Generation of diacylglycerol molecular species through the cell cycle: a role for 1-stearoyl, 2-arachidonyl glycerol in the activation of nuclear protein kinase C-βII at G2/M

Elizabeth M. Deacon1, Trevor R. Pettitt2, Paul Webb1, Timothy Cross1, Hema Chahal1, Michael J. O. Wakeham2 and Janet M. Lord1, *

1MRC Centre for Immune Regulation and 2CRC Institute for Cancer Studies, Birmingham University, Birmingham B15 2TT, UK
*Author for correspondence (e-mail: j.m.lord@bham.ac.uk)

Accepted 14 November 2001

Summary
Protein kinase C (PKC) is a family of 11 isoenzymes that are differentially involved in the regulation of cell proliferation. PKC-βII, a mitotic lamin kinase, has been shown previously to translocate to the nucleus at G2/M and this was coupled to the generation of nuclear diacylglycerol. However, it is not clear how isoenzyme selective translocation and nuclear targeting is achieved during cell cycle. To investigate further the role of nuclear diacylglycerol we measured PKC isoenzyme translocation and analysed diacylglycerol species at different stages of the cell cycle in U937 cells synchronized by centrifugal elutriation. Translocation of PKC-βIII to the membrane fraction, an indicator of activation, occurred at S and G2/M, although PKC-βII was targeted to the nucleus only at G2/M. Levels of nuclear diacylglycerol, specifically tetraunsaturated species, increased during G2/M. By contrast, there were no obvious changes in nuclear phosphatidic acid species or mass. 1-stearoyl, 2-arachidonyl glycerol (SAG), the major polyunsaturated nuclear diacylglycerol, was able to activate classical PKC isoenzymes (PKC-α and β), but was less effective for activation of novel isoenzymes (PKC-δ), in an in vitro PKC assay. We propose that PKC-βIII nuclear translocation during G2/M phase transition is mediated in part by generation of SAG at the nucleus.

Key words: Cell cycle, Protein kinase C, Nucleus, Diacylglycerol, G2/M

Introduction
Protein kinase C (PKC) is a lipid-activated serine/threonine protein kinase consisting of 11 isoenzymes that are differentially regulated by co-factors (Nishizuka, 1995). The functions of the various isoforms do not appear to overlap and an important mechanism in their regulation is selective activation and targeting to distinct sub-cellular locations (Jaken, 1996). For example, PKC-βII expression is required for the proliferation of human leukaemic cells (Thompson and Fields, 1996), while Goss et al. have shown that this isoenzyme translocates to the nucleus at G2/M, concomitant with the phosphorylation of lamin B1 (Goss et al., 1994). Thus PKC-βIII has been proposed as a mitotic lamin kinase involved in the disassembly of the nuclear lamina during mitosis. What is not clear is how PKC-βII is activated selectively and the direction of translocation controlled. The 11 isoforms of PKC can be divided into classical (α, βI, βII, γ), novel (δ, ε, η, θ) and atypical (λ/τ, ζ) subgroups, according to their requirements for activation. PKC-μ forms a fourth group and is also known as protein kinase D. All require association with acidic phospholipids for full activation; the classical PKCs are calcium dependent, whereas both the classical and novel isoformes are activated by diacylglycerol (DAG). The atypical PKCs are not activated by DAG but appear to respond to distinct lipid factors including ceramide. Differential activation and subcellular translocation of specific PKC isoforms could therefore be mediated by the generation of endogenous PKC activators in specific subcellular compartments. Furthermore, these activators would have to show a degree of selectivity towards PKC isoformes within each sub-class.

