Phospholipase C and protein kinase C involvement in mouse embryonic stem-cell proliferation and apoptosis

L. R. Quinlan, S. Faherty and M. T. Kane*

Department of Physiology, National University of Ireland, University Road, Galway, Ireland

Activation of the phosphatidylinositol (PtdIns) signal transduction system involves stimulation of phospholipase C (PLC) by hormones and other agonists to produce two second messengers, the inositol phosphate, $\text{Ins}(1,4,5)\text{P}_3$ which releases calcium from intracellular stores, and diacylglycerol which activates protein kinase C (PKC). This study, using activators or inhibitors of PLC and PKC and a calcium ionophore, examined the role of the PtdIns system in mouse embryonic stem (ES) cells. The PLC inhibitor, U-73122, inhibited ES-cell proliferation and also inhibited PLC activation as evidenced by a decrease in inositol phosphate formation in response to fetal calf serum stimulation. The two PKC activators, the diacylglycerol analogue 1,2, dioctanoyl-sn-glycerol (DOG) and the phorbol ester 12-O-tetra-decanoyl phorbol 13-acetate (TPA),

increased cell proliferation in a dose-dependent manner, as did the calcium ionophore, ionomycin. However, co-stimulation with either ionomycin and DOG or ionomycin and TPA resulted in a reduced number of cells. The PKC inhibitor, bisindolylmaleimide II (Bis II), significantly decreased the number of ES cells, mainly due to increased apoptosis. The possible feedback effect of PKC on PLC was examined by preincubating ES cells with either the PKC inhibitor Bis II or the activator TPA before stimulation of inositol phosphate production with fetal calf serum; preincubation with Bis II increased inositol phosphate formation whereas preincubation with TPA decreased inositol formation. These results indicate that the PtdIns system is involved in the control of ES-cell proliferation and apoptosis.

Introduction

There is considerable evidence that inositol-containing compounds have an important role in preimplantation embryonic development. Studies in the laboratory of the present authors have shown that inositol is essential for the continued cell proliferation and growth of rabbit blastocysts (Kane, 1988; Kane, 1989; Fahy and Kane, 1992). Inositol has also been shown to stimulate the hatching of hamster blastocysts (Kane and Bavister, 1988). One of the fundamental roles of inositol is that it is part of the phosphatidylinositol (PtdIns) signal transduction system (reviewed by Berridge, 1992) which involves receptor-stimulated activation of a PtdIns-specific phospholipase C (PLC) resulting in the hydrolysis of phosphatidylinositol(4,5)bisphosphate ($\text{PtdIns}(4,5)\text{P}_2$) to produce the two second messengers inositol(1,4,5)-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) which acts to release Ca^{2+} from internal stores, and diacylglycerol which, together with the released Ca^{2+} , is involved in the activation of protein kinase C (PKC). It has been shown that mouse (Kane *et al.*, 1992; Higgins and Kane, 2003), rabbit (Fahy and Kane, 1993) and cattle (Hynes *et al.*, 2000) embryos, and mouse embryonic stem (ES) cells

(Duffy and Kane, 1996) incorporate inositol into the phosphoinositides and inositol phosphates of the PtdIns signal transduction system. Other workers have shown that inhibitors of PLC inhibit mouse blastocyst formation (Stachecki and Armant, 1996a) and that calcium release in mouse morulae occurs predominantly through the $\text{Ins}(1,4,5)\text{P}_3$ receptor (Stachecki and Armant, 1996b). The essential role of phosphoinositide compounds in preimplantation development is emphasized by the demonstration that even though exogenous inositol is not necessary for blastocyst formation, knockout of the PLC $\beta 3$ gene prevents development of mouse embryos to the blastocyst stage (Wang *et al.*, 1998).

PKC forms a central control point in many ligand-activated cell-signalling events and is activated by one axis of the PtdIns signal transduction system through diacylglycerol. PKC represents a family of at least 12 serine/threonine kinases that are involved in a myriad of signal transduction events in response to specific hormonal and growth factor stimuli (reviewed by Nishizuka, 1992). One specific aspect of PKC that is relevant to the present study is that PKC has been shown to play a vital role in regulating early events in embryonic development (reviewed by Gallicano *et al.*, 1997a). This role is at least partly linked to its role in regulation of cell-cycle control in both yeast and mammalian cells (reviewed by Livneh and Fishman, 1997). PKC is also

*Correspondence
Email: michael.kane@nuigalway.ie

thought to play an important role in oocyte remodelling at zygote formation in many species (Gallicano *et al.*, 1997b).

