INVESTIGATION OF THE ROLE OF PROTEIN KINASE C (PKC) IN CYTOSTASIS INDUCED BY TUMOUR PROMOTING PHORBOL ESTERS AND RELATED COMPOUNDS.

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Investigation of the role of protein kinase C (PKC) in cytostasis induced by tumour promoting phorbol esters and related compounds.

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Protein kinase C (PKC) is considered to be the major receptor for tumour promoting phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA). These agents evoke a plethora of biological effects on cells in culture. The growth of A549 human lung carcinoma cells maintained in medium fortified with 10% foetal calf serum (FCS) is arrested for 6 days by TPA and other biologically active phorbol esters. In the work described in this thesis, the hypothesis was tested that modulation of PKC activity is closely related to events pivotal for cytostasis to occur. The effect of several phorbol esters, of newly synthesized analogues of diacylglycerols (DAG) and of bryostatins (bryos) on cell growth and ability to modulate activity of PKC has been investigated.
Determination of the subcellular distribution of PKC following treatment of cells with TPA and partial enzyme purification by non-denaturing polyacrylamide gel electrophoresis revealed translocation of enzyme activity from cytosolic to particulate fraction. Chronic exposure of cells to TPA resulted in a time and concentration dependent degradation of enzyme activity. Synthetic DAG and DAG analogues, unable to arrest the growth of cells at non-toxic concentrations, were neither able to affect subcellular PKC distribution nor compete effectively for phorbol ester binding sites at physiologically relevant concentrations. Bryos 1, 2, 4 and 5, natural products, possessing antineoplastic activity in mice, elicited transient arrest of A549 cell growth *in vitro*. They successfully competed for phorbol ester receptors in A549 cells with exquisite affinity and induced a shift in subcellular PKC distribution, though not to the same extent as TPA. Enzyme down-regulation resulted from prolonged exposure of cells to nanomolar concentrations of bryos. *In vivo* studies demonstrated that neither PDBu nor bryo 1 was able to inhibit A549 xenograft growth in athymic mice.
The growth of A549 cell populations cultured under conditions of serum-deprivation was inhibited only transiently by biologically active phorbol esters. Fortification of serum-free medium with EGF or fetuin was able to partially restore sensitivity to maintained growth arrest by TPA. PKC translocation to the particulate cellular fraction and subsequent enzyme down-regulation, induced by TPA, occurred in a manner similar to that observed in serum-supplemented cells. However, total PKC activity and cytosolic phorbol ester binding potential were greatly reduced in the serum-deprived cell population. Western blot analysis using monospecific monoclonal antibodies revealed the presence of PKC-α in both A549 cell populations, with significantly reduced protein levels in serum-deprived cells. PKC-ß was not detected in either cell population.

Keywords: Protein kinase C; A549 human lung carcinoma; phorbol ester; bryostatin; growth inhibition.

To Mum and Dad
with love

Nourishing a youth sublime
with fairy tales of science,
and the long result of time

Lord Tennyson
1809-1892.

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CONTENTS

List of figures.

List of tables.

List of abbreviations.

A549-FCS	serum-supplemented A549 cell population
A549-TPA	cells cultured in excess of 9 weeks in the continued presence of 10nM TPA and more permanently `resistant` to TPA-induced growth-arrest
A549-US	serum-deprived A549 population
Ab	antibody
ADP	adenosine 5`-diphosphate
AFP	alpha fetoprotein
ATP	adenosine 5`-triphosphate
[-^{32}P]ATP	adenosine 5`-[-^{32}P]triphosphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
Bod-3-PE	Bodipy-3-propionyl-13-acetate
Bryo	Bryostatin
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular calcium
cAMP	adenosine 3`5`-cyclic monophosphate
Ci	curies
cpm	counts per minute
DAG	diacylglycerol
1,2-diC_8	1,2-sn-dioctanoylglycerol
d.H_2O	distilled water
DMSO	dimethylsulphoxide
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	ethyleneglycol-bis-(ß-aminoethylether) N,N,N`,N`-tetraacetic acid
EM	electron microscopy / electron micrograph
FACS	flourescence activated cell sorter
FCS	foetal calf serum
g	acceleration due to gravity
G_1	gap 1 phase of cell cycle
G_2	gap 2 phase of cell cycle
G protein	guanine nucleotide binding protein
GTP	guanine 5`-triphosphate
h	hours

IC_{50}	concentration which inhibits cell growth by 50%
IgG	immunoglobulin
IMS	industrial methylated spirits
I $1,4,5P_3$	inositol 1,4,5-trisphosphate
I $1,3,4,5P_4$	inositol 1,3,4,5-tetrakisphosphate
IP	inositol phosphate
i.p.	intraperitoneally
ITS	medium supplement containing selenium, insulin and transferrin
kDa	kilodaltons
KP	potassium persulphate
LC_{50}	concentration yielding 50% cell death
LDH	lactate dehydrogenase
log	logarithm
M	mitotic phase of cell cycle
min	minute
mM	millimolar
μM	micromolar
n	number of experiments
NADH	nicotinamide adenine dinucleotide (reduced form)
NBCS	new born calf serum
NBT	nitro-blue tetrazolium
nM	nanomolar
NP 40	nonidet p 40
OAG	1-oleoyl-2-acetyl-sn-glycerol
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDBu	phorbol-12,13-dibutyrate
[^{3}H]PDBu	[20-^{3}H(N)]-phorbol-12.13-dibutyrate
PDD	phorbol-12,13-didecanoate
4-α-PDD	4-alpha-phorbol-12,13-didecanoate
PDGF	platelet derived growth factor
PE	phosphatidylethanolamine
PI	propidium iodide
PI	phosphatidylinositol
PI_4P	phosphatidylinositol-4-phosphate
$PI_{4,5}P$	phosphatidylinositol-4,5-bisphosphate
PI-PLC	phosphoinositide-specific phospholipase C
PLA_2	phospholipase A_2
PLC	phospholipase C

PLD	phospholipase D
PKC	protein kinase C
pM	picomolar
PTX	pertussis toxin
PS	phosphatidylserine
RA	retinoic acid
RF	riboflavin
RNA	ribonucleic acid
RPA	12-O-retinoylphorbol-13-acetate
rpm	revolutions per minute
S	phase of cell cycle during which DNA is synthesized
s	second
SD	standard deviation
SDS	sodium dodecylsulphate
STSPN	Staurosporin
TBS	Tris buffered saline
TBS-T	Tris buffered saline + Tween 20
TCA	trichloroacetic acid
TGF-α	transforming growth factor-alpha
TGF-ß	transforming growth factor-beta
[^{3}H]TdR	5-[methyl ^{3}H]-thymidine
TEMED	N,N,N`,N`-tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol-13-acetate
Tween 20	polyoxyethylene sorbitan monolaurate
US	ultroser G

Section 1: INTRODUCTION

1.1 Malignant Disease.

Malignant tumours are invasive and destructive with respect to adjacent normal tissue; they metastasize via lymphatic channels or blood vessels to lymph nodes and other tissues in the body causing secondary illness such as cachexia, haemorrhage, and infection. Malignant transformation may arise within any tissue containing cells capable of division, and occurs when completion of differentiation is prevented by cellular interaction with a carcinogenic agent. Thus, a neoplastic cell is blocked or diverted in its differentiation pathway and possesses an heritable insensitivity to growth constraint. Cancer is primarily a disease of aberrant control of proliferation and differentiation. Simplistically, genes controlling these processes are inappropriately and imperfectly expressed. There normally exists a controlled equilibrium between cell proliferation, differentiation and death, a balance which is disrupted in cancer tissue. Cell growth arrest *in vivo* is co-ordinated with expression of the differentiated phenotype. This is the case in certain *in vitro* culture systems, for example, preadipocyte mouse 3T3 cells can be induced to differentiate into fat cells (Scott, 1982). However, cells transformed by carcinogenic agents or oncogenic viruses lose the potential for growth arrest and terminal differentiation (Ruddon, 1987). Potter in 1969 described "oncogeny as blocked ontogeny" as often re-expression of oncofoetal antigens can be detected in association with malignancy. A number of examples exist reporting the ability of malignant cells to differentiate and become non-malignant (Rabson *et al.*, 1987). Pierce (1970) and Brinster (1974) report the spontaneous differentiation to a mature phenotype of murine tumour systems with simultaneous loss of proliferative potential. In a recent observation, Kraft has described the *in vitro* differentiation of chronic lymphocytic leukaemic cells from 15 patients upon administration of the experimental antineoplastic agent, bryostatin 1. Differentiation of certain myeloid leukaemic samples to a macrophage lineage has also been demonstrated (Kraft *et al.*, 1989). The potential reversibility of a malignant phenotype fuels the hypothesis that differentiation inducing therapies may provide a successful approach to malignant disease. To understand the molecular basis of carcinogenesis, there is a need for thorough understanding of the mechanisms controlling cell differentiation and proliferation. These are a major goal of current cancer research and a prerequisite to the development of cancer prevention or cure.

1.2 Therapeutic strategies in malignancy.

There is a desperate need for novel strategies in cancer chemotherapy. The vast majority of drugs currently in the clinic focus on the destruction of rapidly dividing cells. These drugs are highly toxic to normal proliferating cells in the body such as epithelial cells of the gastrointestinal tract, bone marrow cells and cells within hair follicles. The drugs themselves often possess mutagenic properties. Too often, treatment of secondary tumours fails due to emergence of the multidrug resistant phenotype, characterized by the expression of the P-glycoprotein energy dependent efflux pump within the plasma membrane

(Moscow and Cowan, 1988) and/or hyperexpression of the gene encoding dihydrofolate reductase (Haber *et al.*, 1990).

The plasma membrane plays an important role in maintenance of normal cellular behaviour. It provides an interface which transduces signals, permits biochemical processes to occur and allows transport of restricted molecules. Communication with and adherence to other cells are governed by plasma membrane properties. It is at the site of the plasma membrane where proteins involved in the control of proliferation and differentiation are expressed and where oncogene products are found, able to subvert these processes (Hickman, 1988). Indeed, alterations in the biochemical characteristics of the malignant cell surface have been reported, including the appearance of tumour associated cell surface antigens, deviant membrane transport systems, impaired cellular cognition, and increased or aberrant production of oncogene products. Such molecular modifications may be responsible for abnormal growth and behaviour, loss of anchorage dependence and density dependent growth, and invasive potential (Nicolson, 1976).

Evidence will be discussed which suggests that an imbalance of the second messengers, involved in signal transduction may be an important aspect in the transformation of normal cells. Therefore sensible targets to consider in novel cancer chemotherapy development may be membrane components of cell signalling mechanisms. Protein kinase C (PKC) activation has emerged as a pivotal early event involved in the cascade of biochemical processes governing mitogenesis, proliferation and differentiation. Precise elucidation of these mechanisms could conceivably lead to the rational design of novel anticancer agents and new biochemical targets. Evidence for the involvement of inositol phospholipid turnover and the activation of PKC in carcinogenesis will be considered. In particular the identification of this enzyme as the major phorbol ester receptor suggests PKC may be a potential target for therapeutic intervention in malignant disease.

1.3 Protein kinase C (PKC).

1.3.1 Introduction.

PKC is a multifunctional serine/threonine specific, Ca^{2+} and diacylglycerol activated, phospholipid dependent protein kinase, and was first described by Nishizuka and colleagues (Inoue *et al.*, 1977; Takai *et al.*, 1977).

PKC is ubiquitously distributed in tissues and organs of mammals (Kuo *et al.*, 1980), with highest levels found in the brain, followed at much lower levels by spleen, thymus, intestines and lung (Ashendel, 1985). PKC was detected in all vertebrate species tested and was present in organisms of evolutionary lower phyla, the earliest species tested were *Hydra japonica* sea urchin, *Drosophila melanogaste* and the nematode *Caenorhabditas elegans* although none was detected in the bacteria *Bacillus subtilus* (reviewed by Ashendel, 1985). It has been purified to apparent homogeneity from a number of species (Kikkawa *et al.*, 1982 and 1986, Wise *et al.*, 1982). Such conservation of PKC during evolution suggests a biological function which is essential for the survival of multicellular animals.