The inositol 1,4,5 trisphosphate (Ins(1,4,5)P3) signalling system has been shown to be increased during G2/M in regenerating liver (Marino et al., 1992) and local calcium transients have been shown to arise in the perinuclear region prior to mitosis in sea urchin eggs (Wilding et al., 1996). Such a localised increase in calcium flux prior to mitosis will provide the calcium required for activation of the classical PKCs, but with no selectivity of PKC-β over α, or for nuclear localisation of the activated PKC. Murray and Fields have more recently shown that phosphatidylglycerol (PG) is a potent and selective activator of PKC-βII, interacting with a region within the C-terminal catalytic domain required for activation of this kinase at the nucleus (Murray and Fields, 1998). Moreover, PG increases PKC-βII enzyme activity above that seen with phosphatidylserine and DAG alone. However, nuclear PG levels do not change through the cell cycle (Murray and Fields, 1998), leading the authors to suggest that PG is involved in enhancing the selective association of PKC-βII with the nuclear membrane and sustained activation of the enzyme,
while initial activation is achieved by another lipid species, possibly nuclear DAG (Sun et al., 1997). Diacylglycerols are potent activators of PKC that are generated as second messenger molecules from the hydrolysis of membrane phospholipids, primarily phosphatidylglycerol, 4,5 bisphosphate (PtdIns(4,5)P_2) (Noh et al., 1995) and phosphatidylcholine (Exton, 1996). DAG serves as a hydrophobic anchor, localising PKC to the membrane, as well as being involved in enzyme activation (Newton, 1995). Several groups have shown that PtdIns(4,5)P_2 hydrolysis occurs within the nuclear matrix (Cocco et al., 1987; Irvine and Divecha, 1992) and that the nuclear phosphoinositide cycle is distinct, and regulated separately, from the classic plasma membrane cycle (Cocco et al., 1989). Moreover, DAG production from phosphatidylcholine in the nuclear envelope was demonstrated in IIC9 fibroblasts following α-thrombin stimulation (Jarpe et al., 1994). Nuclear translocation of PKC isoenzymes, or the activation of pre-existing nuclear PKCs, may therefore be mediated by the generation of DAG at the nucleus at a specific stage in the cell cycle. The few studies that have attempted to correlate DAG mass with PKC translocation, have not been informative. However, analysis of individual DAG species has revealed that the component molecular species are numerous and the generation of polyunsaturated species does correlate with PKC activation (Pettitt and Wakelam, 1999).

Here we show that the activation of PKC-βII through the cell cycle correlates with the generation of polyunsaturated DAG species at the nucleus, primarily 1-stearoyl, 2-arachidonyl glycerol (SAG). Moreover, SAG showed some selectivity for the activation of classical PKC isoenzymes over nontoxic PKCs in an in vitro kinase assay and this diacylglycerol may represent a significant physiological activator of PKC-βII at G2/M. We propose that the molecular composition of DAG generated by lipid hydrolysis will contribute to differential signalling through the PKC pathway in a variety of cell processes, including the cell cycle.

Materials and Methods

Cell culture and elutriation

U937 promonocytic cells were cultured in RPMI 1640 medium containing 10% foetal calf serum (Gibco-BRL, Paisley, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, Poole, UK), in a humidified, 95% air, 5% CO_2 atmosphere. For cell cycle studies, cells in each stage of the cell cycle were isolated by centrifugal elutriation (Hoppe et al., 1991). U937 cell cultures in exponential growth were separated into three fractions, containing cells in G1, S and G2/M, using a Beckman JE6B elutriator rotor in a JE6NE centrifuge. Cell cycle status and the purity of each fraction were confirmed by staining cells with propidium iodide and FACS analysis. Cell viability was greater than 98%.

Isolation of nuclei

Nuclei were isolated from U937 cells using a rapid method designed to retain the double nuclear membrane and minimise loss of nuclear proteins (Bunce et al., 1988). Briefly, cells were suspended at 10^7 cells/ml in buffer A (50 mM Tris-HCl pH 7.4, 250 mM sucrose, 50 mM KCl, 2 mM MgSO_4, 1 mM dithiothreitol) containing 2% Tween-40 and immediately snap frozen in liquid nitrogen. Cells were then thawed slowly and homogenised with 25 strokes in a Dounce homogeniser. The homogenate was layered onto a cushion of 30% (w/v) sucrose in buffer A in a microfuge tube and spun at 11,000 g for 1 minute (MSE microcentaur). The supernatant was discarded and the nuclear pellet washed three times in buffer A. Purity of nuclei was checked using marker enzymes as described previously (Bunce et al., 1988) and the only contaminating element was endoplasmic reticulum, which was routinely less than 6%.

