin sulfate (150 mg ml^{-1}), β -mercaptoethanol (0.007 mg ml^{-1}), mouse leukaemia inhibitory factor (LIF, 1000 U ml^{-1}) (Williams *et al.*, 1988), and 10% fetal calf serum (FCS). Cells were grown on gelatinized tissue culture flasks and 12-well plates in an incubator maintained at 37°C in an atmosphere of 5% CO_2 in air. In all inositol incorporation studies, ES cells

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were first grown to 80% confluency in 25 cm² flasks, then trypsinized and plated into 12-well plates at a density of 1×10^5 cells ml⁻¹ (2 ml per well). They were allowed to attach overnight and [³H]inositol was added 24 h after seeding.

Experiment 1: investigations of the culture requirements of embryonic stem cells for inositol

These experiments were performed in gelatinized 12-well plates. Cells were grown in a 'low-inositol' medium supplemented with 0, 3, 15, 40, 75 and 375 µmol inositol l⁻¹ to examine the effects of inositol on ES cell proliferation. The concentrations of inositol examined were those previously tested for stimulation of rabbit blastocyst growth (Fahy and Kane, 1992) with the addition of the concentration in the standard DMEM (40 µmol l⁻¹). The cells were seeded at a density of 1×10^5 ml⁻¹ (2 ml per well) and cultured for 48 h, then trypsinized and counted using a haemocytometer chamber. The 'low-inositol' medium consisted of inositol-free DMEM supplemented with 5% FCS plus the standard supplements for ES cell medium. The concentration of inositol in serum is about 32 µmol l⁻¹ (Inamoto *et al.*, 1995).

Embryonic stem cells were also cultured over 2–3 months in an 'inositol-free' culture medium. The inositol-free medium consisted of inositol-free DMEM (GibcoBRL, Life technologies Ltd) supplemented with 5% dialysed serum plus the standard supplements. Dialysed serum, free of inositol, was prepared by dialysing 10 ml FCS over 48 h against three changes of 1 l of MilliQ (Millipore Corporation, Watford) water at 4°C, using Spectrapor membrane tubing (Spectrum Medical Industries Inc., Los Angeles, CA) that had a molecular mass cut off between 6 and 8 kDa. The non-dialysable fraction obtained was filter sterilized both after dialysis and also immediately before use.

In these long-term growth studies in inositol-free medium, the cells at each passage were seeded at high densities by splitting confluent flasks 1:8 to ensure a high rate of cell division, thereby minimizing spontaneous differentiation (Robertson, 1987b). The cells were cultured in 25 cm² flasks and were passaged every 2–3 days for up to 3 months.

Experiment 2: effect of lithium chloride on embryonic stem cell proliferation

Because lithium is known to disrupt development in frog embryos by inhibiting phosphoinositide breakdown and inositol recycling, thus starving the cells of inositol (Busa and Gimlich, 1989), the effect of lithium on ES cell proliferation was examined by culturing cells in a range of LiCl concentrations (0, 2, 5, 10, 15 and 20 mmol l⁻¹) for 48 h. The ability of high concentrations of inositol to counteract the inhibitory effects of LiCl was investigated by culturing the cells for 48 h in 10 mmol LiCl l⁻¹ with 1875 µmol inositol l⁻¹ added to the standard ES medium. The concentrations of lithium and inositol examined were similar to concentrations previously tested in rabbit blastocysts (Fahy and Kane, 1994).

Experiment 3: incorporation of [³H]inositol and [³H]glucosamine into the phosphoinositides

Cells were labelled with [³H]inositol (10 µCi ml⁻¹) or [³H]glucosamine (20 µCi ml⁻¹). Labelling was carried out for 0.5, 2, 4, 8, 18 and 24 h to analyse the degree of incorporation of [³H]inositol into the phosphoinositides, and for 18 h in the case of [³H]glucosamine incorporation.

After labelling, the phospholipids were extracted as described by Roldan and Harrison (1989) except that perchloric acid was substituted for trichloroacetic acid throughout. An aliquot of the sample was taken after acid precipitation for protein determination. Protein determinations were carried out using the microtitre protocol of the Pierce BCA protein assay (Pierce and Warriner, Chester). The extracted phosphoinositides were separated by TLC on pre-coated silica-gel 60 F₂₅₄ plates (E. Merck, Darmstadt). Before use, the TLC plates were sprayed with 1% (w/v) potassium oxalate and were then activated by heating in an oven at 110°C for 1 h. The solvent used was chloroform/methanol/water/concentrated NH₃ (45:35:8:2, v:v). The phosphoinositide spots were located by autoradiography on Hyperfilm-³H (Amersham International plc, Amersham). The film was used as a template and areas corresponding to the phosphoinositides, and all other areas showing the incorporation of radioactivity, were scraped from the TLC plate and placed into scintillation vials. Methanol (1 ml) was added to each vial and the samples were left for 1 h, to elute the lipids from the silica. Scintillation cocktail (5 ml, Beckman Ready Value, Beckman Instruments, High Wycombe) was placed into each vial, which was vortexed and left overnight to equilibrate before counting in a scintillation counter. Incorporation was expressed as d.p.m. per 100 µg of protein.