The present study used mouse ES cells as a model for embryonic growth. ES cells are a stable cell line, which are derived from the inner cell mass of preimplantation embryos. These cells closely resemble their *in vivo* counterparts and thus provide a stable *in vitro* model of embryo growth and development and provide a means whereby specific signalling systems can be investigated. In the data presented here, using activators or inhibitors of PLC and PKC and a calcium ionophore, the possible roles of the PtdIns signalling system and PKC in ES-cell proliferation and apoptosis were examined.

Materials and Methods

ES-cell culture

Mouse ES cells of the CCE cell line were cultured in the standard culture medium for ES cells (Robertson, 1987), Dulbecco's modified Eagles medium (DMEM; Gibco BRL, Life Technologies Ltd, Paisley) supplemented with penicillin G (150 U ml^{-1}), streptomycin sulphate ($150 \mu\text{g ml}^{-1}$), β -mercaptoethanol ($0.007 \mu\text{g ml}^{-1}$), mouse leukaemia inhibitory factor (LIF; 1000 U ml^{-1} , ESGROTM, Gibco BRL) (Williams *et al.*, 1988) and 5% fetal calf serum (FCS). Cells were grown on gelatinized tissue culture flasks and 24-well plates in an incubator maintained at 37°C in an atmosphere of 5% CO_2 in air.

Cell proliferation studies

For all studies except apoptosis, ES cells were first grown to 60% confluency in 25 cm^2 flasks, and then the exponentially growing cells were trypsinized and seeded at $2 \times 10^5 \text{ cells ml}^{-1}$ in 24-well plates in fresh standard DMEM and allowed to attach for 24 h.

For inhibitor studies, after the attachment period, the inhibitor being investigated was added for 24 or 48 h and culture was carried out in standard DMEM plus 10% FCS. For activator studies, the cells were further cultured in standard medium without FCS after the attachment period, supplemented with 0.1% (w/v) essentially fatty-acid free BSA (Sigma-Aldrich, Poole; cell-culture grade) for 24 h in the presence of various concentrations of PKC activators or the calcium ionophore, ionomycin.

When it was necessary to dissolve inhibitors or activators in non-aqueous solvents (ethanol, DMSO), controls were treated with diluent at the same concentration as the highest concentration of inhibitor or activator being examined.

Cell viability was determined by dye exclusion in which cells were stained with ethidium bromide and acridine orange. In studies of cell viability and apoptosis, cells ($10 \times 10^4 \text{ ml}^{-1}$) were grown on Eppen-

dorf CELLocate[®] microgrid coverslips (Eppendorf AG, Hamburg).

Cell proliferation assays

The effects of various PLC and PKC activators and inhibitors on cell proliferation were assayed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5, diphenyl-tetrazolium bromide) assay (Mosmann, 1983; Denizot and Laing, 1986) at the end of a 24 or 48 h treatment period. The MTT salt (Sigma cat no. M2128) was prepared as a 5 mg ml^{-1} stock solution in PBS and used at a working concentration of 1 mg ml^{-1} in DMEM culture medium without phenol red. At the end of the cell-culture treatment period, the cells were incubated in MTT solution for 3 h at 37°C . The solution was removed and the blue formazan product was dissolved with 2-propanol. The absorbance was then read at 550 nm with 690 nm reference. In certain cases cell proliferation was also measured by direct cell counting using a haemocytometer.

Apoptosis assay

The effects of various activators and inhibitors of PLC and PKC on cell viability and apoptosis were measured. The cells were fixed with 2% (w/v) paraformaldehyde and stored under PBS for analysis at the end of the cell-culture treatment period. Cells were stained with either ethidium bromide or acridine orange to measure viability or with the TUNEL *in situ* cell death detection kit from Roche Molecular Biochemicals (Roche Diagnostics Ltd, Lewes) to measure apoptosis. TUNEL-stained cells were counterstained with propidium iodide and apoptotic nuclei were counted using confocal microscopy.