Measurement of DAG mass and analysis of DAG molecular species

Lipids from whole U937 cells and from isolated U937 nuclei were extracted using the method of Bligh and Dyer and total DAG was then measured using a DAG kinase mass conversion assay (Priess et al., 1987) using DAG kinase purchased from Boehringer-Mannheim. Changes in DAG saturation were measured using a silver nitrate TLC methodology. Lipid extracts were [32P]-phosphorylated as for the DAG kinase assay (Priess et al., 1987) in a total volume of 90 μl. The reaction was stopped by addition of 70 μl chloroform/methanol/0.5 M HCl (150:300:20 by volume), left to extract for 10 minutes then mixed with a further 225 μl chloroform followed by 225 μl 0.1 M HCl to split the phases. The upper phase was discarded and the lower organic phase (containing the [32P]phosphatidate now in free acid form) washed with 600 μl chloroform/methanol/water (3:48:47 by volume). After drying, the samples were methylated with a saturated solution of diazomethane in diethyl ether (overnight at room temperature), dried again, resuspended in 20 μl chloroform/methanol (2:1 v/v) and separated by silica TLC developed three times with chloroform/methanol (98:2 v/v). The dimethyl [32P]-phosphatidate band was eluted with chloroform/methanol (2:1 v/v). Finally separation based on double bond number was achieved using TLC plates impregnated with 5% AgNO_3, developed firstly to about half way with chloroform/methanol (93:7 v/v), air dried and then developed to the top with chloroform/methanol (97:3 v/v). Detection and quantification was carried out by phosphorimaging using a Molecular Dynamics PhosphorImager. For analysis of DAG molecular species, lipids from a minimum of 5×10^6 cells and 1×10^6 nuclei were derivatized with 3,5-dinitrobenzoyl chloride and separated by hplc as described previously (Pettitt and Wakelam, 1993).

Assessment of PKC isoenzyme translocation during cell cycle

Cells in G1, S or G2/M, isolated by centrifugal elutriation, were assessed for PKC isoenzyme activation/translocation by measuring association of PKC with the particulate (membrane) fraction (Pongracz et al., 1999). Briefly cells were resuspended at 10^7 cells/ml in hypotonic lysis buffer (20 mM Tris-HCl pH 7.4, 5 mM MgCl_2, 1 mM dithiothreitol, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin) and swollen on ice for 10 minutes. Cells were then homogenized with 25 strokes in a tight fitting Dounce homogeniser. Nuclei were isolated at 1000 g for 10 minutes and cytosol and particulate fractions prepared at 100,000 g for 45 minutes. Nuclear, cytosolic and particulate fractions were assessed for PKC isoenzyme content by western blotting using isoenzyme-specific antibodies (Santa Cruz) and HRP-conjugated secondary antibodies (Santa Cruz). Equal loading of nuclear extracts was confirmed by reprobing blots with an anti-lamin B antibody (N. Chaudhary, RPI, Boulder Co., USA). Blots were developed using enhanced chemiluminescence (ECL, Amersham International).

In vitro PKC enzymatic assay

Activation of recombinant PKC isoenzymes was measured using an in vitro micellar assay, essentially as described previously (Lord and Ashcroft, 1984). Briefly, 200 ng of recombinant human PKC-α, βII, δ or ζ (CN Biosciences, Nottingham, UK) was combined with lipid micelles containing 16 μg/ml phosphatidylserine (PS, Sigma) and 1.6 μg/ml sn1,2 stearoyl, arachidonyl glycerol (Sigma) or PS alone, in
PKC assay buffer (20 mM Tris-HCl pH 7.4, 5 mM magnesium acetate, 0.2 mg/ml histone H1, 50 μM CaCl2). The reaction was started by the addition of 20 μM ATP and 1 μCi per assay tube of [γ-32P]ATP (Amersham International). The reaction was carried at 30°C and terminated after 10 minutes by the addition of ice-cold trichloroacetic acid. Incorporation of radioactivity into histone was determined by scintillation counting and enzyme activity was expressed as pmol of 32P incorporated per minute per mg protein kinase C.