Experiment 4: effect of whole and dialysed serum on formation of ³H-labelled inositol phosphates

After an initial 24 h attachment period, ES cells were cultured in serum-free medium containing 10 µCi [³H]inositol ml⁻¹ for a further 24 h. At the end of this 24 h labelling period, cells were stimulated by the addition of 10% whole FCS or dialysed FCS either for 2 or 20 min. LiCl (10 mmol l⁻¹) was also added before the end of the labelling period, for the final 20 min of labelling when stimulation with FCS was for 2 min and for the final 30 min when stimulation was for 20 min. Appropriate controls without FCS stimulation but with LiCl added for 20 and 30 min were also carried out. LiCl was added to cause accumulation of metabolized inositol phosphates, mainly as inositol monophosphates, and thus facilitate detection (Berridge *et al.*, 1982).

At the end of stimulation 10% (w/v) perchloric acid was added to stop all reactions, and inositol phosphates were extracted and separated as described by Harrison *et al.* (1990). Protein determinations were carried out as described for Expt 3. Inositol phosphates were separated by HPLC anion-exchange chromatography on a Pharmacia Mono Q HR5/5 chromatographic column (Meek and Nicoletti, 1986; Fahy and Kane, 1993) using an Na₂SO₄ gradient (see Fig. 4). The Mono Q column and elution system was standardized using nucleotides and tritiated inositol phosphate markers. The nucleotide

markers were AMP, ADP, ATP, adenosine tetraphosphate, GMP, GDP, GTP and guanosine tetraphosphate at 4–10 nmol per injection and the inositol phosphate standards were the HPLC-pure [^3H]inositol phosphates, $\text{Ins}(4)\text{P}$, $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (NEN, Dupont, Germany) at about 2 nCi per injection. The nucleotide markers were also used to spike all samples from embryo extracts as a check on the repeatability of standardization and sample chromatographic runs. All runs were monitored at 254 nm to detect the nucleotides. Flow rate was 1 ml min^{-1} and 1 ml fractions were collected, counted in a scintillation counter and results expressed as d.p.m. per 100 μg of protein.

Statistical analysis

Experimental data were analysed by analysis of variance (Steel and Torrie, 1960) followed, where appropriate, by a two-tailed Dunnett's test to compare each treatment with controls.

Results

Experiment 1: the culture requirement of embryonic stem cells for inositol

Embryonic stem cells grew rapidly in the standard culture medium, dividing every 18–20 h, a normal growth rate for ES cells (Robertson, 1987b). The long term growth capacity of ES cells in both the 'low-inositol' and 'inositol-free' media was found to be unaffected as compared with the normal medium. The cells cultured in modified media proliferated at a similar rate to those cultured in standard ES cell medium with $40 \mu\text{mol inositol l}^{-1}$. It was possible to maintain the cells in undifferentiated culture for 2–3 months in the 'inositol-free' medium, at which time the study was terminated. Jackson and Shin (1982) considered a cell line to be capable of inositol-independent growth if they could be cultured successfully for three passages and in these studies this criterion was exceeded by a wide margin.

The effect of supplementing the 'low-inositol' medium with inositol at concentrations of 0, 3, 15, 40, 75 and $375 \mu\text{mol l}^{-1}$ on the proliferation of ES cells cultured for 48 h had no effect on ES cell proliferation ($P > 0.05$, data not shown).

Experiment 2: effects of lithium chloride on embryonic stem cell proliferation

Lithium chloride significantly ($P < 0.05$) inhibited ES cell proliferation at all concentrations from 2 to 20 mmol l^{-1} (Fig. 1a). This inhibitory effect was not reversed by the addition of excess ($1875 \mu\text{mol l}^{-1}$) inositol in the culture medium (Fig. 1b).