Inositol phosphate assay

Unless otherwise stated, the general strategy to assess the effects of various activators and inhibitors of PLC and PKC on PtdIns-specific PLC formation of inositol phosphates in ES cells was as follows. After the 24 h attachment period, $2.5 \mu\text{Ci}$ [$2\text{-}^3\text{H}$]inositol ml^{-1} was added for a further 24 h in FCS-free inositol-free DMEM. Treatment with activators or inhibitors was carried out during the labelling period; timing of treatments is outlined below under specific experiments. In all cases, lithium chloride (10 mol l^{-1} final concentration) was added 25 min before the end of the labelling period to facilitate detection of labelled inositol phosphates by preventing their breakdown to free inositol (Berridge *et al.*, 1982). After a further 10 min the PtdIns system was stimulated by the addition of 10% FCS. At the end of the 24 h labelling period (15 min later), the reaction was stopped with 5% (w/v) perchloric acid and the radiolabelled inositol phosphates extracted as described by Duffy *et al.* (1998). Extracted inositol phosphates were

Fig. 1. The effects of the phospholipase C inhibitor U-73122 and its inert analogue, U-73433, on the number of mouse embryonic stem (ES) cells in culture. (a) Effect of U-73122 (0: $\square$; 1: hatched ; and 10 $\mu\text{mol l}^{-1}$: $\blacksquare$) for 24 and 48 h on the number of ES cells as determined by haemocytometric counting. Values are means $\pm$ SEM for eight replicates. (b) Effect of U-73122 on ES cells over 24 h, as measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5, diphenyl-tetrazolium bromide (MTT) cell-proliferation assay. Absorbance values at 550 λ –690 λ are means $\pm$ SEM for eight replicates. (c) Effect of U-73433 for 24 h on ES cells as measured by the MTT proliferation assay. Absorbance values at 550 λ –690 λ are means $\pm$

separated by standard methods using Dowex anion exchange chromatography (Berridge *et al.*, 1983; Duffy *et al.*, 1998) and their incorporation into the inositol monophosphate (InsP₁) and the >InsP₁ fractions was quantified by liquid scintillation counting. [³H]inositol was obtained from Amersham International plc (Amersham), and NEN, Dupont de Nemours (Bad Homburg).

Statistical analysis

All experiments were analysed by ANOVA followed in some experiments by a comparison of treatment means with the control using the Bonnferroni–Dunn test.

Results

Experiment 1: effect of the PLC inhibitor U-73122 on number of ES cells, the induction of apoptosis and inositol phosphate formation

The effects on the number of cells of 24 h incubation with U-73122 and its inert analogue, U-73433 (1, 5 and 10 $\mu\text{mol l}^{-1}$), were measured by the MTT assay; effects of 24 and 48 h incubation with U-73122 (1 and 10 $\mu\text{mol l}^{-1}$) were also measured by haemocytometric cell counting. There was a significant effect ($P < 0.001$) of the PLC inhibitor U-73122 and duration of incubation with U-73122 on ES-cell proliferation (Fig. 1a,b). U-73122 reduced the number of ES cells over 24 and 48 h as measured by haemocytometric cell counting (Fig. 1a) and as determined by the MTT assay when measured over 24 h (Fig. 1b). The inert analogue U-73433 had no effect on the number of cells ($P > 0.05$) over the same concentration range (Fig. 1c).

The effects of 24 h incubation with U-73122 (0.5, 1, 5 and 10 $\mu\text{mol l}^{-1}$) on apoptosis and cell viability were examined. Viability assays showed that $\geq 98\%$ cells were viable at the end of the treatment period and that U-73122 had no effect on apoptosis (Fig. 2). The increases in apoptosis observed over the various time periods were acceptable for cell lines in culture (Han *et al.*, 2000), reaching a maximum of approximately 6% at 24 h. The failure of U-73122 to increase the incidence of dead or apoptotic cells coupled with its effect in reducing the number of cells relative to the control after 24 and 48 h incubation indicates that U-73122 reduced the number of cells in culture by inhibiting ES-cell proliferation.