Indirect immunostaining for PKC-βII
For analysis of PKC isoenzyme subcellular localisation, cells were indirectly immunostained as cytopsins, after air-drying for 2 hours and fixation in ice-cold acetone, as previously described (Pongracz et al., 1999). Primary antibody was affinity purified and raised in rabbits against peptides in PKC-βII (Santa Cruz). Anti-rabbit IgG-FITC conjugated antibody (Dako, UK) was used as the secondary antibody and fluorescence was visualised using a confocal microscope (MRC 500, BioRad).

LC-MS analysis of phosphatidic acid (PA)
Lipids were extracted from cell or nuclei pellets by vigorous mixing with methanol (1 ml containing 2 nmol 12:0/12:0-phosphatidic acid (PA) internal standard) followed by chloroform (2 ml). After standing for 10 minutes, phases were split by addition of 0.88% KCl in 0.1 M HCl (1 ml) and the upper aqueous phase discarded. The lower organic phase was washed with 1 ml methanol/0.88% KCl in 0.1 M HCl (1:1 v/v) then dried under a stream of nitrogen before resuspending in a small volume of chloroform/methanol (2:1 v/v). The total lipid extract was separated and characterized by LC-MS (QP8000alpha, Shimadzu) using a Luna silica column (3μ, 2.0×150nm; Phenomenex, UK) with a solvent gradient of chloroform/methanol/water/ammonia (90:9.5:0.5:0.32 by volume) over 40 minutes at 0.35 ml/minute. PA was detected in negative electrospray ionisation (ESI) mode (nitrogen flow; 4 l/minute, CDL: 300°C, probe high voltage; ~5 kV) with a retention time of approximately 30 minutes.

Results
Translocation of PKC isoenzymes at different stages of the cell cycle
Centrifugal elution was used to isolate cells at G1, S and G2/M stages of the cell cycle from cultures of U937 cells in exponential growth. This method allowed us to synchronise cells without the use of cell cycle blocking agents, which might perturb cell membranes and give artefactual results. FACS analysis of propidium iodide-stained cells confirmed that the fractions isolated contained cells that were predominantly at G1, S or G2/M (data not shown). Cells from each of the cell cycle phases were lysed and the distribution of each PKC isoenzyme between the cytosol and particulate fractions determined. We have reported previously that U937 cells contained significant levels of PKC-α, βI, βII, δ and ζ, with lower but detectable levels of PKC-ε (Pongracz et al., 1995). Fig. 1 shows that PKC-α was located predominantly within the cytosol of G2/M cells, but translocated to the membrane at G1 and to a lesser extent at S phase. As association of PKC with cell membranes is an indicator of enzyme activation (Kraft and Anderson, 1983), these data indicate that PKC-α was activated predominantly during G1. By contrast, PKC-βII was associated routinely with the membrane fraction only at S and G2/M, with greater association at G2/M (Fig. 1). The third isoenzyme showing cell cycle regulation was PKC-ζ, this PKC was present only in the cytosol during G1 and S phase and translocated to the membrane during G2/M. PKC-δ (Fig. 1), PKC-β and PKC-ε (data not shown) did not show any change in subcellular location with cell cycle.