Experiment 3: incorporation of [^3H]inositol and [^3H]glucosamine into phosphoinositides

Extraction of the phospholipids of ES cells after incubation with [^3H]inositol followed by separation by TLC consistently

Fig. 1. (a) The effect of LiCl on embryonic stem cell proliferation. Embryonic stem cells were cultured in various LiCl concentrations for 48 h. (b) The effect of a high concentration of inositol ($1875 \mu\text{mol l}^{-1}$ versus $40 \mu\text{mol l}^{-1}$) on cell proliferation in the absence (□) and presence (■) of 10 mmol l^{-1} LiCl for 48 h. All values are means of numbers of cells $\pm$ SEM for 12 wells.

showed four spots after autoradiography (Fig. 2, lane 1). Of the four spots that consistently incorporated [^3H]inositol, three were positively identified as PtdIns , $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ by their co-migration with lipid standards. The regions on the TLC plate that corresponded to the three standards on the autoradiograph were scraped and counted using a scintillation counter. The incorporation of inositol into the three phosphoinositides reached a steady state after 18 h (Fig. 3). The incorporation of inositol into both $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ as a proportion of the total incorporation into the phosphoinositides was similar to that reported for other cells and tissues (Downes *et al.*, 1989).

The remaining spot which did not co-migrate with any of the three phosphoinositide standards was tentatively identified as a glycosyl phosphoinositide, based on its migration position between PtdIns and $\text{PtdIns}4\text{P}$. Incorporation of [^3H]inositol into this spot did not level off after 18 h (Fig. 3). This region has been reported as the position for a glycosyl phosphoinositide,

Fig. 2. Autoradiogram showing the TLC separation of phosphoinositides extracted from [^3H]inositol (lane 1) and [^3H]glucosamine-labelled embryonic stem cells (lane 2) after incubation with [^3H]inositol ($10 \mu\text{Ci ml}^{-1}$) or [^3H]glucosamine ($20 \mu\text{Ci ml}^{-1}$) for 24 h. Lipid identification in the case of the three phosphoinositides (PtdIns, PtdIns4P and PtdIns(4,5) P_2) was based on co-migration with lipid standards visualized by iodine staining. The fourth spot in lane 1 was tentatively identified as an inositol glycan-containing phosphoinositide, based on its incorporation of both [^3H]inositol and [^3H]glucosamine and its elution position between PtdIns and PtdIns4P.

an inositol glycan-containing lipid. Embryonic stem cells were incubated with [^3H]glucosamine for 24 h to determine if it would be incorporated into the unidentified lipid, as it is a constituent component of these glycolipids. The lipid into which most [^3H]glucosamine was incorporated had a similar R_F value to that of the unidentified lipid (Fig. 2, lane 2), providing strong evidence that this spot represents a glycosyl phosphoinositide.

Experiment 4: effect of whole serum and dialysed serum on inositol phosphate formation

Embryonic stem cells, labelled in serum-free medium for 24 h, were stimulated by the addition of either 10% whole or dialysed FCS for 2 and 20 min. Peaks of [^3H]inositol incorporation were identified as inositol mono-, bis-, tris-, tetrakis- and pentakisphosphates in control and serum stimulated cells (Fig. 4). The largest peak of incorporation was into inositol pentakisphosphate. Owing to the overlap in the elution position of Ins(1,4,5) P_3 and the Ins(1,3,4) P_3 in most profiles, the data for these two inositol phosphates are presented together as the Ins P_3 peak. However, it was clear from the elution positions that most of the Ins P_3 peak was due to the presence of Ins(1,3,4) P_3 . Also because the tetrakisphosphate peak may include Ins(1,3,4,6) P_4 , which elutes very close to Ins(1,3,4,5) P_4 (Wong *et al.*, 1992), data for this peak are presented as Ins P_4 .

Fig. 3. Time course of incorporation of [^3H]inositol into PtdIns (■), PtdIns4P (●), PtdIns(4,5) P_2 (○) and into a putative glycosyl phosphoinositide (▲) after incubation with $10 \mu\text{Ci}$ [^3H]inositol ml^{-1} for 0.5, 2, 4, 8, 18 and 24 h.

Because there were no significant differences in the effects of dialysed versus whole serum on [^3H]inositol incorporation into inositol phosphates by the ES cells, only the results for dialysed serum are presented. The addition of dialysed serum caused significant increases ($P < 0.05$) in the sizes of all inositol phosphate peaks except the Ins(1,3) P_2 and the Ins P_5 peak (Fig. 5).