The effects at the same concentrations of U-73122 and U-73433 on radiolabelled inositol phosphate formation in the presence and absence of FCS were determined. U-73122 significantly ($P < 0.001$) inhibited both FCS-stimulated (Fig. 3a) and basal unstimulated (Fig. 3b)

SEM for eight replicates. * $P < 0.05$, ** $P < 0.001$, significantly different from value for no addition of inhibitor at same time point.

Fig. 2. Effect of concentration of the phospholipase C inhibitor U-73122 and duration of incubation (4: □; 8: ▨; 12: ▩; and 24 h: ■) on apoptosis in mouse embryonic stem cells. Values are means $\pm$ SEM for six replicates.

inositol phosphate formation in a dose-dependent manner. The highest concentration ($10 \mu\text{mol l}^{-1}$) caused a decrease of approximately 58% in FCS-stimulated inositol phosphate formation and 22% in unstimulated formation. The inert analogue U-73433 had no effect on inositol phosphate formation (results not shown). On the basis of the ability of U-73122 to inhibit PLC activity, these results provide evidence that PLC has a controlling role in ES-cell proliferation.

Experiment 2: effects of PKC activators, DOG and TPA, on ES-cell proliferation

The effects on ES-cell proliferation of incubation for 24 h with the diacylglycerol analogue, 1,2, dioctanoyl-sn-glycerol (DOG), at concentrations of 10, 100 and 1000 nmol l^{-1} and the phorbol ester 12-*O*-tetra-decanoyl phorbol 13-acetate (TPA) at concentrations of 1, 10 and 50 nmol l^{-1} were measured by the MTT assay. The effects of stimulation with DOG were compared with stimulation with 10% FCS.

Incubation with DOG had a significant ($P < 0.001$) effect on ES-cell proliferation (Fig. 4a). DOG (10 nmol l^{-1}) increased cell proliferation by approximately 15% whereas 100 nmol l^{-1} DOG increased cell proliferation by approximately 40%. The highest concentration used, 1000 nmol l^{-1} , had no effect on cell proliferation. However, FCS had a much greater stimulatory effect on ES-cell proliferation than the optimal concentration of

DOG ($P < 0.001$); FCS increased cell proliferation 2.7-fold as compared with the 0 control.

TPA had a significant ($P < 0.001$) effect on ES-cell proliferation (Fig. 4b). TPA (1 nmol l^{-1}) increased cell proliferation by approximately 35%, whereas higher concentrations reduced the number of cells. TPA (50 nmol l^{-1}) reduced the number of cells by approximately 23%. These results support the idea that PKC is involved in the control of ES-cell proliferation.

Experiment 3: effect of the calcium ionophore alone and in combination with DOG or TPA on ES-cell proliferation

The effect of incubation for 24 h with the calcium ionophore, ionomycin (0.1 , 1 and $10 \mu\text{mol l}^{-1}$) on ES-cell proliferation was measured by the MTT assay.

Co-stimulation studies involving dual treatment with ionomycin (0.1 and $1 \mu\text{mol l}^{-1}$) and DOG and TPA over the concentration range used in Expt 2 were also performed and cell proliferation was again measured by the MTT assay. Ionomycin had a significant effect ($P < 0.001$) on ES-cell proliferation (Fig. 5a). Ionomycin ($0.1 \mu\text{mol l}^{-1}$) increased cell proliferation by approximately 67%, whereas $1 \mu\text{mol l}^{-1}$ ionomycin increased cell proliferation by approximately 96%. However, the number of cells in $10 \mu\text{mol l}^{-1}$ ionomycin was decreased markedly, relative to the $1 \mu\text{mol l}^{-1}$ concentration, indicating that higher concentrations were less active in

Fig. 3. Effect of the phospholipase C inhibitor U-73122 on (a) fetal calf serum-stimulated and (b) basal unstimulated inositol phosphate formation. Values are means $\pm$ SEM for eight replicates. * $P < 0.05$, ** $P < 0.001$, significantly different from value for no addition of inhibitor.

stimulating cell proliferation or else caused some cell death.