PKC-βII translocation was investigated further by isolation of nuclei and western blotting of nuclear extracts. Some PKC-βII was detected in the nuclear fraction at G1 and S phase but was significantly increased at G2/M (Fig. 2A). The ratio of PKC-βII to nuclear lamin B was calculated to allow for unequal loading of gels and confirmed increased association of this isoenzyme with the nucleus at G2/M (Fig. 2B). PKC-βII translocation to the nucleus was also confirmed by indirect immunostaining. Cells in G1, S and G2/M were indirectly immunostained using an isoenzyme-specific antibody to PKC-βII and immunofluorescence analysed by laser scanning confocal microscopy. PKC-βII immunoreactivity was present in the cytosol and nucleus at G1 and S and the fraction of the isoenzyme associated with the nucleus increased during G2/M (Fig. 2C). Counterstaining of cells with propidium iodide allowed for quantification of the degree of coincidence between FITC-immunofluorescence (i.e. PKC) and PI (i.e. DNA) staining. This analysis showed that association of PKC-βII with the nucleus was highest during G2/M, with a 21.6±3% increase in nuclear PKC-βII compared with G1. Our data thus confirm the nuclear translocation of PKC-βII during G2/M phase transition (Goss et al., 1994).

DAG mass in nuclei at different stages in the cell cycle
The DAG content of whole U937 cells at G1, S and G2/M...
stages of the cell cycle and nuclei isolated from these cells, was measured using a mass conversion assay. DAG mass in whole cells was very similar across the cell cycle, with only a modest increase in DAG at G2/M (388±42 pmoles/10^6 cells) compared with G1 phase cells (351±48 pmoles/10^6 cells), confirming published data (Thompson and Fields, 1996). Nuclear DAG levels were significantly greater at G2/M compared with nuclei from cells in G1 or S phase (Fig. 3A). The change in nuclear DAG levels can account for the small increase in whole cell DAG seen at G2/M and is therefore unlikely to result from contamination of nuclear preparations with whole cells. These data are expressed as pmoles of DAG per 10^6 cells or nuclei and could potentially be influenced by the different size of the cells as they progress through cell cycle. However, expressing DAG mass as a ratio of total phospholipid gave the same trend, with a lesser but significant increase in nuclear DAG at G2/M (5.77±0.6 pmol DAG/pmol phospholipid) relative to G1 (3.34±0.3 pmol DAG/pmol phospholipid; P<0.05).

Changes in DAG molecular species in nuclei during cell cycle
Measurement of changes in DAG mass are of limited value in interpreting differential changes in the activation status of specific PKC isoenzymes through the cell cycle, as eight of the known isoenzymes are responsive to DAG. In addition, measurements of DAG mass could mask significant changes in individual molecular species and although most DAG species can activate PKC in vitro, we have already shown that polyunsaturated DAGs are probably the physiological activators of PKC in vivo (Wakelam, 1998).

Silver nitrate TLC analysis of nuclear DAG (following [32P]-phosphorylation and diazomethane methylation to form dimethyl [32P]-phosphatidate) revealed that mono- and di-unsaturated DAGs were the major unsaturated species in the nucleus (Fig. 3B). There was a fall in the level of monounsaturated species during G2/M as a percentage of total nuclear DAG, although the absolute mass did not change significantly. However, a corresponding and significant increase in polyunsaturated species was seen during G2/M, specifically in the tetra-unsaturated DAGs, both as a percentage of total nuclear DAG (Fig. 3B) and in absolute mass, suggesting that physiologically relevant DAGs were generated at the nucleus through the cell cycle. To obtain further information on the nature of these DAG species, nuclear lipid extracts were 3,5-dinitrobenzoyl derivatized and then separated by HPLC. The major nuclear DAG species identified were 14:0/16:0, 14:0/18:1n-9, 16:0/16:0, 16:0/18:1n-9, 16:0/18:2n-6, 18:0/18:0, 18:0/18:1n-9, 18:0/18:2n-6 and 18:1n-9/18:1n-9, while the predominant polyunsaturated species was shown to be the tetraunsaturated 1-stearoyl, 2-arachidonyl glycerol (18:0/20:4n-6; SAG). The mass of this DAG doubled during G2/M (2.05-fold increase compared with G1 levels), correlating with the nuclear translocation of PKC-bII. The actions of SAG on PKC-bII were therefore investigated further.