1.3.2 Molecular heterogeneity of PKC.

Molecular cloning and enzymological analysis have revealed that multiple subspecies of PKC exist. To date, 7 closely related isozymic forms of PKC have been identified and it is anticipated that additional forms are yet to be discovered (Nishizuka, 1988). Initially, the screening of bovine brain cDNA libraries demonstrated 3 related but non-identical isoforms derived from unique genes (Coussens *et al.*, 1986; Parker *et al.*, 1986). Screening of the human foetal brain library confirmed the multiplicity of the PKC species (Coussens *et al.*, 1986). The cDNAs of PKC-α, PKC-ß and PKC-γ were mapped onto chromosomes 17, 16 and 19 respectively. Further, PKC-ß exists in 2 forms; PKC-ßI and -ßII derived from the alternate splicing of a single gene (Ono *et al.*, 1987). Recently, additional members of the PKC family have been identified, termed PKC-δ, PKC-ε and PKC-ζ (Ono *et al.*, 1988). An additional cDNA clone termed -ε` has been isolated, differing from -ε only by a short sequence at the 5`-terminal end region (Ono *et al.*, 1988). Other PKC forms closely related to PKC-ε, termed nPKC (Ohno *et al.*, 1988) and nPKC-ε (Akita *et al.*, 1990) have also been isolated.

1.3.3 Structure of PKC.

All PKC subspecies identified to date exist as a single polypeptide chain whose molecular weight ranges between 76 and 85kD. The structure of PKC is represented schematically in figure 1. PKCs-α, -ßI, -ßII and -γ each possess 4 constant (C_1-C_4) and 5 variable (V_1-V_5) regions. PKC-δ, -ε and -ζ differ only in that they lack the second conserved (C_2) region (Ono *et al.*, 1988). PKC-ßI and -ßII subspecies differ from each other in a short range of approximately 50 amino acid residues at their carboxy terminal extreme (V_5). The enzyme may be defined functionally as comprising two structural domains. The carboxy terminal, of 380 residues and Mr 51kD contains the catalytic domain (Parker *et al.*, 1986). Within this domain lie two conserved regions, C_3 and C_4 and two variable regions, V_4 and V_5. The third conserved region, C_3, possesses a Gly-Gly--Gly sequence (residues 346-351) which beside lysine 368 forms part of the nucleotide binding site. Other sequence stretches within this domain share high homology with all serine, threonine and tyrosine specific kinases (Parker and Ullrich, 1987). The 26kD hydrophilic amino terminal of PKC possesses the regulatory domain and membrane binding functions (Kishimoto *et al.*, 1983). Direct evidence has demonstrated that the DAG, phorbol ester, phospholipid and Ca^{2+} binding sites are located within this regulatory domain (Huang and Huang, 1986; Cazaubon *et al.*, 1989). It has been demonstrated that the conserved region C_2 confers enzyme dependence upon Ca^{2+} for phorbol ester binding activity (Ono *et al.*, 1989). The regulatory domain contains the pseudosubstrate prototype which maintains the catalytic domain of PKC in an inactive state through the occupation of the peptide substrate binding site (House and Kemp, 1987). Toward the amino terminus of the regulatory domain, within the conserved region C_1, in all but PKC-ζ isozyme there is a region characterized by

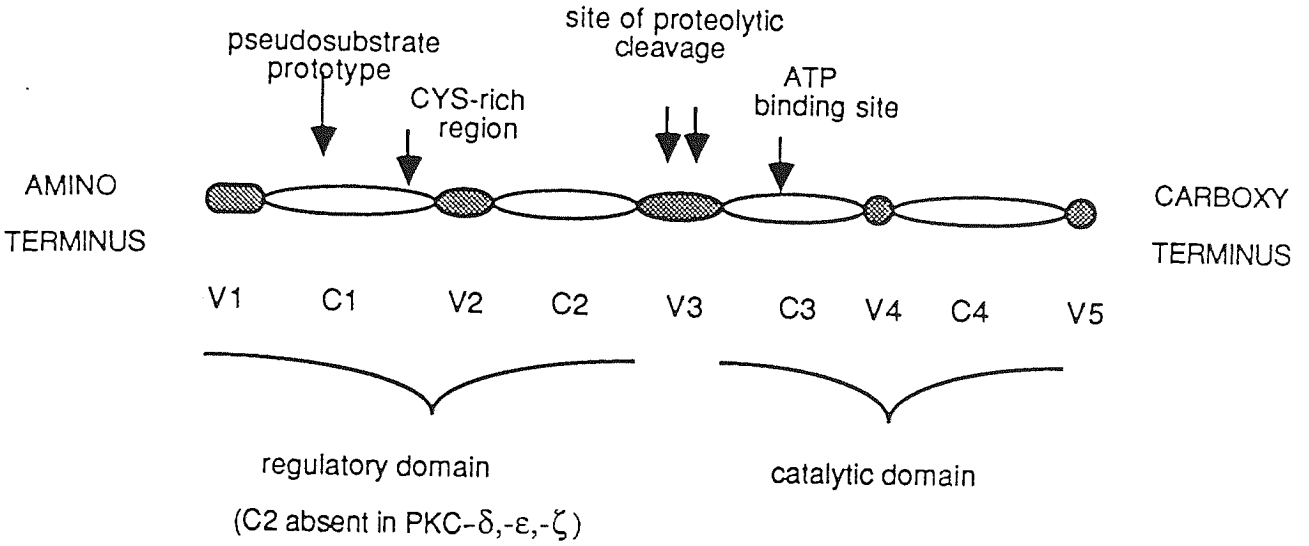


Figure 1. Schematic diagram of Protein kinase C.
The amino terminal regulatory domain and the carboxy terminal catalytic domain are depicted.Between these domains lies the "hinge" region (V3) susceptible to proteolytic attack.

a tandem repeat and within each repeat unit are 6 precisely conserved cysteine residues (Parker and Ullrich, 1986). PKC-ζ contains a single such sequence (Ono *et al.*,1989). The cysteine rich motif exhibits similarities to a number of DNA binding proteins, where such structures have been proposed to form divalent metal ion coordination complexes (Berg, 1986) and demonstrates significant homology to the "cysteine fingers" of steroid receptors; therefore it is speculated that these sequences may play a role in binding DNA. Various deletions and point mutations in this domain have been constructed (Ono *et al.*, 1989). Phorbol ester binding analysis demonstrated that the cysteine rich sequence was essential for PKC to bind phorbol ester. The cysteine rich sequence (C_1) and protein kinase sequence, are both highly homologous among the PKC family. Major regions of heterology within the regulatory domain are the two sequences either side of the cysteine repeats (V_1 and V_2) and the site susceptible to proteolytic attack (V_3) (Parker and Ullrich, 1987).

1.3.4 Differential tissue expression.

The use of cDNA probes and selective antisera has allowed the analysis of the relative expression of PKC genes. Initially it appeared that PKC-γ was exclusive to the brain and spinal cord with highest expression associated with the hippocampus, cerebral cortex, amygdala and cerebellum (Knopf *et al.*, 1986; Nishizuka, 1989). PKC-γ has since been discovered in a number of cell types for example in FDC-P1 myeloid cells (Ways *et al.*, 1990). The -γ subspecies is associated with most membranous structures present throughout the cell except for the nuclear membrane. PKCs with ßI and ßII sequence display differential expression in the brain and many other tissues including endocrine glands. Normally, activity of PKC-ßII exceeds that of ßI isozyme. PKC possessing the α sequence is widely expressed. Co-expression of PKC-α and -ß to varying extents is observed in most tissues including liver, kidney, spleen and testis and a variety of cell lines (Melloni *et al.*, 1989; Sawamura *et al.*, 1989; Beh *et al.*, 1989; reviewed by Nishizuka 1988) with all permutations observed. These subspecies, by light microscopy resolution, illustrate distinct intracellular location, dependent upon the state of differentiation or proliferation. With respect to the distribution of the sequences encoding δ, ε and ζ isozymes; Northern blot analysis has indicated that of those tissues tested PKC-δ has been found in spleen, kidney lung and brain; PKC-ζ was found mainly in the brain but additionally in kidney and lung; mRNA hybridizing with the ε cDNA probe was found in the brain (Ono *et al.*, 1988) and additionally in the heart and lung (Schaap *et al.*, 1989). Such differential tissue type distribution of isozymes, together with the sequential expression of PKC polypeptides observed during development (Yoshida *et al.*, 1988) suggests that the nature of the cellular response may be governed by PKC species expressed. Evidence suggesting the attribution of specific isozymes to specific functions has been obtained by Schaeffer *et al.* (1989). These authors have isolated and characterized

a novel PKC from *Drosophila melanogaster*. Transcripts are expressed solely in photoreceptor cells; thus a role in visual adaptation is proposed.

1.3.5 Activation of PKC.

A quarternary structure for PKC activation has been described. For the execution of phosphotransferase activity, PKC requires Ca^{2+} and an acidic phospholipid such as phosphatidylserine (PS) or phosphatidylinositol (PI). 1,2-diacylglycerol (DAG) generates 2 effects: in the presence of phospholipid, DAG permits the activation of PKC by Ca^{2+} at concentrations normally too low for enzyme activation; secondly, DAG and Ca^{2+} together activate PKC to a greater extent than Ca^{2+} alone. The binding of DAG or phorbol esters to PKC is on a 1:1 basis, but the interaction of enzyme with phospholipid is complex (Hannun *et al.*, 1986). PKC subspecies exhibit subtle individual characteristics in modes of activation and kinetic properties.

PKC-ε is Ca^{2+}-independent (Schaap *et al.*, 1989). In certain conditions activation of PKC-γ when compared to that of -α or -ß was less dependent on Ca^{2+} (Marais and Parker, 1989; Jaken and Kiley, 1987). However, using a preparation of pure lipid micelles, PKC-ß was more sensitive to activation by DAG than PKC-α or -γ and demonstrated substantial activity without added Ca^{2+} in the presence of DAG and phospholipid (Nishizuka, 1988; Marais and Parker, 1989). Zn^{2+} inhibited the catalytic activity of the 3 isoforms tested (α, ß and γ) in the presence of Ca^{2+}, but in its absence, Zn^{2+} was able to activate PKC- γ but not PKC-ß or -α (Sekiguchi *et al.*,1988). PKC-γ is activated at μM concentrations by arachidonic acid in the absence of DAG and phospholipids. The ß and α isoforms respond to higher concentrations of arachidonic acid but only in the presence of elevated Ca^{2+} (Sekiguchi, 1988). PKC-δ, -ε, and -ζ have demonstrated reduced dependency on DAG and phospholipid when compared with α, ß and γ isoforms (Ono *et al.*, 1989). Thus, the potential can be appreciated for selective isozyme activation *in vivo* .

1.3.6 Enzyme translocation.

The accepted mechanism of action of agonist-induced PKC activation is the "mobile-receptor" hypothesis. That physiological activation of PKC is accompanied by translocation of the inactive enzyme from cytosol to cellular membranes was first demonstrated by Kraft and Anderson (1983). 1,2-diC_8 has been shown to induce a discrete but transient translocation of PKC to the plasma membrane (Issandou and Darbon, 1988). Tumour promoting phorbol esters caused a rapid, tight and lengthened association of PKC activity with the plasma membrane (Kraft and Anderson, 1983). If PKC is only active when associated with membranes, the implication is that only membrane proteins may be phosphorylated (Nishizuka, 1984). Activity at the plasma membrane is compatible with PKC's function in signal transduction. However, enzyme translocation to other cellular compartments has been reported. Insulin induced PKC translocation to the cytosol; it is

conceivable that certain key regulators of glycolysis may be influenced herein (Chiarugi, 1989). Although in 1986 Nishizuka reported none or seemingly little PKC present in the nucleus it became an issue of some controversy as to whether nuclear events induced by TPA or other PKC agonists were mediated directly by this enzyme. There is accumulating evidence pertaining to the presence of PKC at the nuclear membrane. Reports have described agonist induced PKC translocation from cytosol to the nuclear membrane thus implicating this enzyme in the regulation of gene expression (Cambier *et al.*, 1987). PKC-α and -ß have been located within the nuclear environment. Following activation of quiescent NIH 3T3 cells with phorbol ester or PDGF, redistribution from cytosol to the nuclear matrix of PKC has been observed (Olah *et al.*, 1990). Specific cellular distribution of PKC during cell cycle has been demonstrated by Kuo *et al.* (1986). In HL60 cells, the primary locales of PKC during G_1 and S phase were the plasma membrane and cytoplasm. During the G_2 phase, PKC was highly restricted to the nucleus. Translocation of PKC to the nuclear compartment during this phase in cell cycle during which RNA synthesis occurs would imply certain control in ensuing gene expression. Bryostatin 1 and interleukin-3, both mitogens for FDC-P1 haematopoietic cells, were shown to mediate rapid translocation of PKC to the nuclear envelope where serine specific phosphorylation of several nuclear envelope polypeptides including lamin B occurred (Fields *et al.*, 1989 and 1988). Phosphorylation of the nuclear lamins A, B and C *via* a PKC-dependent mechanism in cultured vascular smooth muscle cells has been reported subsequent to angiotensin II stimulation with the ensuing induction of c-*fos* expression (Tsuda and Alexander, 1990).