There was no significant effect of either ionomycin or DOG in the ionomycin–DOG co-stimulation experiment or of ionomycin–TPA in the ionomycin–TPA experiment. However, there was a significant interaction ($P < 0.001$) between ionomycin and DOG (Fig. 5b) and between ionomycin and TPA (Fig. 5c). These significant negative interactions were due to the fact that the responses to DOG and TPA were decreased by increasing concentrations of ionomycin. Whereas both DOG and

Fig. 4. Effect of protein kinase C activators on mouse embryonic stem-cell proliferation as determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5, diphenyl-tetrazolium bromide (MTT) cell-proliferation assay. (a) Effect of the diacylglycerol analogue 1,2, dioctanoyl-sn-glycerol (DOG) and fetal calf serum. (b) Effect of the phorbol ester, 12-*O*-tetra-decanoyl phorbol 13-acetate (TPA). Absorbance values are means $\pm$ SEM for eight replicates for both activators. * $P < 0.01$, ** $P < 0.001$, significantly different from value for no addition of activator.

TPA were stimulatory in the absence of ionomycin, only the lowest concentration of DOG (10 nmol l⁻¹) in the presence of 0.1 µmol ionomycin l⁻¹ resulted in a slight increase in cell proliferation (Fig. 5b). All other concentrations of DOG and all concentrations of TPA (Fig. 5c) tested in the presence of either concentration of ionomycin reduced the number of cells.

Fig. 5. Effects of the calcium ionophore, ionomycin, both alone and in combination with the protein kinase C activators 1,2, dioctanoyl-sn-glycerol (DOG) or 12-*O*-tetra-decanoyl phorbol 13-acetate (TPA) on mouse embryonic stem (ES)-cell proliferation as measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5, diphenyltetrazolium bromide (MTT) cell-proliferation assay. (a) Effect of ionomycin on cell proliferation. Absorbance values are means $\pm$ SEM

These results provide evidence that calcium may have a key role in ES-cell proliferation.

Experiment 4: effects of the PKC inhibitor bisindolylmaleimide II on number of ES cells and incidence of apoptosis

The effect of 24 h incubation with bisindolylmaleimide II (Bis II) at 0.5, 5 and 10 $\mu\text{mol l}^{-1}$ or its inert analogue Bis V at 0.5, 5, 10 and 20 $\mu\text{mol l}^{-1}$ on the number of cells was examined by MTT assay. The effects on cell viability and extent of apoptosis of incubation with Bis II (0.5, 5 and 10 $\mu\text{mol l}^{-1}$) for 24 h were also determined.

Bis II at concentrations $\geq 5 \mu\text{mol l}^{-1}$ significantly ($P < 0.001$) depressed the number of ES cells in a dose-dependent manner as assessed by the MTT assay (Fig. 6a); 10 $\mu\text{mol l}^{-1}$ Bis II caused a 26% decrease in the number of ES cells over 24 h. The inactive analogue Bis V had no effect on the number of ES cells over the same period (Fig. 6b). Viability assays showed $\geq 98\%$ cells to be viable.

There was a significant effect ($P < 0.001$) of Bis II and duration of incubation on apoptosis of ES cells; the highest concentration of Bis II (10 mmol l^{-1}) increased the percentage of apoptosis approximately fivefold (Fig. 7). Thus, the effect of Bis II in reducing the number of ES cells in culture may not be due to an anti-proliferative effect but to its effect in increasing apoptosis.

Experiment 5: effect of PKC activation or inhibition on inositol phosphate formation

The effect of acute inhibition or activation of PKC, before FCS treatment, on FCS stimulation of inositol phosphate formation was examined. Cells were pre-incubated with PKC inhibitor Bis II (0.5, 5 and 10 $\mu\text{mol l}^{-1}$) or its inert analogue Bis V for 30 min before the end of the 24 h labelling period for the study of acute PKC inhibition. Cells were pre-incubated with PKC activator TPA (100 nmol l^{-1}) either 1 or 2 h before the end of the 24 h labelling period for the study of acute PKC activation.