PA changes
Since termination of DAG signalling is believed to be through the action of diacylglycerol kinase (DAGK), resulting in the formation of PA, this lipid was also analysed for cell-cycle-
SAG was found to be less efficient in the activation of the novel PKC isoenzyme PKC-δ (Fig. 4). SAG is therefore a potent activator of PKC and shows some specificity for classical PKC isoenzymes.

**Discussion**

We have investigated PKC isoenzyme translocation and diacylglycerol generation in U937 cells synchronised by centrifugal elutriation. Translocation of PKC-βII to the membrane fraction, an indicator of activation, occurred at S and G2/M, although PKC-βII was targeted to the nucleus only at G2/M. Levels of nuclear diacylglycerol, specifically tetraunsaturated species, increased during G2/M correlating with nuclear translocation of PKC-βII. Furthermore, addition of 1-stearoyl, 2-arachidonyl glycerol, the major polyunsaturated nuclear diacylglycerol, to recombinant PKC isoenzymes gave a potent activation of classical PKC isoenzymes α and βII, with only minimal activation of PKC-δ. We propose that the generation of tetraunsaturated diacylglycerol at the nucleus, specifically SAG, contributes significantly to the selective activation and nuclear translocation of PKC-βII during G2/M phase (Goss et al., 1994).

The data reported here address the question of selective activation of PKC isoenzymes during cell cycle and are novel in several respects. Unlike the majority of reports in the literature concerning cell cycle changes in lipid generation (Divecha et al., 1997) or PKC activation (Goss et al., 1994), we have not used pharmacological agents to arrest cells at a particular stage of the cell cycle. Such studies have synchronised proliferating cells either by serum starvation, or with agents such as hydroxyurea, aphidicolin or nocodazole. These extreme methods will almost certainly affect cell processes, including signalling pathways. More importantly, following release from growth arrest, cells do not remain synchronised and subtle changes in lipid generation during each cell cycle stage could be missed due to lack of synchronisation. Therefore in the studies reported here, cells were separated into the different stages of the cell cycle by centrifugal elutriation, which causes minimal cellular perturbation. In addition, DAG mass and individual DAG molecular species were analysed in whole cells and nuclei isolated from cells at G1, S and G2/M. The data reveal distinct patterns of DAG species generation and PKC isoenzyme activation through the cell cycle.

Our results show that PKC-βII was most active in the G2/M phase, accompanied by increased nuclear localisation. These observations confirm the findings of Fields and co-workers, who have reported the association of PKC-βII with the nucleus at G2/M (Thompson and Fields, 1996) and its role in mitosis as a mitotic lamin kinase (Goss et al., 1994). Their studies were

---

**Effect of 1-stearoyl, 2-arachidonyl glycerol on PKC isoenzyme activity in vitro**

To determine whether the elevation of SAG in the nucleus at G2/M might contribute to the selective activation of PKC-βII seen during cell cycle we tested the ability of SAG to activate PKC isoenzymes using an in vitro kinase assay and recombinant human PKC isoenzymes. The results (Fig. 4) show that SAG could activate classical PKCs in vitro, with PKC-α responding as well as PKC-βII to SAG. In addition, SAG was found to be less efficient in the activation of the novel PKC isoform PKC-δ (Fig. 4). SAG is therefore a potent activator of PKC and shows some specificity for classical PKC isoenzymes.

---

**Discussion**

We have investigated PKC isoenzyme translocation and diacylglycerol generation in U937 cells synchronised by centrifugal elutriation. Translocation of PKC-βII to the membrane fraction, an indicator of activation, occurred at S and G2/M, although PKC-βII was targeted to the nucleus only at G2/M. Levels of nuclear diacylglycerol, specifically tetraunsaturated species, increased during G2/M correlating with nuclear translocation of PKC-βII. Furthermore, addition of 1-stearoyl, 2-arachidonyl glycerol, the major polyunsaturated nuclear diacylglycerol, to recombinant PKC isoenzymes gave a potent activation of classical PKC isoenzymes α and βII, with only minimal activation of PKC-δ. We propose that the generation of tetraunsaturated diacylglycerol at the nucleus, specifically SAG, contributes significantly to the selective activation and nuclear translocation of PKC-βII during G2/M phase (Goss et al., 1994).