1.3.7 Enzyme down-regulation.

Prolonged exposure of cells to phorbol esters results in down-regulation of PKC activity directly due to loss of the PKC polypeptide (Rodriguez-Pena and Rozengurt, 1984; Blackshear *et al* 1985). This phenomenon is common to many cell types and accompanied by no change in the PKC-mRNA expression nor the rate of PKC synthesis (Mizuguchi *et al.*, 1988; Isakov *et al.*, 1990). Phorbol esters promote prolonged association of activated PKC at the membrane, a process which initiates proteolytic degradation of the enzyme possibly due to the exposure of the proteolytically sensitive "hinge" domain (V_3) (Kishimoto *et al.*, 1983; Young *et al.*, 1987). Hydrolytic cleavage of intact PKC by the Ca^{2+} dependent, neutral protease calpain occurs at specific sites in the V_3 region releasing the catalytically active fragment protein kinase M (PKM), Mr 51kD (Kikkawa *et al.*, 1982; Kishimoto *et al.*, 1983). Release of this active species may allow the protein kinase domain unregulated access to substrates not normally functioning through the inositol phosphate pathway. PKC is believed to be activated *in vivo* by both mechanisms; reversibly by allosteric cofactors and irreversibly, following limited proteolysis (O`Brian and Ward, 1989). Calpain exclusively targets activated forms of PKC, displaying differential activity toward the different isozymes: PKC- γ was very susceptible, whereas PKC-α displayed greater resistance to proteolysis (Kishimoto *et al.*, 1989). Indeed, differential rates of down-regulation between isoforms have been established (Ase *et al.*, 1988; Cooper *et al.*,

1989; Huang *et al.*, 1989). In human neutrophils, modification by calpain of PKC-ß yielded a Ca^{2+}/phospholipid-independent form which retained 50% of the original activity, but PKM formed from PKC-α retained full catalytic activity (Pontremoli *et al.*, 1990). These workers supported the hypothesis that PKM is a temporary intermediate of down regulation, and have suggested that further down regulation is carried out by neutral serine proteinase (Pontremoli *et al.*,1990). Kishimoto *et al.*, (1989) have also demonstrated the rapid removal from cells of PKM. The physiological relevance of down-regulation remains unclear. The regulatory fragment may play a part in the control of gene expression, since it contains the cysteine rich DNA binding motif. A role has been ascribed to this phenomenon in certain cases of uncontrolled proliferation (O`Brian and Ward 1989).

1.3.8 Substrates for PKC.

Protein phosphorylation is an established major general mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli (Hidaka *et al.*, 1984). The biological functions of diacylglycerol (DAG) are manifest through protein phosphorylations catalysed by PKC. PKC catalyses the transfer of the gamma phosphate group of ATP to seryl and threonyl residues (Nishizuka, 1984; Kikkawa and Nishizuka, 1986). PKC is capable of phosphorylating many proteins *in vitro*, for example Rhodopsin (Schaeffer *et al.*, 1989), retinoid binding proteins, vinculin, filamin, polyoma virus middle T antigen, the alpha subunit of the inhibitory GTP binding protein and the delta subunit of the acetylcholine receptor (reviewed by Ashendel, 1985). However, the identification of physiologically significant biological substrates of this enzyme *in vivo* is essential to uncover the precise role of PKC in signal transduction. Evidence suggests there is a precedent for isozyme specific or isozyme favoured substrates.

The EGF receptor has been identified as a physiologically significant target for PKC mediated phosphorylation resulting in a decreased number of high affinity binding sites and reduced tyrosine (tyr) specific protein kinase activity of the receptor (Cochet *et al.*, 1984). Moreover, Ido *et al.* (1988) have demonstrated that PKC-α reacts with the EGF receptor more rapidly than PKC-ß or -γ. The tumour promoters TPA, and teleocidin as well as synthetic DAG have stimulated phosphorylation of the Rous sarcoma gene product, $pp60^{src}$, itself a tyr kinase. There is evidence supporting the *in vivo* phosphorylation of the ribosomal protein S6 by PKC and $pp60^{src}$ in cells in which phorbol esters are mitogenic. Evidence for physiological phosphorylation of myosin light chain and platelet protein (Mr 40-47kD) has been obtained. Phosphate was incorporated into the latter after treatment with DAG, TPA, arachidonic acid, collagen or thrombin (reviewed by Ashendel, 1985). Two 27kD stress proteins have been shown to be physiological substrates of PKC (Regazzi *et al.*, 1988). The time course of their phosphorylation closely paralleled the rapid subcellular redistribution of PKC and its subsequent down-regulation, during TPA-induced growth inhibition of human mammary carcinoma cells. Darbon *et al.* (1990) have demonstrated a distinct pattern of protein phosphorylation following PKC activation by TPA in cells which show opposing growth responses to TPA. Common to both cell lines was the observed

phosphorylation of the 27kD and 28kD stress proteins.

Isozymes possess differential sites and rates for autophosphorylation as well as displaying different catalytic activities toward a range of substrates; moreover, *in vivo*, diversity thus generated may be exaggerated by the action of phosphatases (Marais and Parker, 1989). PKC-γ and -α primarily autophosphorylated at a serine residue, PKC-ß autophosphorylated at both serine and threonine residues (Huang *et al.*,1986). PKC- γ most rapidly underwent autophosphorylation (Ido *et al.*, 1988). Schaap *et al.* (1989) have proposed that PKC-ε may selectively phosphorylate a specific set of intracellular proteins in response to agonist-induced degradation of lipids which generate DAG independent of a Ca^{2+} mobilizing signal.

The phosphorylation of nuclear proteins by PKC has been proposed as one of the important steps by which the PKC signalling pathway regulates agonist induced nuclear events (Tsuda and Alexander, 1990). The significance of enzyme translocation to, and lamina phosphorylation at the nuclear envelope has been described. Several nuclear enzymes have been identified as *in vitro* targets for PKC catalysed phosphorylation including DNA topoisomerase I; resulting in a two- to three-fold increase in its activity. Topoisomerase I transiently nicks a single DNA strand, catalyzing topological manipulations such as the relaxation of supercoiled DNA molecules, and is associated with transcriptionally active genes (Samuels *et al.*, 1989). DNA topoisomerase II from *Drosophila* has been reported as a target for PKC catalysed phosphorylation (Sahyoun *et al.*, 1986). Topoisomerase II is associated with the interphase nuclear matrix and with the mitotic chromosome scaffold and is one of the enzymes which modulates DNA topology (Pienta *et al.*,1989). PKC has been found to phosphorylate DNA methyltransferase *in vitro* (De paoli-Roach *et al.*, 1986). This enzyme alters gene expression by changing DNA methylation patterns. Chuang *et al.* (1989) have proposed a possible mechanism by which PKC may regulate gene expression. These authors have demonstrated *in vitro* phosphorylation of RNA polymerase II by PKC, an important event in the initial stages of RNA synthesis.

1.3.9 Inhibitors of PKC.

Inhibitors of PKC have been identified such as polyamines, adriamycin, quercetin, the bee venom peptide mellitin, polymyxin B and 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7). Many compounds mediate inhibitory properties *via* interaction with the catalytic domain of PKC, based on their ability to compete for ATP binding sites, for example the nucleoside analogue, sangivamycin (Loomis and Bell, 1988). Aminoacridines affect both the catalytic and regulatory sites of PKC (Hannun and Bell 1988). Sphingolipids are a class of PKC inhibitors which interact with the lipid-binding domain and antagonize catalytic activity, possibly by decreasing the availability of PS and thereby preventing effective interaction between enzyme and substrate (Bazzi and Nelsestuen, 1987; Tritton and Hickman,1990). Some enzyme inhibitors have been employed experimentally to investigate the role of PKC in certain systems. However caution during interpretation of these data

must be heeded as these inhibitors are neither potent nor specific for PKC. More recently the potent PKC inhibitor staurosporin (STSPN) has been isolated. This microbial alkaloid with antifungal activity binds to the catalytic domain of PKC inducing enzyme translocation to the membrane. It binds with little effect on the regulatory domain and does not inhibit phorbol ester binding, neither is it competitive with membrane phospholipids, but competition is observed with ATP (Tamaoki *et al.*, 1986). Five closely related compounds, the calphostins, isolated from the fungi *Cladosporium cladosporioides*. display specificity and high affinity for PKC. The calphostins are reported to interact with the regulatory domain of PKC and have exhibited cytotoxicities against various tumour cells, proportional to the degree of PKC inhibition (Kobayashi *et al.*, 1989).
The specific enzyme inhibitors discussed latterly provide excellent tools to investigate more accurately mechanisms in which PKC activation has a proposed role.

1.3.10 Biological function of PKC.

The mechanisms by which extracellular ligands that interact with membrane receptors elicit biological responses involve the generation of second messengers. The activation of PKC following receptor-mediated hydrolysis of inositol phospholipids is now established as an important mechanism *via* which signals from certain polypeptide hormones, growth factors, neurotransmitters and light are transduced to the interior of the cell. Thus, the action of PKC is implicated in the control of a diverse spectrum of cellular processes. Isoenzyme specificity with respect to substrate proteins, effector dependence and localization suggest that PKC subspecies as well as demonstrating overlapping roles are likely to fulfil unique functions *in vivo*.
The response of phospholipids to the stimulation of cell surface receptors in certain excretory tissues such as pancreas was initially recognized by Hokin and Hokin in 1953. Acetylcholine was demonstrated to induce a rapid incorporation of ^{32}P into PI and phosphatidic acid (PA). Later, it became evident that this incorporation was a result of enhanced phospholipid breakdown and resynthesis in stimulated cells; thus the hypothesis arose of a phospholipid response in receptor function. Michell subsequently postulated that phospholipid breakdown may be related to Ca^{2+} gating. When, in 1977 PKC was discovered (Inoue *et al.*, 1977), no importance was assigned to the enzyme in signal transduction. In 1980, Nishizuka and coworkers established convincingly that PKC is a Ca^{2+} activated, phospholipid dependent enzyme, firmly linked to signal transduction, by demonstrating the direct activation of PKC by DAG, an early, transient product of PI turnover (Kishimoto *et al.*, 1980). Separate from, but acting in synergy with DAG-stimulated PKC activation, is the triggered release of intracellular Ca^{2+}. These 2 second messengers were demonstrated by Berridge and Irvine (1984) to be products of a bifurcating signal transduction pathway, following inositol lipid breakdown and are responsible for mediating many diverse cellular responses. That PKC possesses a pivotal role in signal transduction was first demonstrated in the release of serotonin from platelets (Kaibuchi *et al.*, 1983). Subsequently, the importance of PKC activation has been demonstrated in the modulation of membrane ion conductance within the cell by