Preincubation with Bis II caused a dose-dependent increase ($P < 0.001$) in FCS-stimulated inositol phosphate formation (Fig. 8a). Bis II (10 $\mu\text{mol l}^{-1}$) caused an increase of approximately 26% in inositol phosphate formation. The inactive analogue Bis V had no effect on inositol phosphate formation (Fig. 8b). Preincubation with TPA (100 nmol l^{-1}) before FCS stimulation caused

for eight replicates. (b) Effect of co-incubation with DOG (0: $\square$; 10: ▨ ; 100: ▩ ; and 1000 nmol l^{-1} : $\blacksquare$) and ionomycin on ES-cell proliferation. Absorbance values are means $\pm$ SEM for four replicates. (c) Effect of co-incubation with TPA (0: $\square$; 1: ▨ ; 10: ▩ ; and 50 nmol l^{-1} : $\blacksquare$) and ionomycin on ES-cell proliferation. Absorbance values are means $\pm$ SEM for four replicates. * $P < 0.001$ significantly different from value for no addition of activator.

Fig. 6. Effect of bisindolylmaleimides (Bis II and Bis V) on mouse embryonic stem cells as measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5, diphenyl-tetrazolium bromide (MTT) cell-proliferation assay. (a) Effects of the protein kinase C inhibitor, Bis II. (b) Effect of the inert analogue Bis V. Values for absorbance are means $\pm$ SEM for eight replicates for both (a) and (b). * $P < 0.01$, ** $P < 0.001$, significantly different from value for no addition of inhibitor.

a decrease ($P < 0.001$) in inositol phosphate formation (Fig. 8c). Preincubation with TPA for 1 and 2 h caused an approximate 26% and 35% decrease in inositol phosphate production, respectively.

Fig. 7. Effect of concentration of the protein kinase C inhibitor bisindolylmaleimide II and duration of incubation (4: $\square$; 8: diagonal lines ; 12: cross-hatch and 24 h: $\blacksquare$) on apoptosis in mouse embryonic stem cells. Values are means $\pm$ SEM for six replicates over three blocks.

These results clearly demonstrate that the PtdIns system is active and tightly regulated via PKC activity in ES cells; increased PKC activity tended to downregulate and decreased PKC activity to upregulate the PtdIns system.

Discussion

Previous work in this laboratory (Duffy and Kane, 1996) has demonstrated that components of the PtdIns signal transduction system are present in ES cells. Demonstrating the presence of PtdIns signal components in ES cells does not, however, prove that the system is essential for ES-cell proliferation. In examining the role of the PtdIns system in ES-cell proliferation in the present work, activators or inhibitors of PLC and PKC and a calcium ionophore were used to investigate the importance of PLC, PKC and intracellular calcium in ES-cell proliferation.

Studies of the role of PLC in cell function are hindered by the absence of a suitable specific cell permeable activator of PLC. In the absence of such an activator, the specific PLC inhibitor, U-73122 (Powis and Phil, 1994; Wang *et al.*, 1994) was used in the present study. The fact that U-73122 significantly reduced the number of ES cells in a dose-dependent manner ($\leq 1 \mu\text{mol l}^{-1}$) but did not decrease cell viability or increase the incidence of apoptotic cells, shows that the effect of U-73122 involved a specific inhibition of ES-cell proliferation and not a general cytotoxic or apoptotic effect. Over 24 h, cell proliferation was inhibited and over 48 h it was abolished by U-73122. U-73122 too had a similar dose-dependent

Fig. 8. The effect of acute inhibition or activation of protein kinase C (PKC) on fetal calf serum-stimulated inositol phosphate formation. Effect of preincubation (a) with the PKC inhibitor, bisindolylmaleimide II (30 min); (b) preincubation with the inert

analogue bisindolylmaleimide V (30 min); and (c) preincubation with the PKC activator 12-*O*-tetra-decanoyl phorbol 13-acetate (TPA; 1 or 2 h). In all cases, values are means $\pm$ SEM for eight replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from value for no addition of inhibitor or activator.

inhibitory effect on the formation of inositol phosphates stimulated by FCS. Evidence for the specificity of U-73122 action on PLC was provided by the fact that the very similar but inert analogue U-73343 (Bleasdale *et al.*, 1990; Powis and Phil, 1994) had no effect on either ES-cell proliferation or inositol phosphate formation. These results indicate that PLC has a controlling role in ES-cell proliferation. As ES cells are derived from the inner cell mass of the blastocyst, this work on the effect of PLC inhibition in ES cells is in agreement with the work of Stachecki and Armant (1996a) showing that the regulation of blastocyst formation in mice is mediated through a PLC-dependent pathway that can be inhibited by U-73122.