Discussion

The results reported here indicate that mouse ES cells can incorporate *myo*-inositol into the phosphoinositide and inositol phosphate components of the PtdIns signal transduction system and that activity of this system in ES cells is stimulated by serum growth factors. There is also evidence of the formation of a glycosyl phosphoinositide which may be part of an inositol glycan signalling system or a protein anchoring system (Saltiel, 1991). Our results also indicate that ES cells do not have a requirement for exogenous inositol in the culture medium.

[^3H]inositol was incorporated into four phosphoinositides. Three of these were positively identified as PtdIns, PtdIns4P and PtdIns(4,5) P_2 by their co-migration with lipid standards on TLC. The incorporation of inositol into both PtdIns4P and PtdIns(4,5) P_2 , as a proportion of the total incorporation into the phosphoinositides, was similar to that reported for other cells and tissues (Downes *et al.*, 1989). Cells can obtain inositol from three principal sources: uptake of inositol from the

Fig. 4. HPLC profiles showing the effect of stimulation of embryonic stem cells by dialysed fetal calf serum (dFCS) on accumulation of [3 H]inositol in inositol phosphates. (a) Control; (b) 2 min dFCS; and (c) 20 min dFCS. Based on their elution at the same positions as inositol phosphate standards, these peaks were identified: peak 1, InsP_1 ; peak 3, $\text{Ins}(1,4)\text{P}_2$; peak 5, $\text{Ins}(1,4,5)\text{P}_3$ and peak 6, $\text{Ins}(1,3,4,5)\text{P}_4$. Based on their positions relative to the inositol phosphate and nucleotide standards, these peaks were more tentatively identified: peak 2, $\text{Ins}(1,3)\text{P}_2$; peak 4, $\text{Ins}(1,3,4)\text{P}_3$ and peak 7, inositol pentakisphosphate.

extracellular medium, *de novo* biosynthesis, and recycling of the products of PtdIns turnover as inositol phosphates are broken down first to inositol monophosphate and then to free inositol. Lithium blocks the recycling process and causes accumulation of inositol phosphates, mainly inositol monophosphate (Sherman, 1989). Phosphoinositide synthesis occurs via *de novo*

Fig. 5. The effect of stimulation of embryonic stem cells by dialysed fetal calf serum (dFCS) on accumulation of [3 H]inositol in inositol phosphates (no dFCS, $\square$; 2 min dFCS, $\square$; 20 min dFCS, $\blacksquare$; 40 min dFCS, $\blacksquare$). Values are mean d.p.m. $\pm$ SEM per 40 μg protein for two replicates for the 2 min stimulation and four replicates for the 20 min stimulation periods. The control no stimulation treatment is based on three replicates. Significantly different from the control treatment: * $P < 0.01$; ** $P < 0.001$.

synthesis of PtdIns from inositol and CMP-phosphatidate followed by formation of $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ by the action of specific kinases that phosphorylate the 4- and 5-position of PtdIns and $\text{PtdIns}4\text{P}$ (Downes *et al.*, 1989). Incorporation of inositol into phosphoinositides can also occur by the exchange of free and lipid-bound inositol, as has been described for turkey erythrocyte membranes (McPhee *et al.*, 1991). The results presented here indicate that even though ES cells do not require extracellular inositol for growth, they can transport inositol from the extracellular medium, and that the enzymes responsible for the synthesis of the components of the PtdIns system are present and active in growing ES cells.

The other lipid into which inositol was incorporated ran on TLC between PtdIns and $\text{PtdIns}4\text{P}$, a position that has been described for a glycosyl phosphoinositide which can be either a protein anchor or a precursor for an inositol glycan second messenger which is released by hormone stimulation (Saltiel and Cuatrecasas, 1986; Saltiel *et al.*, 1986). This identification was confirmed by the fact that glucosamine, a component of inositol glycans, was incorporated into a lipid spot with the same R_F value. The question of whether the glycan was part of a glycosyl phosphoinositide protein anchor (Low *et al.*, 1986) or the inositol glycan second messenger system (Saltiel and Cuatrecasas, 1986) was unresolved. The incorporation of ethanolamine should be examined in ES cells as it is present only in the inositol glycan of protein anchors (Saltiel, 1991) and not the second messenger inositol glycan.