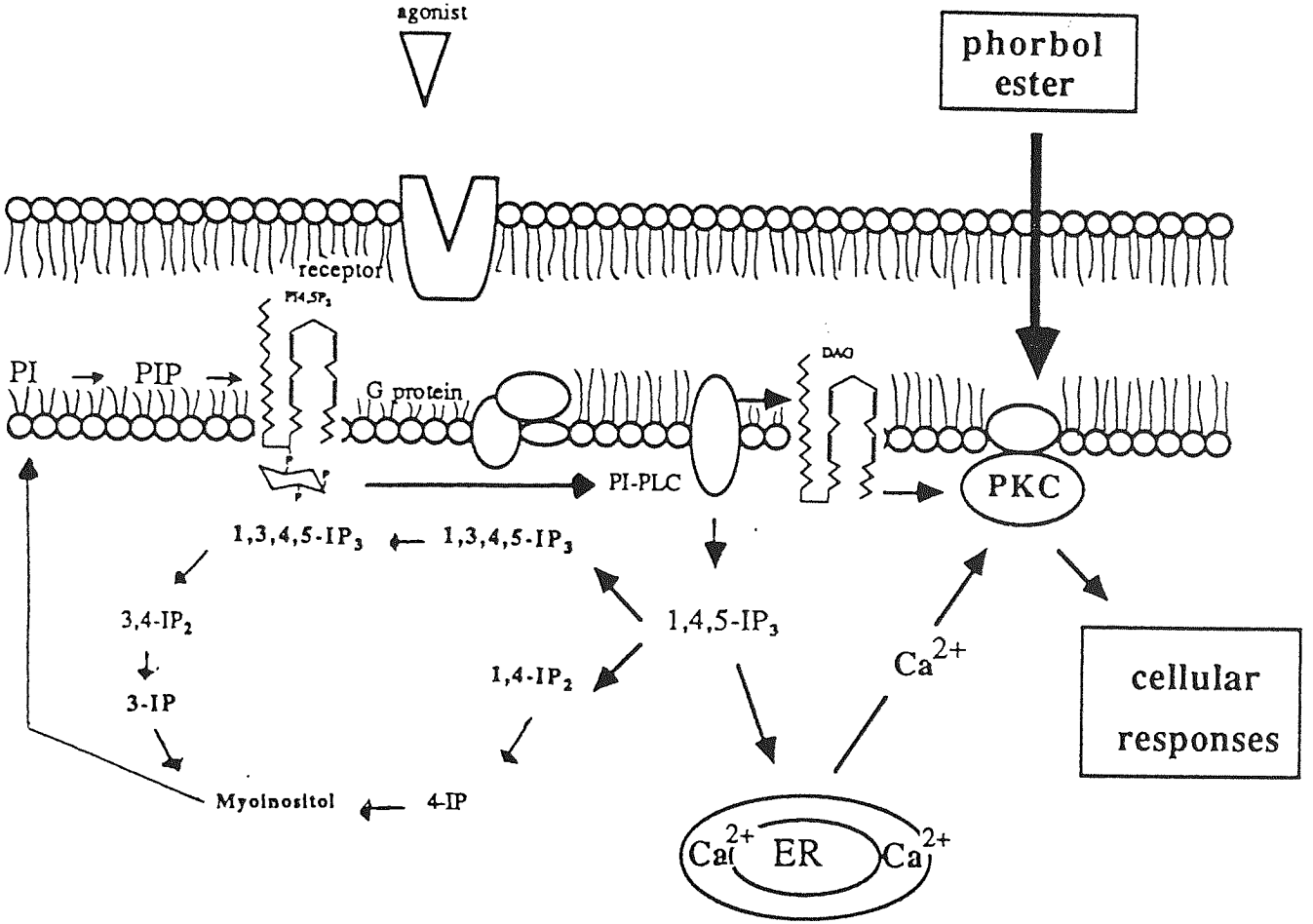
phosphorylating ion exchange proteins (Nishizuka, 1986), smooth muscle contraction and the oxidative burst of granulocytes. This enzyme has been reported to play a key role in processes such as cell ageing and memory (Burgoyne, 1989). The action of PKC is implicated in the stimulation of certain hormone and neurotransmitter secretion (reviewed by Nishizuka, 1986). Administration of the purinoreceptor agonist ATP to type II alveolar cells results in an increase in IP_3, Ca^{2+} mobilization, PKC activity and the subsequent secretion of PC surfactant (Rice *et al.*, 1990). The release of histamine, T and B lymphocyte activation, metabolism and phototransduction also necessitate the action of PKC.

This enzyme has been reported to play a central role in mitogenesis and cell proliferation, for example during the stimulation of quiescent fibroblasts (Rozengurt *et al.*, 1984). Susa *et al.* (1989) have concluded that activation of PKC contributes to mitogenicity in quiescent Swiss 3T3 cells. The haematopoietic growth factors interleukin 3, GM-CSF (granulocyte/ macrophage-colony stimulating factor), M-CSF and G-CSF each induce the translocation of PKC from the cytosol to the membrane (Powis *et al.*, 1990). However, that PKC is necessary or required for a mitogenic response has been disputed (van Corven *et al.*, 1989). Quiescent fibroblasts, despite the loss of functional PKC, fully retained the ability to proliferate in response to lysophosphatidic acid (LPA). The activation of PKC is reported to be an essential component of differentiation in many systems (Blumberg, 1980). A role for PKC has been proposed in programmed cell death (McConkey *et al.*, 1989). A wealth of evidence exists heavily implicating PKC in the genesis and promotion of malignant disease. The accumulating bias toward a positive correlation between aberrant PKC and neoplasia will be discussed in section 1.7.

1.4 Phosphoinositide signal transduction.

Inositol phosphates represent only a minor portion of mammalian cell membrane phospholipid. Yet a wide range of extracellular messengers evoke phosphoinositide breakdown, initiating a signalling cascade which generates messages to activate many cellular functions. An outline of the inositol phosphate signalling pathway is illustrated diagrammatically in figure 2. Agonist-induced receptor activation is coupled through a GTP-binding protein to the stimulation of inositol phospholipid specific phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$), positioned in the inner leaflet of the plasme membrane. The cleavage of $PI4,5P_2$ generates 2 second messengers, thus activating two distinct signalling pathways. Inositol 1,4,5-trisphosphate ($I1,4,5P_3$) liberates Ca^{2+} from intracellular endoplasmic reticulum stores, thereby increasing the intracellular concentration of free Ca^{2+} and leading to the activation of Ca^{2+} dependent enzymes. DAG, enriched in stearic and arachidonic acids, is the second product of this bifurcating mechanism. Normally absent from plasma membranes, DAG is produced only transiently, it functions as the endogenous activator of PKC and upon binding to the enzyme, increases its affinity for Ca^{2+}, transiently and reversibly activating PKC (Nishizuka, 1986). Two futile PI/$PI4,5P_2$ cycles maintain a substrate supply for PLC-

Figure 2. Phosphoinositide signal transduction.
Agonists bind to the external receptor to stimulate the hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP_2). The reaction is catalysed by inositol phosphate-specific phospholipase C (PI-PLC) and generates diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3). The latter binds to a specific receptor on the endoplasmic reticulum with the subsequent release of calcium (Ca^{2+}). Ca^{2+} and DAG are required, to varying extents for the activation of Protein kinase C (PKC) isozymes.



catalysed hydrolysis. DAG may be converted back to PI, hydrolysed by DAG lipase with the release of arachidonic acid and monoacylglycerol or phosphorylated by DAG kinase to PA (Berridge and Irvine, 1984).

1.5 Generation of diversity within the signalling cascade.

The signalling mechanism is far more complex than initially recognized. Both the DAG and IP branches involve several alternative sources of, and potential targets for metabolites. There are many points of interaction with other signalling pathways and many points of origin for the generation of diversity and selectivity for the ensuing cellular responses.

At least 20 inositol phosphate isomers have been identified in cells, including inositol pentakis- and -hexakisphosphates, thus $I1,4,5P_3$ is not the only inositol phosphate possessing a regulatory role. The recently discovered inositol phosphates $PI3P$, $PI3,4P_2$ and $PI3,4,5P_3$ generated from the phosphorylation of PI, $PI4P$ and $PI4,5P_2$ respectively by the enzyme phosphatidylinositol-3-kinase, may possess regulatory roles specifically within their intracellular locale. It has been proposed that $PI3P$ may regulate the inositol lipid cascade by mobilizing discrete hormone insensitive pools of PI thus amplifying a normal hormonal response (Whitman and Cantley, 1988).

Following the mobilization of $[Ca^{2+}]^i$, $I1,4,5P_3$ is metabolized either by dephosphorylation to $I1,4P_2$ or by phosphorylation to $I1,3,4,5P_4$. $I1,3,4,5P_4$ may be dephosphorylated by 5-phosphomonoesterase to $I1,3,4P_3$ or by a PKC regulated 3-phosphomonoesterase to $I1,4,5P_3$ thus providing an additional pathway for the formation of Ca^{2+}- mobilizing second messenger (Oberdisse *et al.*, 1990). Recently, it was demonstrated that $I1,3,4,5P_4$ antagonized the action of $I1,4,5P_3$, by stimulating the sequestration of cytosolic Ca^{2+} into intracellular stores (Hill *et al.*, 1988).

1-stearoyl-2-arachidonyldiacylglycerol, the primary DAG species of PI hydrolysis serves as a source of arachidonic acid, which is subsequently metabolized to prostaglandins, leukotrienes and other eicosanoid mediators. One such eicosanoid is able to mediate PI turnover linked neurotransmitter response. Arachidonic acid has been shown to evoke phosphoinositide (PI) turnover and accumulation of IP_3 through the activation of PI specific PLC in astrocytes (Murphy and Welk, 1989). Prostaglandins produced in response to PDGF may serve to elevate cellular cAMP levels (reviewed by Whitman and Cantley, 1988).

The multiplicity of isoforms of PKC suggests differential susceptibility to signals (Nishizuka, 1988). It was noted previously that PKC-ε may become selectively activated following the production of DAG in the absence of Ca^{2+} fluxes and thus may operate to transduce signals from more than one pathway (Schaap and Parker, 1990).The variable regions seen in each subspecies may play key roles in governing the individual enzymological characteristics and possibly their specific localization and function. The main region of sequence heterogeneity occurs in DAG and in phospholipid binding sites of

PKC. It has been suggested that different isoenzymes may be activated by DAG that differ in their fatty acid chains (Coussens *et al.*, 1986). Isoforms have demonstrated preferential conditions for activation and favoured protein targets. Evidence is emerging to suggest that phospholipid environments are specific for each intracellular locale. Indeed, growth factor receptors, IP metabolism and the generation of DAG have been discovered within internal membranes. In addition, Da silva *et al.* (1990) have observed phospholipid free activation of PKC mediated by phorbol esters and have demonstrated an altered pattern of substrate specificity dependent upon conditions of activation. Data presented in this report indicated that isoenzymes of PKC may be activated at least partially without binding to membrane phospholipid, potentially providing additional substrates. Moreover, differential sensitivity of PKC isozymes to inactivation by acidic phospholipids has been demonstrated (Huang and Huang, 1990). PS, PA, phosphatidylglycerol and cardiolipin; activators of PKC in the assay of protein phosphorylation, in the absence of divalent cations were able to inactivate γ, ß and α isozymes with respective decreasing susceptibility by binding to the catalytic domain of PKC. In the presence of divalent cations, suppression of this interaction and of enzyme inactivation was observed, interaction of PS with the regulatory domain was favoured, preferentially activating PKC.

There are 12 forms of the α subunit, an intrinsic component of high molecular weight G proteins and investigations are underway regarding smaller molecular weight G proteins (Powis *et al.*, 1990). Their widespread distribution suggests a diverse role in signal transduction or intracellular trafficking.

Four isozymes of inositol phospholipid specific PLC have been identified (Homma *et al.*, 1989, Pann-Ghill *et al.*, 1988), exhibiting tissue and cell type specificity in their expression, suggesting diverse functions for PLC isozymes in single cells. Furthermore, there are multiple species of PLC. In addition to PI specific PLCs discussed, PC and PI-glycan specific PLC exist, the latter is activated in response to insulin. This cleavage generates DAG and IP glycan. Farese *et al.* (1987) have observed in addition to a transient rise in DAG as a result of PLC activity, sustained, elevated *de novo* DAG synthesis in response to insulin.

In 1981, it was reported by Mufson *et al.* that phorbol esters also stimulate the hydrolysis of phosphatidylcholine (PC) (reviewed by Exton, 1990) Glatz *et al.* (1987) have provided evidence to suggest that this effect is mediated by PKC. Following enzyme down regulation, no activation of PC specific PLC by phorbol ester was observed. Arachidonic acid or lyso-phosphatidylcholine (lyso-PC) are produced by phospholipase A_2 (PLA_2) catalysis of PC hydrolysis. Lyso-PC exerts dual regulation over the activation of PKC. At concentrations below 20µM PKC is stimulated, but above 30µM lyso-PC inhibits PKC (Oishi *et al.*, 1988). Studies of PC breakdown also vindicate the activities of PLC and PLD yielding DAG and PA. Multiple forms of PC specific PLC and PLD exist. Hydrolysis by PLD is more rapid, hence PA increases more rapidly than DAG and itself gives rise to DAG through the action of PA phosphohydrolase. PA has also been shown to activate PIP_2 PLC *in vitro* and to stimulate PIP_2 hydrolysis in intact cells (Moolenar, 1986). In

addition, PA may inhibit adenylate cyclase and lead to arachidonic acid release *via* the activation of PLA_2. Tumour promoting phorbol esters and exogenous DAGs, *via* the activation of PKC, promote PC hydrolysis in many cell types mainly by PLD (Mufson, 1981). Pai *et al.* (1990) have suggested that PLD is positively regulated by PKC. They demonstrated that over expression of PKC-ßI enhanced PLD activation by phorbol esters in rat fibroblasts. Increased PC breakdown yielded increased DAG formation. Growth factors such as EGF and PDGF stimulate PC hydrolysis, Ca^{2+} increases the breakdown of PC to PA and DAG. Platelet activating factor (PAF) stimulates the accumulation of DAG *via* Ca^{2+} and PKC mediated breakdown of PC (Uhing *et al.*, 1989). Hormonally stimulated, rapid hydrolysis of PC, potentiated by purinergic receptors leads to prolonged generation and accumulation of DAG containing higher concentrations of palmitic, oleic and linoleic acids than DAG species released from $PI4,5P_2$ hydrolysis. Experiments with hepatocytes or endothelial cells incubated with labelled fatty acids indicated that the source of accumulating DAG in response to vasopressin or thrombin was not solely $PI4,5P_2$, but also PC (Ragab-Thomas, 1987).