In ES cells U-73122 inhibited basal concentrations of inositol phosphate formation (that is, in the absence of FCS stimulation). This finding indicates the presence of an as yet unknown autocrine or paracrine factor produced by the ES cells themselves that stimulates phosphoinositide turnover, causing the system to be constitutively active in ES cells.

The two second messengers produced by activation of PLC action, diacylglycerol and $\text{Ins}(1,4,5)\text{P}_3$, stimulate PKC and increase intracellular calcium (Berridge, 1992); the PKC activators, DOG and TPA, and a calcium ionophore, ionomycin, were used to mimic these actions on ES cells (Berry and Nishizuka, 1990).

The results with DOG and TPA support the hypothesis that PKC is involved in the control of ES-cell proliferation. DOG at concentrations of 10 and 100 nmol l^{-1} and TPA at 1 nmol l^{-1} stimulated cell proliferation. However, the results also showed that super-activation of PKC led to growth arrest, as 1000 nmol l^{-1} DOG was slightly growth-inhibitory and TPA was inhibitory at 10 and 50 nmol l^{-1} ; this effect is presumably through PKC downregulation (Kraft and Anderson, 1983; Kishimoto *et al.*, 1989).

Ionomycin at 0.1 and 1 $\mu\text{mol l}^{-1}$ caused a significant increase in cell proliferation, which was abolished at 10 $\mu\text{mol l}^{-1}$ as its effect tended to become growth-inhibitory or cytotoxic. Treatment with ionomycin causes a rapid and sustained increase in intracellular calcium (Hanson and Ziegler, 2001) without the requirement for an external stimulus to open specific calcium channels. Thus, the ionomycin treatment bypasses the role of $\text{Ins}(1,4,5)\text{P}_3$ in releasing calcium from internal stores. The majority of effects elicited by calcium are via interaction with the calcium-binding protein calmodulin (Billingsley *et al.*, 1990). Whatever the mechanism involved in the present study, the results indicate that calcium is a key messenger in ES-cell proliferation.

Co-stimulatory studies were also performed, involving dual treatment with ionomycin and DOG or TPA. Only 10 nmol DOG l^{-1} in the presence of 0.1 μ mol ionomycin l^{-1} resulted in a slight increase in cell proliferation. All other concentrations of DOG and all concentrations of TPA tested in the presence of either concentration of ionomycin proved to be growth-inhibitory or toxic. Co-incubation with ionomycin in effect reduced the concentrations of both activators required to produce a growth-inhibitory response. As PKC is a known target for both DOG and TPA and calcium is necessary for the activation of PKC (Takai *et al.*, 1979; Quest, 1996), one explanation for these results is that there is a fine balance between intracellular calcium concentrations and the concentration of DOG or TPA required to activate or downregulate PKC. However, other effects of calcium, not related to PKC, may also be involved.

Whereas the results with PKC activators provided strong evidence for a role for PKC in controlling ES-cell proliferation, the results with the PKC inhibitor Bis II alone are more complex. Although Bis II reduced the number of ES cells as measured by the MTT assay in a dose-dependent manner, it also increased the incidence of apoptosis; the highest concentration of Bis II (10 μ mol l^{-1}) induced significant (16%) apoptosis and this increase in apoptotic cells is sufficient to account for much if not all the decrease in the number of cells measured by the MTT assay. The inert analogue Bis V had no effect in the MTT assay. The MTT assay measures cell metabolic activity including mitochondrial activity (Denizot and Laing, 1986; Pechhold and Kabelitz, 1998) and thus can reflect not just changes in cell proliferation but also decreases in cell activity or cell death due to cell apoptosis or necrosis. There was no effect of Bis II on cell death as measured by live–dead staining in the present study. This result is not unusual; Savickiene *et al.* (1999) found that the PKC inhibitor calphostin C caused considerable apoptosis (26%) in HL-60 cells even though cell viability as measured by a dye-exclusion assay was high ($\geq 98\%$).