The data reported here address the question of selective activation of PKC isoenzymes during cell cycle and are novel in several respects. Unlike the majority of reports in the literature concerning cell cycle changes in lipid generation (Divecha et al., 1997) or PKC activation (Goss et al., 1994), we have not used pharmacological agents to arrest cells at a particular stage of the cell cycle. Such studies have synchronised proliferating cells either by serum starvation, or with agents such as hydroxyurea, aphidicolin or nocodazole. These extreme methods will almost certainly affect cell processes, including signalling pathways. More importantly, following release from growth arrest, cells do not remain synchronised and subtle changes in lipid generation during each cell cycle stage could be missed due to lack of synchronisation. Therefore in the studies reported here, cells were separated into the different stages of the cell cycle by centrifugal elutriation, which causes minimal cellular perturbation. In addition, DAG mass and individual DAG molecular species were analysed in whole cells and nuclei isolated from cells at G1, S and G2/M. The data reveal distinct patterns of DAG species generation and PKC isoenzyme activation through the cell cycle.

Our results show that PKC-βII was most active in the G2/M phase, accompanied by increased nuclear localisation. These observations confirm the findings of Fields and co-workers, who have reported the association of PKC-βII with the nucleus at G2/M (Thompson and Fields, 1996) and its role in mitosis as a mitotic lamin kinase (Goss et al., 1994). Their studies were
performed on cells that were synchronised by treatment with aphidicolin and then harvested at regular intervals to assess nuclear localisation of PKC-βII. By the time the population taken to be G2/M were harvested 8 hours later, the cells were no longer synchronised. Although their G2/M fraction contained approximately 50% of cells in G2/M, as assessed by FACS analysis, a significant proportion of cells were in S phase (Hocovar and Fields, 1991). However, the data reported here show that although PKC-βII showed increased membrane association during S phase, significant translocation to the nucleus only occurred during G2/M.

Although the studies of Fields and co-workers have determined the involvement of PKC-βII in the regulation of cell cycle, they have not identified mechanisms to fully explain the differential activation and nuclear translocation of this isoenzyme during cell cycle. The process of selective activation of PKC isoenzymes, eight of which are responsive to the physiological activator diacylglycerol, is fundamental to the PKC signalling pathway, but remains poorly understood. Murray and Fields identified a nuclear factor that stimulated PKC-βII activity to levels 3-6 times greater than those achieved by optimal concentrations of calcium, DAG and PS (Murray et al., 1989) and have shown subsequently that this factor is phosphatidylglycerol, PG (Murray and Fields, 1998). However, levels of PG at the nuclear membrane do not change through the cell cycle and are thus insufficient alone to mediate the selective nuclear targeting and activation of PKC-βII during G2/M. Our data suggest that activation of PKC-βII through cell cycle may also involve the generation of polyunsaturated DAG species, specifically 1-stearoyl, 2-arachidonyl glycerol. DAG species rich in stearate and arachidonate indicate phospholipase C (PLC)-mediated hydrolysis of PtdIns(4,5)P2 (Divecha et al., 1991) and a nuclear PI-PLC activity has been identified that is active during G2 (Sun et al., 1997). Moreover, inhibition of nuclear PI-PLC led to a decrease in nuclear DAG and cell cycle arrest in G2 (Sun et al., 1997). SAG generated at the nucleus is therefore likely to arise from the hydrolysis of PtdIns(4,5)P2. Although the increase in tetraunsaturated species at G2/M were modest, we propose that SAG acting together with PG, could mediate the targeting of PKC-βII to the nucleus at G2/M. Increases in nuclear polyunsaturated DAG during G2/M have also been observed in the human keratinocyte Hakat cell line (T.R.P., unpublished), demonstrating that this phenomenon is reproducible and not unique to the hematopoietic cells.