PC hydrolysis generates species of DAG which act as a source of arachidonic acid, may activate PLA_2, translocate DAG kinase to membrane bound compartments and are very effective activators of PKC. It is likely that DAG derived from PC hydrolysis produces different cellular effects from that resulting from PIP_2 breakdown. Activation of PKC following PC hydrolysis occurs without mobilization of Ca^{2+}. PKC activation without inositol lipid hydrolysis is observed in stem cells exposed to interleukin 3 (Il-3) (Whetton *et al.*, 1988). It is postulated that PC hydrolysis is involved in cellular control mechanisms which require prolonged activation of PKC. These may include regulation of transcription, events related to cell growth and differentiation, control of ion channel activity, changes in neurons concerned with memory, receptor function alterations involved in desensitization and other long term adaptations of cells to stimuli (reviewed by Exton, 1990).

It has recently been reported that sphingosine, an inhibitor of PKC activity activates PLD, potently elevating the levels of PA, but decreasing DAG levels (Lavie and Liscovitch, 1990). Evidence suggests that PLD mediated PC hydrolysis may lead to a selective mixture of DAG and ether-linked diglycerides. The latter derivatives have been reported to inhibit PKC (Hii *et al.*, 1989).

Interaction between PKC and adenosine 3`5`-cyclic monophosphate (cAMP) signal transduction pathways has been reported in a number of systems (Nishizuka, 1986; Cambier *et al.*, 1987; Quillam *et al.*, 1989). Summers *et al.* (1988) have established that phorbol ester activated PKC reduces the rate of decay of adenylate cyclase activity, thus enhancing cAMP production. Both adenylate cyclase and cAMP concentration increase substantially during embryonic induction of neuroectoderm. Enhancement of adenylate cyclase requires PKC activation, indicating crosstalk (Otte *et al.*, 1989). In certain systems activation of PKC has inhibited and desensitized the adenylate cyclase system, for example in rat hepatocytes TPA inhibits glucagon stimulated adenylate cyclase activity (Heyworth *et al.*, 1984). Conversely, signal induced breakdown of inositol phospholipids has been

profoundly blocked by cAMP in some tissues such as platelets, lymphocytes and mast cells (Kaibuchi *et al.*, 1982).

It has been demonstrated that the phospholipid, lyso-PA is able to stimulate 3 signal transduction pathways: PLA_2, PI-specific PLC and a pertussis toxin sensitive G_i protein are simultaneously activated, the latter mediating inhibition of adenylate cyclase; resulting in the stimulation of quiescent fibroblasts (van Corven *et al.*, 1989). Certain hormones and regulatory peptides that activate PI specific PLC require the addition of a synergizing peptide growth factor to evoke a mitogenic response, for example, bradykinin and vasopressin require the presence of insulin for the stimulation of 3T3 cell growth (Rozengurt *et al.*, 1979).

The transduction of biological signals comprises an intricate biochemical network. Such complexity within the phosphoinositide signalling cascade is certainly of physiological significance, to selectively generate, amplify, or perpetuate messages encoding a diverse repertoire of cellular responses.

1.6 PKC as a target for tumour promotion.

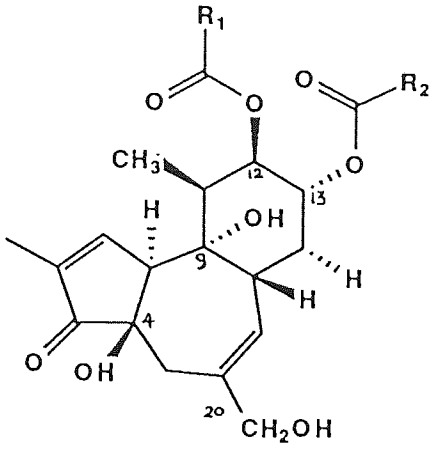
1.6.1 Tumour promoting phorbol esters.

In 1941, Berenblum stated that "croton oil causes a marked augmentation of carcinogenesis when applied at weekly intervals to the skin of mice in conjunction with a very dilute solution of benzpyrene in acetone." This led him to the concept that chemically induced cancer involves two distinct steps: initiation and promotion. The 2 stage protocol of mouse skin carcinogenesis is now well documented (Boutwell, 1964 and Van Duuran, 1969) and the most well characterized initiation-promotion system, though other studies have indicated that cancers of the breast, colon, bladder and liver also develop as a consequence of initiating and promoting events.

Typically, tumour initiation is brought about by a single application of a "pure" initiator such as urethrane, or a subcarcinogenic dose of an agent possessing both initiator and promoter properties such as polycyclic hydrocarbon benzo(a)pyrene. With relevance to human malignancy, initiation is rapid and involves a permanent heritable alteration in genotype; most likely by interaction of the initiating agent with DNA to induce mutations, gene arrangements or amplification. The incidence of cancer in patients with DNA repair deficiencies is increased. One could hypothesize that these cells are more prone to tumour initiation.

Oil obtained from the seeds of *Croton tiglium* possesses powerful tumour promoter activity. The active components were identified in 1967 by Hecker as diesters of the diterpene alcohol phorbol, the most potent of which is 12-O-tetradecanoylphorbol-13-acetate (TPA), the structure of which is demonstrated in figure 3. Returning to the mouse skin model; tumour promotion is carried out by repeated application (3 times weekly) of phorbol ester such as TPA. Benign papillomas appear within 12-20 weeks and following 1 year of such a protocol, squamous cell carcinomas have evolved in between 40-60% of cases. The application of tumour promoter alone or prior to the initiating agent does not generally lead to the occurrence of malignant tumours. TPA is able to induce neoplastic

Figure 3. Structure of a) Phorbol ester
b) Bryostatin



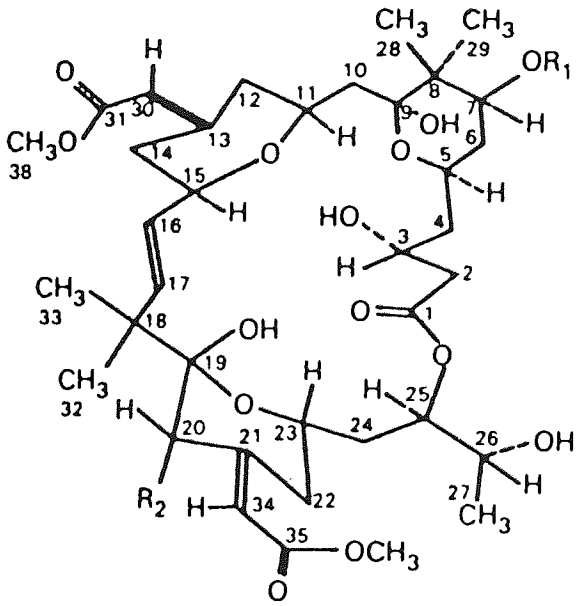
TPA

$R_1 = -(CH_2)_{12}CH_3$

$R_2 = -CH_3$

PDBu

$R_1 = R_2 = -(CH_2)_2CH_3$



Bryo	R_1	R_2
1	$COCH_3$	$OCO(CH)_4(CH_2)_2CH_3$
2	H	$OCO(CH)_4(CH_2)_2CH_3$
4	$COCH_2CH(CH_3)_2$	$OCOCH_2CH_2CH_3$
5	$COCH_2CH(CH_3)_2$	$OCOCH_3$

transformation of fibroblasts from humans with adenomatosis of the colon and rectum. These individuals, whose cells exist in the initiated state due to a dominant mutation, are genetically prediposed to cancer (Kopelovich *et al.*, 1979; Kopelovich and Bias, 1980). Membrane-mediated tumour promotion is itself multistaged and termed collectively as tumour progression. Promoters are subdivided by their potencies into stage I; for example the Ca^{2+} ionophore A23187, stage II; for example mezerein and 12-O-retinoylphorbol-13 -acetate, or complete tumour promoters; for example TPA (Slaga *et al.*, 1980; Fürstenberger *et al.*, 1981). Promoting agents act as mitogens for the transformed cell type leading to a heterogeneous tumour population. In the aetiology of human cancer, tumour promotion may take years. Promoting agents are most likely present in our environment. Implications in human cancer are profound, especially in the light of the recent isolation of a new tumour promoting phorbol ester from the seed oil of *Jathropa curcas L.* (family Euphorbicceae). The oil is intended to be produced in large quantities in Thailand for use as a substitute for diesel oil and as an ingredient in commercial printing ink. This may disastrously result in exposure of high populations to tumour promoters (Hirota *et al.*, 1988). Thus, phorbol esters initially became the object of intense research interest on the basis of their potent activity as mouse skin tumour promoters (Boutwell 1974). In addition, TPA, when administered to cells elicits a vast plethora of responses similar to those of hormones and neurotransmitters. Evidence at this time suggested that the primary site of action of the tumour promoting phorbol esters was located on cell surface membranes. Driedger and Blumberg (1980) demonstrated the specific nature of phorbol ester binding to chick embryo fibroblasts in culture. The specific binding of [^{3}H]PDBu was saturable, high affinity, specific for biologically active phorbol esters, reversible, stable in a manner characteristic of protein and linearly related to the amount of protein in the assay. In 1982, it was found that TPA could substitute for DAG and activates PKC directly (Castagna *et al.*, 1982). Shortly afterwards, Neidel *et al.* (1983) copurified the phorbol ester receptor and PKC to homogeneity and it was concluded that PKC was the prime target for the tumour promoting phorbol esters. (Sando and Young, 1983; Leach *et al.*, 1983; Ashendel *et al.*, 1983). As such, the phorbol esters have proved to be valuable pharmacological tools with which to identify physiological systems in which PKC is involved and to study mechanisms of signal transduction and carcinogenesis. That PKC is the sole phorbol ester receptor has been disputed in a recent report by Hashimoto and Shudo (1990). These authors have described the existence of a TPA-specific binding protein in the cytosolic fraction of HL60 cells, which translocates to the nucleus upon treatment with TPA. As there are genes whose transcription is induced by TPA, it has been proposed that this cytosolic-nuclear tumour promoter specific binding protein (CN-TPBP) might play a role in the action of tumour promoters (Hashimoto and Shudo, 1990).

1.6.2 Additional methods of tumour promotion.

In addition to the phorbol esters, other tumour promoting agents have been isolated and found to induce the same spectrum of biological responses as do phorbol esters with potencies similar to TPA; examples include the indole alkaloid teleocidin from *streptomyces*

and aplysiatoxin from *Lyngiba gracilis* (reviewed by Ashendel, 1985). Resiniferatoxin, first described in 1979 by Hergenhahn *et al.* has been found to possess extremely potent irritant qualities; however, this compound has been reported to be non tumour promoting (Zur Hausen *et al.*, 1979); furthermore, evidence has established that the mechanism of action is not *via* PKC (Dreidger and Blumberg, 1980). Unsaturated fatty acids possess tumour promoting ability. Such agents, like phorbol esters potently stimulate phospholipid free activation of PKC, potentially providing additional substrates for this enzyme which may at least in part account for promoting activity (Da Silva *et al.*, 1990). Other established mechanisms able to mediate tumour promotion include abrasion and oxidative stress and substantial evidence exists to support a role for PKC. A number of promoters such as TPA, hydrogen peroxide, phenobarbitol, teleocidin, mezerein and tissue wounding cause the generation of reactive oxygen species. Lipid peroxidation by oxygen free radicals in biological membranes may stimulate PKC thereby initiating the cascade of events comprising tumour promotion (O`Brian *et al.*, 1988). Moreover, PKC activators such as TPA and OAG have been shown to intensify the detrimental actions of radical-inducing agents (von Ruecker, *et al.*, 1989). PKC, however is not the direct target for all tumour promoters for example okadaic acid (Haystead *et al.*,1989) and calyculin A (Suganumo *et al.*, 1990), powerful inhibitors of protein phosphatases-1 (PP1) and -2A (PP2A). Evidence suggests that PP1 and PP2A are the major phosphatases which reverse the phosphorylation events catalyzed by PKC. The level of phosphorylation of any isolated protein reflects the dual regulation of both kinases and phosphatases. It can be hypothesized therefore that treatment with either TPA, okadaic acid or calyculin A would lead to increased phosphorylation of proteins critical for tumour promotion.