It is possible that PKC inhibition in ES cells may directly stimulate apoptosis; it is also possible that the increased apoptosis may be triggered by inhibition of cell proliferation resulting from PKC inhibition. There is considerable controversy over the role of PKC in apoptosis; both the inhibition and activation of PKC isoforms have been correlated with induction of apoptosis (Han *et al.*, 2000). One hypothesis is that reduced PKC activity leads to apoptosis and that the increased incidence of apoptosis seen after PKC activation is due to a resulting downregulation of PKC (Leszczynski, 1996). However, there are at least twelve different PKC isozymes and there is evidence that some are anti-apoptotic and some pro-apoptotic (reviewed by Dempsey *et al.*, 2000). There is also evidence that in some tissues there are distinct PKC isozymes for cell proliferation and apoptosis (Weller *et al.*, 1999). There are differences in the

expression and distribution of the various PKC isozymes throughout mammalian tissues and certain isoforms are localized to specific tissues (Nishizuka, 1992; Hofmann, 1997; Kanashiro and Khalil, 1998). These differences in PKC isozyme distribution and activity may explain the results of Ward *et al.* (2000), who found that the PKC inhibitor, staurosporine but not Bis I (an analogue of Bis II), induced apoptosis in mouse embryos over 24 h.

The specificity of protein kinase inhibitors in general, including some bisindolylmaleimides, has been questioned. Davies *et al.* (2000) found that some bisindolylmaleimides inhibit protein kinases other than PKC (Bis II was not examined); in contrast, Han *et al.* (2000) found that the bisindolylmaleimides appear to be specific for PKC.

Cross-talk between signal transduction systems and between components of signalling systems is common (Houslay, 1991; Bourne, 1995; Zamponi *et al.*, 1997). The present study used Bis II to inhibit PKC and TPA to activate PKC to examine the possible feedback regulation PKC may exert on PLC activity (Pfeilschifter *et al.*, 1989; Boarder and Challiss, 1992). Inhibition of PKC by preincubation with Bis II caused a dose-dependent increase in inositol phosphate formation. The opposite situation, activation of PKC with TPA, caused a decrease in inositol phosphate formation. These studies demonstrate that increased PKC activity downregulates PLC activity in ES cells. It has been known for some time that PKC phosphorylates the cytoplasmic domain of cell membrane receptors leading to their downregulation. PKC has also been shown to phosphorylate G-proteins and thereby inactivate them (Orellana *et al.*, 1987; Smith *et al.*, 1987). Whatever the site of action, PKC activity modulates the production of inositol phosphates in ES cells.

In summary, the work presented here indicates that the PtdIns signalling system is essential for normal proliferation of ES cells and may also be involved in control of apoptosis. A limitation of the approach used in this study is its reliance on the use of pharmacological inhibitors and activators with their associated problems of specificity. However, the general conclusion that the PtdIns system plays an important role in ES cells is strengthened by the fact that the evidence is based on the use of a number of inhibitors and activators.

The PtdIns system in ES cells appears to be constitutively active possibly through the action of some autocrine or paracrine factor. Although the system appears to be essential for ES-cell proliferation, activation of the system is probably insufficient to stimulate normal ES-cell proliferation. Both ionomycin treatment to increase intracellular calcium and DOG or TPA treatment to activate PKC stimulated ES-cell proliferation but not to the same extent as FCS treatment. Burdon *et al.* (2002) described how cell proliferation, self renewal and differentiation in ES cells are controlled by at least three signalling systems: the JAK–STAT, the

phosphatidylinositol-3-OH kinase, and the extracellular-signal-related kinase (ERK) systems. The PtdIns signalling system and PLC can activate the ERK system, which is a complex and diverse system (van Biesen *et al.*, 1996; Dhanasekaran *et al.*, 1998). Although the present study indicates that PtdIns system activity is essential for ES-cell proliferation, it is but one of a number of systems involved in ES-cell control.

As ES cells are derived from the inner cell mass of the blastocyst and therefore may be a useful model for inner cell mass development, these studies support the results of other studies (Kane *et al.*, 1986; Kane, 1988; Stachecki and Armant, 1996a,b), indicating that the PtdIns signalling pathway could play an important part in the regulation of blastocyst growth and development.

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