In an attempt to pick out the signalling DAG from the non-signalling background we applied the methodology of D’Santos et al. (D’Santos et al., 1999), which uses the endogenous nuclear DAGK to specifically [32P]-phosphorylate the nuclear DAG, the [32P]-PA can then be analysed by silver nitrate TLC. A short (5 minute) incubation might be expected to phosphorylate the signalling DAG at a faster rate than nonsignalling DAG since, in vivo, one or more DAGKs must have the capacity to rapidly and selectively phosphorylate signalling DAG and thus terminate DAG signalling. Unfortunately, while exogenous DAG could be phosphorylated by the endogenous nuclear DAGK, indicating an active kinase, only trace amounts of endogenous nuclear DAG were phosphorylated, far too little for species analysis (data not shown). This suggests differences in nuclear DAGK activity and/or regulation between the U937 cells used here and the murine erythroleukemia (MEL) cells used in the D’Santos work. A complementary LC-MS approach to investigate nuclear PA species changes failed to detect reproducible differences over the cell-cycle; however, changes may not be detectable since our earlier work suggested that polyunsaturated PA formed from signalling polyunsaturated DAG may be metabolised more rapidly than the PA derived from other sources, thereby preventing accumulation of signalling DAG-derived PA (Pettitt et al., 1997). An alternative explanation is that the DAG may be metabolised in the nuclei through a different pathway, for example the action of CTP:phosphocholine cytidylyltransferase activity directly generating PC. The consequence of this would be that the nuclear DAG is not available for phosphorylation.

Current models of classical PKC isoenzyme activation suggest that association with membranes induces clustering of acidic phospholipids such as PS and PG (Bazzi and Nelsestuen, 1991) and the role of DAG is to increase affinity of PKC for the membrane (Murray and Fields, 1998). The role of diacylglycerol in the activation of novel PKCs is less clear, as recent studies have shown that DAG enhanced association of PKC-β with PS-containing micelles, but had a much lesser effect on PKC-ε (Medkova and Cho, 1998). The inability of SAG to induce membrane association of PKC-δ, or significant activation in vitro, would concur with these data and support the suggestion that DAG may not be a key activator of the novel PKCs in vivo (Medkova and Cho, 1998). As SAG was able to activate PKC-α in vitro, generation of this DAG alone would not be expected to produce the selective translocation and sustained activation of PKC-βII seen during G2/M. However, if our results are considered together with those of Murray and Fields (Murray and Fields, 1998), we can propose a model in which increased generation of SAG at the nucleus during G2/M induces the initial association of PKC-βII with the nuclear membrane, followed by clustering of PG that then causes firm PKC binding and enhanced activation. Targeting of PKC isoenzymes to distinct intracellular sites is crucial for their role in cell regulation and the translocation and docking mechanisms involved appear to vary. PG, working in concert
with SAG, may thus represent a lipid membrane anchor for PKC-βII at the nucleus.

In conclusion, we propose that the generation of different DAG molecular species at specific sites within the cell contributes to specific PKC isoenzyme activation and translocation during cell cycle and possibly other cell processes influenced by PKC. Activation by DAG coupled with association with isoenzyme-specific docking elements, such as PG, will add a further level of selectivity to PKC activation.

These studies were supported by funds from the Leukaemia Research Fund (E.M.D. and T.C.), EU grant Mas3CT970156 (P.W.) and a Wellcome Trust programme grant held by M.J.O.W. (T.R.P.).

References
Divecha, N., Banfi, H., Tregus, J., Vann, L. R., Irvine, R. F. and D’Santos, C. (1997). Nuclear diacylglycerol, the cell cycle, the enzymes, and a red herring (or how we came to love phosphatidylcholine). *Biochem. Soc. Trans.* **25**, 571-575.