1.6.3 Biological effects of tumour promoting phorbol esters.

The tumour promoting phorbol esters evoke pleiotropic responses upon cells; a great number of which are predicted to result from phosphorylation of many different proteins by PKC. TPA, the most potent tumour promoting phorbol ester discovered to date is an amphipathic diterpene ester which rapidly incorporates into the plasma membrane and diffuses to all intracellular compartments. Such agents are able to induce cellular misperception by affecting membrane cognitive processes. Consistent with this are the phorbol ester-induced alterations in cell surface receptors for EGF, insulin, transferrin for example, as well as their interference with junctional cell-cell communication. Many of the biological changes are related to the ability of phorbol esters to promote growth of initiated cells *in vivo*, and are reminiscent of the transformed phenotype. In culture there is a correlation between the effects elicited by phorbol esters and their potencies as promoters in the mouse skin model. Effects on cultured cells include transformation of mouse embryo fibroblasts treated with UV light (Mondal, 1976); reversible inhibition of terminal differentiation, for example in erythroleukaemia cells (Fibach, 1979); induction of terminal differentiation accompanied by growth inhibition, for example in the human HL60 promyelocytic cell line (Kraft *et al.*, 1986); stimulation of DNA synthesis and cell proliferation for example in quiescent 3T3 cells, BALB/c, chick embryo fibroblasts; in

bovine and human lymphocytes phorbol esters act as mitogenic stimuli (reviewed by Diamond *et al.*, 1980 and Blumberg, 1980); cell growth inhibition (reviewed by Gescher, 1985).

1.6.4 Growth inhibitory properties of phorbol esters.

Inhibition of growth is attained in many cell lines following their treatment with nM concentrations of phorbol ester, often, as in HL60s, as a result of terminal differentiation (reviewed by Vandenbark and Niedel, 1984). Ultimately these cells go on to die. Similarly, in the EBV-negative BJAB and Ramos cell lines, cell death follows growth inhibition by TPA (Bechet and Guetard, 1983). Cultured fibroblasts of individuals with hereditary adenomatosis of the colon and rectum exhibit growth inhibition in response to certain concentrations of TPA (Kopelovich and Bias, 1980). Phorbol esters act differentially upon epidermal subpopulations *in vitro* by inducing differentiation in certain cells and stimulating proliferation of other cell populations (Yuspa *et al.*, 1982; Reiners and Slaga, 1983). This would seem to reflect its function as a tumour promoter, to increase the probability of survival of the malignant phenotype by selective clonal expansion of initiated cells. Experimental evidence supports this hypothesis. Normal epidermal cells but not initiated ones are induced to terminally differentiate in response to phorbol ester treatment (Hartley *et al.*, 1985 and Yuspa *et al.*, 1986). Similarly, cultured normal bronchial cells differentiate following exposure to phorbol ester, whereas many lung carcinoma lines do not (Sanchez *et al.*, 1987). Olsson *et al.* (1985) report profound morphology changes, loss of proliferative potential *in vitro* and tumourigenicity *in vivo* in the RH-SCL-L11 squamous-cell and RH-SCC-R10 small-cell lung carcinomas following TPA treatment. The FGF and insulin induced proliferation of NIH/3T3 cells is potently arrested by TPA (Yamamoto *et al.*, 1988). In a similar vein, it has been demonstrated that TPA inhibits the proliferation of murine B-cells stimulated by lipopolysaccharide or anti-IgM (Germolec *et al.*, 1988). Increasing evidence suggests that TPA may elicit opposite responses in normal cells and malignant cells of the same lineage, possibly reflecting a differential susceptibility between continually cycling cells and resting cells to a critical activation event. Non-tumourigenic mouse melanocytes are dependent upon TPA for continued growth (Bennet *et al.*, 1987) metastatic melanoma cells however are growth arrested following TPA treatment (Huberman and Callaham, 1979; Coppock *et al.*, 1990). Similarly, the growth of SV40 transfected human melanocytes became retarded in the presence of TPA (Melber *et al.*, 1989). Additionally, TPA has been shown to promote cellular proliferation of normal human B lymphocytes; yet the growth of 3 B lymphoma lines was profoundly inhibited by TPA (Beckwith *et al.*, 1990). Osborne *et al.* (1981) demonstrated that TPA induced growth inhibition in MCF7, ZR75-1 and MDA-MB-231 human breast cancer lines. Arrest of MCF7 cell growth was accompanied by cell hypertrophy and stimulation of protein synthesis, but upon removal of TPA, was reversible. However after 10-12 days exposure to TPA, cells detached from culture dishes and cell death ensued. Reversible phorbol ester-induced growth inhibition has also been observed in the M5076 murine macrophage tumour cell line (Goode and Hart, 1990). Resumption of proliferation was associated with

removal of TPA or phorbol dibutyate (PDBu). TPA evokes non-toxic antiproliferative responses in the A431 human epidermoid cell line (Smith *et al.*, 1983), HeLa cells (Süss *et al.*, 1972) and vascular smooth muscle A7r5 cells (Owen, 1985). Unusually, in both the SVK14 cell line, SV40 transformed human foreskin keratinocytes (McKay *et al.*, 1983) and the A549 human lung carcinoma line (Gescher, 1985) growth arrest by phorbol ester is only transient, in the continued presence of TPA, cells appear to become insensitive following several days exposure.

The role of PKC in the induction of growth inhibition by phorbol esters is discussed in section 4.2.

1.7 Further evidence supporting a role for PKC in malignant disease.

1.7.1 Bryostatins and other agents whose target appears to be PKC.

During 1965 a systematic programme was initiated to evaluate marine invertebrates and arthropods as new sources of potentially useful anticancer drugs (Pettit *et al.*, 1989). It was reported in 1970 by Pettit and coworkers that the marine bryozoan *Bugula neritina* contained potent antineoplastic components. Subsequently, 17 structurally related macrocyclic lactones, the Bryostatins (bryos), have been purified from *Bugula neritina* and *Amanthia convoluta*, and found to exhibit anti-neoplastic activity against mouse lymphocytic leukaemias, P388 and L1210 aswell as a reticulum cell sarcoma Walker carcinosarcoma 256 in the rat (Pettit *et al.*, 1982). Bryos are a novel class of experimental anticancer agents which are able to activate PKC and bind to the phorbol ester receptor(s) with exquisite affinity. It has been demonstrated that bryos share a number of properties with tumour promoting phorbol esters for example the activation of human polymorphonuclear leucocytes (Berkow and Kraft, 1985). In addition, bryos act as mitogenic stimuli for quiescent Swiss 3T3 fibroblasts (Smith *et al.*, 1985). However, in many cell types, bryos fail to mimic but rather antagonize the phorbol ester induced response. For example in HL60 cells, bryo 1 was able to activate PKC and cause enzyme translocation, but unable to induce either monocytic differentiation or a fall in the expression of c-*myc* RNA levels and indeed blocked monocytic differentiation induced by TPA (Kraft *et al.*, 1986). Bryo 1 has also blocked phorbol ester induced differentiation in colon cancer cells (McBain *et al.*, 1988). In Friend erythroleukaemia cells, PDBu suppressed hexamethylene-bisacetamide-induced differentiation, bryo 1 blocked the phorbol ester response, thus restoring differentiation (Dell`Aquila *et al.*, 1987). Bryo 1 itself is inactive as a complete tumour promoter and only a very weak stage II tumour promoter. Simultaneous administration of bryo and TPA in a complete tumour promotion protocol demonstrated that bryo 1 suppressed the promoting ability of the phorbol ester (Hennings *et al.*, 1987). Thus, the nature of the bryo response appears as something of a paradox; although an activator of PKC with comparable potency to TPA, bryo 1 inhibits certain typical responses of TPA. Moreover, bryos share certain properties with PKC inhibitors: staurosporine has been shown to inhibit the metastatic ability of clinically invasive human bladder carcinoma cells (Schwartz *et al.*, 1990). The antimetastatic properties of bryo 1 in mice with B16 melanomas has been published (Schuchter *et al.*,

1989). Additionally, bryo 1 has demonstrated its ability *in vitro*. to stimulate bone marrow stem cells (May *et al.*, 1987) and evoke prompt and persistent macrophage-like differentiation of CML cells obtained from the peripheral blood of patients (Lilly *et al.*, 1990). Thus, bryos present favourably as potentially exciting anticancer agents, and phase 1 clinical trials upon bryo 1 are currently being designed.

STSPN, a potent inhibitor of PKC failed to inhibit the TPA-mediated differentiation of primary keratinocyte cultures, but itself elicited a much more potent effect: 100% terminal differentiation was obtained folowing 24h treatment with 10nM STSPN. Moreover, 2 murine benign neoplastic keratinocyte cell lines, neither of which respond to TPA, differentiated in response to STSPN (Dlugosz *et al.*, 1990). These workers have suggested that STSPN may provide a useful antitumour agent *in vivo*.

Another class of potential chemotherapeutic agents whose target of action is PKC are the calphostins. The calphostins are specific inhibitors of PKC. As discussed, 5 related compounds have been isolated and characterized. Each have demonstrated cytotoxic activity against MCF-7 and HeLa S_3 cells to a degree consistent with their potencies as PKC inhibitors, suggesting that this is the specific mechanism responsible for cytotoxic action. Antitumour activity of the calphostin complex is presently being investigated in detail (Kobayashi *et al.*, 1989).

A further class of PKC inhibitors, bisnaphthalene sulfonamides have displayed growth inhibitory properties upon a human melanoma cell line (Powis *et al.*, 1990). These compounds modulated the catalytic activity of PKC and inhibited phorbol ester binding with potencies which correlated with antiproliferative potential.

1.7.2 Oncogene action.

Oncogene products can be divided into 4 general categories, based on their biochemical function or cellular location: 1) members of the *src* gene family which code for a tyr-specific protein kinase activity and are able to stimulate phospholipid phosphorylation; 2) *ras* gene family encoding a guanine 5`-triphosphate (GTP)-binding activity; 3) protein products of certain oncogenes possess sequence homology with growth factors or growth factor receptors; 4) *myc, myb* and *fos* nuclear gene products which may function as chromatin activating proteins.

There is evidence to suggest that a number of oncogenes from each class, and transforming gene products interact with the PKC pathway. It is intriguing to speculate that transformation of cells results from subversion of normal signal transduction. Berridge hypothesized that an imbalance of 2nd messengers may be responsible for the cancerous state. The *sis* product (highly homologous to the B-chain of PDGF) and *erb B* are reported to enhance PI turnover. Cellular transformation by v-*fes* and v-*fms* is also associated with increased PI metabolism as well as a large increase in membrane associated PI-specific PLC (reviewed by Whitman and Cantley, 1988). There appear to be 2 signal pathways initiated by the oncogenic $p21^{ras}$ protein. Its incorporation by scrape loading into mouse fibroblasts in the presence of insulin and insulin-like growth factor-1 led to morphological transformation and DNA synthesis. There was also slow phosphorylation of

PKC. Down-regulation of PKC with phorbol esters abolished the stimulation of DNA synthesis but did not affect morphological changes by H-*ras*. $p21^{ras}$ transformed cell lines possess elevated levels of cellular DAG through the stimulation of PLA_2 catalyzed hydrolysis of PC or PE (Preiss *et al.*, 1986). Haliotis *et al.* (1990), have demonstrated that the expression of *ras* oncogene in C3H $10T_{1/2}$ fibroblasts leads to the down-regulation of PKC and have concluded that this mechanism may be the basis of transformation by c-Ha-*ras*. It has been observed in T cells that stimulation of PKC resulted in an immediate activation of $p21^{ras}$ proteins. It was subsequently suggested that these proteins may act as down-stream effectors of PKC (Cantrell *et al.*, 1990). *Src, ros, abl* and the polyoma middle T antigen stimulate PI kinase, leading to elevated levels of substrate for PLC (Macara *et al.*, 1984, Fleischman *et al.*, 1986). Moreover, PI3P, formed *via* an association between middle T antigen/ c-*src* and PI 3-kinase may mobilize discrete hormone insensitive pools of PI and thus amplify normal hormonal responses (Whitman and Cantley, 1988). The possibility arises that these oncogene products are able to contribute to cellular transformation by causing prolonged, uncontrolled stimulation of inositol lipid turnover, producing a continual supply of DAG and I145 P_3. Not least in this contribution is that down regulation of PKC activity has been observed in cells transfected with Ha-*ras*, Ki-v-*ras*, v-*src* and v-*fms*.

PKC phosphorylates the protein product of Ki-*ras* (Ballester *et al.*, 1987), the membrane associated oncogene product $pp60^{src}$ (Gould *et al.*, 1985) and a number of growth factor receptors including those for transferrin and interleukin II (Nishizuka, 1986). The EGF and the insulin receptors are PKC substrates that are related to the c-*erb B* and c-*ros* oncogene products. The receptor for EGF is phosphorylated on the threonine-654 residue, a key site on the cytoplasmic face of the receptor close to the protein kinase domain (Hunter *et al.*, 1984; Hunter, 1987). Phorbol esters thus inhibit both the binding of EGF to its specific receptor and EGF catalyzed receptor autophosphorylation. Certain human malignancies possess elevated levels of receptors for EGF or EGF-like growth factors; among these are ovarian, cervical, renal, lung, breast tumours and glioblastomas (Ruddon, 1987). The A431 human epidermal carcinoma cell line has a greatly enhanced number of specific EGF receptors due to a severalfold amplification of the EGF receptor gene (Liberman *et al.*, 1985).