Stimulation of serum-starved ES cells with either whole or dialysed serum caused large increases in the accumulation of inositol mono-, bis-, tris- and tetrakisphosphates. These results are consistent with the hypothesis that the serum growth factors stimulate the phospholipase C-dependent hydrolysis of PtdInsP_2 to $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol. The fact that there was not a large increase in the $\text{Ins}(1,4,5)\text{P}_3$ peak is not surprising since $\text{Ins}(1,4,5)\text{P}_3$ is rapidly metabolized (Wang *et al.*, 1995). There are two possible metabolic fates for the

Ins(1,4,5) P_3 signal: (i) dephosphorylation to Ins(1,4) P_2 , and (ii) phosphorylation to Ins(1,3,4,5) P_4 which is then dephosphorylated to Ins(1,3,4) P_3 (Majerus *et al.*, 1988). LiCl blocks the hydrolysis of both Ins(1,4) P_2 to Ins4P, and Ins(1,3,4) P_3 to Ins(3,4) P_2 , by inhibiting the action of the inositol polyphosphate 1-phosphorylase (Sherman, 1989). Therefore, when the phospholipase C-dependent hydrolysis of PtdIns P_2 is activated in the presence of LiCl, both the inositol phosphates, Ins(1,4) P_2 and Ins(1,3,4) P_3 will accumulate, as will inositol monophosphates. Thus, the marked increase in the inositol monophosphate, Ins(1,4) P_2 and Ins(1,3,4) P_3 peaks in response to stimulation with serum provides strong evidence for phosphoinositide signalling in ES cells in response to serum stimulation.

The fact that the inositol pentakisphosphate peak was the largest peak, comprising approximately 60% of the total incorporation of [3H]inositol, was not surprising since the inositol pentakis- and hexakisphosphates are the most abundant of the inositol phosphates in the cell lines and tissues examined to date (Berridge and Irvine, 1989). The absence of inositol hexakisphosphate was probably due to the length of the labelling period, as inositol incorporation into the hexakisphosphates is a slower process than for the other inositol phosphates (Menniti *et al.*, 1993).

Previous studies on the inositol requirements of mammalian cells (Eagle *et al.*, 1957; Furuya *et al.*, 1971; Jackson and Shin, 1982) have shown that cell lines vary greatly in their requirement for inositol for survival and growth. From the results presented here, ES cells would appear to fit into the group that does not require exogenous inositol in the culture medium. This does not indicate that inositol and the phosphatidylinositol system are unimportant in ES cells but that, like many other cell types, ES cells may have their own *de novo* synthetic pathway for inositol, most probably from glucose-6-phosphate (Sherman, 1989). These results correlate with the situation in mouse blastocysts (from which these ES cells are derived) where two-cell embryos develop to the blastocyst stage in media without inositol (Brinster, 1963). However, it contrasts markedly with the situation in rabbit blastocysts, where blastocyst expansion and DNA synthesis requires inositol in the culture medium (Kane, 1989; Fahy and Kane, 1992).

The inhibitory effects of lithium on ES cell proliferation presented here agree with previous studies both in mouse and rabbit embryos. It has been shown (Izquierdo and Becker, 1982) that lithium reduces cell numbers and particularly ICM cell numbers in preimplantation mouse embryos cultured to blastocysts. In rabbit embryos lithium decreased DNA synthesis and blastocyst expansion (Fahy and Kane, 1994). Studies on rat embryo development (Klug *et al.*, 1992) showed similar effects of lithium.

A major effect of lithium is that it inhibits dephosphorylation of several inositol phosphates generated during phosphoinositide signalling (Burgess *et al.*, 1985; Sherman, 1989), thereby trapping inositol into biosynthetically useless molecules and reducing the cellular inositol pool for PtdIns resynthesis. This action of lithium reduces the supply of PtdIns-related molecules and consequently inhibits PtdIns-related signalling. However, if the lithium inhibition is specific for the PtdIns system, it should be possible to abolish it by supplying excess inositol in the culture medium (assuming the inositol is taken up freely by the cells) and in the present study

this did not occur. It is possible that if we had examined other concentrations of lithium and inositol, it might have been possible to demonstrate reversal of the growth inhibitory effects of lithium. However, in similar studies in intact rat (Klug *et al.*, 1992) or rabbit (Fahy and Kane, 1994) embryos using a range of lithium and inositol concentrations it was not possible to demonstrate such a reversal of the growth inhibitory effect of lithium. The failure of excess inositol to reverse the inhibitory effects of lithium could be due to failure of exogenous inositol to enter ES cells or embryos at a high enough rate or to other effects of lithium not involving inositol metabolism. There are reports that lithium can block GTP binding to membranes of rat cortex (Avissar *et al.*, 1988), alter gene expression (Dobner *et al.*, 1988) and affect phospholipase A_2 (Huang and Detwiler, 1986).

In summary, the results described here provide strong evidence that phosphoinositide signalling is active in ES cells and may be involved in the regulation of ES cell proliferation by serum growth factors.

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