The relationship between EGF and signalling *via* inositol lipid hydrolysis appears complex. In a number of cell lines EGF rapidly stimulates the breakdown of inositol phospholipids and indeed some of the actions of EGF mimic those of the tumour promoting phorbol esters. Both may act as mitogens and stimulate the loss of density dependent growth. EGF has been reported to promote tumour formation after treatment of mouse skin with an initiating carcinogen (Rose *et al.*, 1976). It has been hypothesized that tyr phosphorylation of PLC is involved in the activation of inositol phospholipid hydrolysis. Three isozymes of PI specific PLC have been identified as possessing tyr phosphorylation sequences and EGF receptor specific phosphorylation of PLC II has been demonstrated (Nishibe *et al.*, 1989). The truncated receptor protein *erb B* possesses sequence homology with the cytoplasmic EGF receptor domain, therefore it is not subject

to shut off by down regulating mechanisms normally triggered by growth factor binding and thus may be constitutively active as a protein kinase.

PKC substrates include proteins that are phosphorylated on tyr residues by oncogene products for example vinculin, and P-36, a major protein tyr kinase substrate are both phosphorylated by PKC and $pp60^{src}$ (Gould *et al.*, 1985). It has emerged that 2 regions of PI specific PLC exhibit significant amino acid sequence similarities to the products of various tyr kinase related oncogenes including *yes, src, fgr, abl, fps, fes,* and *tck,* raising the possibility that PLC and cytoplasmic tyr kinases are modulated by common cellular components (Pann-Ghill *et al.*, 1988).

The activation of PKC has been implicated in the induction of certain genes; for example c-*fos*, c-*myc*, prolactin and collagenase gene expression, and the suppression of others; for example glycophorin A and B. Treatment with TPA, in cell lines for which it is mitogenic leads to transcription of the nuclear proto-oncogenes c-*myc* and c-*fos,* mimicking early events in growth factor stimulation (Whitman and Cantley, 1988). Differentiation associated disappearance of the c-*myc* protein product was observed in HL60 cells 4h after TPA treatment (Gailani *et al.*, 1989). Goode *et al.* (1990), have demonstrated elevated expression of c-*fos* oncogene mRNA in M5076 cells in response to agents which activate PKC; yet only those activators which inhibited M5073 cell growth induced a marked increase in c-*fos* protein. It is attractive to speculate that through the action of PKC, the expression of genes related to the action of growth factors may be perturbed. Certain genes whose transcription is induced by TPA, including c-*fos,* (Hata *et al.*, 1989) and TGF-ß (Kim *et al.*, 1989) possess a palindromic TPA-responsive element (TRE) in the 5`-flanking region (Hashimoto and Shudo, 1990). TRE is the binding site for the transcriptional factor AP-1 (activator protein), also inducible by TPA. Hata and coworkers have shown that the protein kinase activity of PKC is required and sufficient for TRE-mediated transcriptional activation (Hata *et al.*, 1989). TPA may also stimulate the transcriptional activity of the SV40 enhancer through AP-1 (Melber *et al.*, 1989).

Intriguing evidence is emerging to substantiate the possibility that mutated forms of PKC may be oncogenic. Multiple growth abnormalities and disturbances in gene expression have been demonstrated in cells which stably overexpress PKC. C3H/10T$^{1}/_{2}$ murine fibroblasts stably expressing a full length cDNA encoding rat PKC-ßI contain 3-11 fold greater PKC activity, are morphologically altered and grow to 4-fold saturation density with decreased adhesiveness. These cells also illustrate constitutive and inducible alterations in the levels of 2 PKC regulated genes, phorbin and TPA-R1 (Krauss *et al.*, 1989). A previous report from the same laboratory demonstrated between 20 and 53 fold increase in PKC activity in rat fibroblast lines overexpressing PKC-ßI. In addition to the abnormalities discussed, these cells were able to form colonies in soft agar (Housey *et al.*, 1988). Altered growth regulation, higher saturation densities, reduced dependence on serum for growth and enhanced tumourigenicity have been described in NIH 3T3 fibroblasts transfected with PKC-γ cDNA. (Persons *et al.*, 1988). The incomplete nature of the transformation phenotype observed in PKC transfectants may be because PKC alone cannot generate all

the signals for cellular transformation. However, expression of a mutant PKC-α in normal Balb/c 3T3 fibroblasts resulted in cell transformation, as judged by formation of dense foci, anchorage independent growth and ability to induce solid tumours when inoculated into nude mice (Megidish and Mazurek, 1989). Thus, disturbances observed in normal PKC expression and point mutations within the primary structure of PKC may contribute toward the transduction or generation of oncogenic signals. C-*raf* codes for a serine/threonine protein kinase which possesses cysteine rich sequences in its N-terminal regulatory domain homologous to the cysteine rich region in the regulatory domain of PKC. In the activated oncogene v-*raf,* the cysteine rich region is deleted and it has been inferred that the catalytic domain of PKC may function as an activated oncogene (Muramatsu *et al.*, 1989), a possibility consistent with studies of constitutively active PKC. It has been reported that upon phorbol ester stimulation of NIH 3T3 cells, a rapid activation of and redistribution from cytosol to nucleus of the *raf*-1 protein kinase occurs. Cells which have been transformed by the v-*raf* oncogene are found to have high levels of the v-*raf* truncated, active kinase constitutively present in the nuclear fraction (Olah *et al.*, 1990). Thus it is suggested that *raf*-1 translocation and activation may be an important step in mitogenic signal transduction from plasma membrane to nucleus.

1.7.3 PKC and human malignancy.

Clear evidence has arisen endorsing a role for aberrant PKC activity in certain human malignancies. O`Brian *et al.* (1989) have demonstrated elevated PKC activity in 8 of 9 spontaneous breast tumour surgical specimens, when compared with PKC activity in normal breast tissue from the same patient. It is hypothesized therefore that raised levels of PKC activity may provide a potential marker for breast malignancy. In several cultured human breast cell lines including the MCF 7 line, there is evidence to suggest that PKC is necessary for cell growth (Fabbro *et al.*, 1986). Furthermore, the synthetic antioestrogen, tamoxifen, which antagonizes human breast cancer *in vivo* is a PKC inhibitor within the micromolar range (O`Brian *et al.*, 1985), and can be administered to patients with low toxicity at concentrations that inhibit PKC. Taken together, these data suggest a role for PKC in human breast carcinogenesis and potential for selective PKC inhibitors as chemotherapeutic agents.

Further data to support PKC as a potential target, in this case as an inhibitor of tumour invasion and metastasis, was obtained from the following observation. Elevated PKC levels (2-3 fold) have been discovered in invasive human bladder carcinoma cells when compared with cells derived from non-invasive human bladder carcinoma. Moreover, invasion of the former through an artificial basement membrane was inhibited by STSPN (Schwartz *et al.*, 1990).

Animal model studies of colon, stomach and hepato-carcinogenesis have indicated that bile acids are tumour promoters. Epidemiological studies have correlated increased colon cancer incidence with high fat diets, believed to be as a result of free fatty acids and bile acids in the colonic lumen (reviewed by O`Brian and Ward, 1989). Certain bile acids, at concentrations present in the lumen, have been shown to activate PKC directly or indirectly

by stimulating PLC-catalyzed production of DAG. This may be highly significant biologically, as direct activation of PKC by bile acids may contribute to tumour promotion. In rat colonic epithelial cells, proliferation has been stimulated by TPA or the bile acid deoxycholate, thus it appears that PKC activation serves as a positive signal for colonic epithelial cell growth (Craven and DeRubertis, 1987). When compared to adjacent normal colonic mucosa, human colon carcinoma biopsies, expressed reduced PKC activity (Guillem *et al.*, 1987), consistent with the observed enzyme down regulation in certain oncogene transformed cell lines (Weyman *et al.*, 1988). Moreover, Choi *et al.* (1990) have obtained evidence revealing that PKC may function as a tumour suppressor in HT29 colon cancer cells. Derivatives of this cell line stably overexpressing PKC-ßI displayed a significant decrease in tumourigenicity in nude mice.

1.7.4 Implications for PKC in multi drug resistance.

PKC has been implicated in the emergence of the multidrug resistant (MDR) phenotype. Exposure to a cytotoxic drug, for example adriamycin allows a tumour cell population to acquire a multidrug resistant phenotype, providing such cells with cross resistance against structurally diverse cytotoxic natural products such as vinca alkaloids, epipodophyllotoxins and anthracyclines (Moscow and Cowan, 1988). Fine *et al.* have established a positive correlation between phorbol ester-induced PKC activation and the acquisition of an MDR phenotype. Exposure, for 5 minutes of drug sensitive MCF-7 breast cancer cells to phorbol dibutyrate (PDBu), followed by the addition of cytotoxic drug for 2.5 h resulted in an MDR phenotype. Furthermore, this study demonstrated that PDBu enhanced drug resistance in MCF-7 cells already possessing the MDR phenotype and increased levels of PKC activity in these cells. Inhibitors of PKC, for example sphingosine, mediated increased intracellular drug accumulation and inhibited clonal proliferation in adriamycin resistant human MCF-7 breast carcinoma cells (Sachs *et al.*, 1990). Transfection of PKC-α into MCF-7 cells transfected with the mdr 1 gene increased several fold drug resistance (Yu *et al.*, 1990). Drug resistant 2780 human ovarian carcinoma cells contained a 2-3 fold increase in total PKC activity when compared to their naive counterparts (Ming Yang *et al.*, 1989) and displayed significantly increased phorbol ester binding potential (Anderson *et al.*, 1990). Studies have been performed describing direct correlation between the level of PKC activity in a series of 4 murine fibrosarcoma cell lines and the degree to which they express resistance to adriamycin (O`Brian *et al.*, 1989). As to its specific role in the typical mdr phenotype, PKC has been reported to enhance phosphorylation of the P-glycoprotein (Hamada *et al.*, 1987) and decrease intracellular drug accumulation, moreover, increased activation of PKC (by PDBu) enhances resistance and P-glycoprotein phosphorylation (Yu *et al.*, 1990). In addition, Michaeli *et al.* (1990) have correlated elevated levels of membrane associated PKC-ß activity with an atypical mdr phenotype.
It has been demonstrated that TPA, during a single step selection assay for methotrexate (MTX) resistance in cultured mouse fibroblasts, causes a 100-fold increase in the incidence of MTX-resistant colonies, an effect shown to be a result of gene amplification (Barsoum and Varshavsky, 1983). Thus it is postulated that tumour promoters may alter gene

expression by favouring gene re-arrangements and gene amplification events in initiated cells that already possess damaged DNA and have a propensity for genetic instability. It has already been noted that DNA topoisomerase II, one of the enzymes that modulates DNA topology, by association with the interphase nuclear matrix and with the mitotic chromosome scaffold (Pienta *et al.*, 1989), is a cellular substrate for TPA-stimulated PKC (Sahyoun *et al.*, 1986). The expression of an altered DNA topoisomerase II in a mdr human leukaemic cell line has been reported (Suttle *et al.*, 1990). Thus, PKC may modulate the level of resistance to certain anticancer drugs and may serve as an important pharmacological tool for reversing this process.

The biochemical pathways comprising the mechanistic interactions implicated between PKC activity and the toxicity induced by chemotherapeutic agents are not clearly understood. Evidence apparently contradictory to the work described above have been presented by Posada *et al.* (1989). Activation of PKC in mouse sarcoma cells, by short exposure to TPA enhanced the cytotoxic potential of adriamycin as well as its ability to invoke DNA damage. Enzyme down-regulation partially protected the cells from these adverse cellular effects. Similarly, pre-treatment of HeLa cells with TPA caused a 9-fold increase in cellular sensitivity to *cis* -diamminedichloroplatinum (II), associated with a time and concentration dependent increase in cellular platinum content (Basu *et al.*, 1990).

1.8 The A549 human lung carcinoma cell line.

This thesis is concerned specifically with the growth inhibitory properties of phorbol esters and certain members of the bryostatin family (figure 3), using as a model, the A549 human lung adenocarcinoma cell line.The A549 human lung carcinoma cell line was derived in 1972 from explant culture of an alveolar cell carcinoma from a 58 year old Caucasian male, following right lower lobectomy (Girard *et al.*, 1973). These cells, at low and high passage levels were classified as type II alveolar (Lieber *et al.*, 1976); the cell type responsible for the production, storage and secretion of pulmonary surfactant; the phosphatidylcholine rich protectorant of the alveolar surface, imperative for normal lung function. Karyological data confirmed the human origin of this cell line and suggests that the tumour was derived from a single cell.

In 1985 it was demonstrated that the tumour promoting phorbol esters provoke potent arrest of A549 cell growth in a manner directly proportional to their potency in the mouse skin model of tumour promotion (Gescher and Reed, 1985). Incubation of cultures with 10nM TPA caused complete inhibition of cell growth for 5-6 days, after which time, whilst still in the presence of phorbol ester, cell growth returned at a rate indistinguishable from control cultures. However, it was observed that upon trypsinization and reseeding, the treated cells were rendered sensitive to phorbol esters once more. Phorbol ester-induced growth arrest was accompanied by a dramatic alteration in cell morphology; flat, polygonal cells became rounded and convex following treatment with growth inhibitory concentrations of phorbol esters. If cells were subcultured in the continual presence of 10nM TPA, it was found that they gradually became refractory to the growth inhibitory properties. Following 9 weeks exposure, TPA was unable to retard cell growth and cells

maintained their resistance after trypsinization and reseeding. The variant population was termed A549-TPA. It has been demonstrated by Dale (1989) that resistance is a property of the whole A549 population and not a consequence of the emergence of a resistant clone. It was hypothesized that PKC may possess a role in A549 cell growth arrest (Gescher and Reed, 1985). This hypothesis was investigated employing [^{3}H]PDBu to detect specific phorbol ester binding. Down-regulation of phorbol ester receptors was observed during the initial stages of growth inhibition such that after 24h pretreatment with 100nM PDBu, binding was reduced to 38% of controls. After 6 days treatment, receptor binding capacity was 75% of controls. The A549-TPA variant cell line possessed 77% of the phorbol ester binding sites compared to naïve parent populations. Thus, these preliminary studies concluded that down-regulation in receptor binding may be an important component in the events by which phorbol esters cause growth inhibition.

In 1988, it was demonstrated in human A549 lung carcinoma cells that the antineoplastic agents bryo 1 and 2 were able to mimic and antagonize phorbol ester responses. Intriguingly, these bryos have been shown to induce a very transient inhibition of growth at specific concentrations yet, at doses exceeding this, abolition of their own growth inhibition and that caused by phorbol ester has been reported (Dale and Gescher, 1989). Growth inhibition was accompanied by the characteristic morphology change, but this too was transient even in the presence of TPA.

1.9 Aims of this project.

The global goal of this project is concerned with investigating the viability of the hypothesis, which states that PKC may provide a potential target in anticancer therapy. The diverse biological responses induced by the tumour promoting phorbol esters are widely believed to be mediated by PKC. In this study we wished to elucidate the precise role of PKC in phorbol ester-induced A549 growth inhibition. To this end, several methods for measuring enzyme activity have been explored. The subcellular distribution of PKC activity in treated cells was examined by quantitative assay following non denaturing PAGE. In addition, specific phorbol ester binding potential was determined in crude cytosolic extracts and on confluent cell monolayers.

Cells have been routinely cultured in serum-free, chemically defined conditions, allowing further characterization of the growth arrest induced by tumour promoting phorbol esters. Experiments were performed to try to define specific conditions which support maintenance of TPA-induced growth inhibition. Cytosolic phorbol ester binding and subcellular distribution of PKC in response to phorbol ester treatment was examined in this serum-starved A549 population.

Phorbol esters are able to inhibit the growth of a number of malignant cell lines (section 1.6.5). Identification of structural components of the pharmacophore responsible for this property may lead to the rational design of novel anticancer agents. An ongoing aim of work within this project is to identify novel PKC activators which possess cytostatic properties. A number of synthetic, rationally designed phorbol ester and DAG analogues have been biologically tested; their potential to cause cytostasis versus cytotoxicity, and

ability to compete for phorbol ester receptors has been evaluated. During the course of this study, a novel and rapid technique for the measurement of phorbol ester binding in individual viable cells, has been developed using the fluorophore Bodipy-3-propionyl-13-acetate (Bod-3-PE) and multiparameter flow cytometry.

Antineoplastic bryos are potent activators of PKC. We wished to examine the nature of A549 cell growth arrest elicited by bryos 1,2,4 or 5 and to investigate the hypothesis that bryos 4 and 5, like bryos 1 and 2 (Dale and Gescher, 1989) are able to evoke phorbol ester antagonist as well as agonist growth responses. Competitive binding assays were performed to determine the affinity of bryos for the phorbol ester receptor. Subcellular enzyme distribution was determined following exposure of cells to bryos in order to examine and directly compare PKC activation and enzyme down-regulation in response to these different enzyme activators. Preliminary cell culture experiments were conducted in the presence of STSPN in order to aid dissection of events induced by bryo or TPA, critically linked to PKC activation.

Using antibodies specific to the PKC subtypes α and ß, Western blot experiments were conducted to probe for the presence of these isozymes in A549 human lung carcinoma cell populations; hence, immunodetectable protein could be compared with functional enzyme activity and phorbol ester binding. This technique was also employed to test the hypothesis that activators of PKC possess preferential isozyme affinity, thus mediating differential responses.

In the light of the imminent introduction of bryo 1 to phase 1 clinical trial, preliminary studies have been undertaken to assess the effect of PDBu and bryo 1 on the growth of transplanted A549 cells *in vivo*. Experiments were conducted to evaluate changes in phorbol ester receptor binding potential in various tissues of the body, following treatment of experimental animals with PDBu; with a view to conduct future investigations after treatment of experimental animals with bryo 1.

Section 2: MATERIALS.

2.1 Sources of chemicals.

Sigma Chemical Company Limited, Poole, Dorset, England.

Adenosine 5`-triphosphate (ATP) (sodium salt), aprotinin, bovine albumin, bromophenol blue (sodium salt), 1,2-sn-dioctanoylglycerol (1,2-di$C_8$), disodium ethylenediamine tetraacetic acid ($Na_2$EDTA), ethyleneglycol-bis-(B-aminoethylether)-N,N,N`,N`-tetraacetic acid (EGTA), fetuin, glucose, glycine, leupeptin, magnesium nitrate, N,N,N`,N`-tetramethylethylene diamine (TEMED), nicotinamide adenine dinucleotide, reduced form (NADH), nonidet NP-40, 1-oleoyl-2-acetyl-sn-glycerol (OAG), phorbol 12,13-dibutyrate (PDBu), 4-α-phorbol 12,13-didecanoate (PDD), phosphatidylserine (PS), potassium persulphate, retinoic acid (all trans), riboflavin, sodium azide, sodium pyruvate, 12-O-tetradecanoylphorbol-13-acetate (TPA), triton-X-100, tris base (Trizma).

Gibco Limited, Paisley, Glasgow, Scotland.

Hams F-12 nutrient media, L-glutamine, new born calf serum (NBCS), penicillin, streptomycin, trypsin, Ultroser-G (US).

Fisons PLC, Loughborough, Leicestershire, England.

Dimethylsulphoxide (DMSO), glacial acetic acid, glutaraldehyde, glycine, hydrochloric acid, Optiphase MP scintillation fluid, magnesium chloride, methanol, phosphoric acid, sodium cacodylate, sodium chloride, sodium hydroxide.

BDH Chemicals Limited, Poole, Dorset, England.

Amaranth dye (technical grade), calcium chloride solution (1M), B-mercaptoethanol, phenol red, protamine sulphate, sodium dodecylsulphate (specially purified)

ICN Flow, High Wycombe, Buckinghamshire, England.

ITS premix preparation combining insulin, transferrin and selenium.

Fluka Chemicals Limited, Glossop, Derbyshire, England.

Acrylamide (electrophoresis pure), N,N`methylenebisacrylamide (electrophoresis pure). sodium dihydrogen phosphate.

Bio-Rad Laboratories Ltd, Watford, Hertfordshire, England.

Bradford dye reagent, bovine serum albumin protein standard.

Aldrich Chemical Company, Gillingham, Dorset, England.

Trichloroacetic acid.

Whatman Labsales Ltd, Croydon Surrey, England.

17 Chr chromatography paper.

Millipore UK Ltd, Harrow, Middlesex, England.

Millipore 0.2μM filters.

Boehringer Corporation Ltd, Lewes, Sussex, England.

96 well microtitre plates, U form.

New England Nuclear, Du Pont (UK) Ltd, Southampton, Hampshire, England.

[20-^{3}H(N)]-phorbol-12,13-dibutyrate (10-20 Ci/mmol), [G-^{32}P]ATP (6000 Ci/mmol).

Amersham International Plc, Amersham, Buckinghamshire, England.

5-[methyl ^{3}H]-thymidine (5 Ci/mmol), Hybond-C nitrocellulose, blotting detection kit for mouse antibodies, rainbow markers.

British Bio-technology Ltd, Cowley, Oxfordshire, England.

Platelet-derived growth factor, transforming growth factor ß.

Oxoid Laboratories Ltd, Basingstoke, Hampshire, England.

Phosphate buffered saline tablets.

Other chemicals

Bryostatins 1, 2, 4 and 5 were gifts provided by Dr. G.R. Pettit, Arizona State University, Tempe, Arizona, USA. Purified bovine brain PKC, was donated by Dr. P.J. Parker, Ludwig Institute for Cancer Research, London. Diacylglycerol (DAG) and phorbol ester analogues were synthesized by Dr.C.A. Laughton, Cancer Research Campaign Medicinal Chemistry Laboratories, Aston University, Birmingham.

Tissue culture materials.

50ml and 250ml culture flasks, 35mm six well multidishes, 140mm petri dishes (Nunclon, Denmark). Class II Gelair BSB 3 microbiological safety cabinet with unidirectional laminar downflow, gassing incubator (Flow laboratories, Irvine, Scotland). 30ml sterile universals, 7ml sterile bijoux, sterile pipettes (Sterilin Limited, Feltham, England).

2.2 Solutions, buffers and experimental reagents.

2.2.1 Tissue culture reagents.

Ultraser G (US).

US media supplement was dissolved in sterile d.H_2O (20ml). An aliquot of 10ml was added to 500ml media such that cells were routinely cultured in media supplemented with 2% US.

Fetuin.

1g Fetuin was dissolved in sterile d.H_2O (10ml). An aliquot of 2ml was added to 100ml media such that cells were routinely cultured in media containing 2mg/ml Fetuin.

ITS premix

ITS medium supplement was dissolved in sterile d.H_2O (25ml). 5ml was added to 500ml media such that cells were routinely cultured in media supplemented in 1% ITS.

Phorbol esters and Bryostatins.

Phorbol esters and bryostatins were stored frozen as 1mM stock solutions at -20°C. When required, further dilutions were made in DMSO as appropriate. Diluted stock solutions which were thawed and frozen frequently were discarded after 4 weeks.

DAGs and synthetic analogues.

Stock solutions of DAGs and analogues synthesized in these laboratories were dissolved in DMSO for tissue culture purposes and chloroform for use in the mixed micelle assay. Preparation was immediately prior to use.

Growth factors.

Human platelet-derived growth factor (PDGF), transformiong growth factor ß (TGF-ß) or

epidermal growth factor (EGF) (1μg) was diluted in a solution of 4mM HCl containing 1mg/ml bovine albumin and stored as a stock concentration of 50nM at -20°C.

Versene solution, 10 x stock.

20 PBS tablets

0.742g Na_2EDTA

0.1g phenol red

to 200ml with $d.H_2O$

pH was adjusted to 7.4 using 1M sodium hydroxide, the solution was then filtered, autoclaved and stored at 4°C.

Trypsin-versene solution.

10ml versene stock solution

10ml 10X trypsin solution

80ml $d.H_2O$

the solution was filtered and stored at 4°C.

Phosphate buffered saline.

5 PBS tablets

to 500ml $d.H_2O$

pH was adjusted to 7.4, if necessary,and the solution stored at 4°C.

Acid fixative.

10ml acetic acid

50ml methanol

to 100ml with $d.H_2O$

Solutions for the LDH cytotoxicity assay.

3.5mM NADH.

9.92mg NADH

to 4ml with $d.H_2O$

The solution was prepared immediately prior to use and kept on ice until used.

32mM sodium pyruvate.

14.08mg sodium pyruvate

to 4ml with $d.H_2O$

The solution was prepared immediately prior to use and kept on ice until used.

2.2.2 Stock reagents for quantitative assay of PKC by PAGE.

20% (w/v) triton X-100.

20g triton X-100

to 100ml with $d.H_2O$

The solution was dissolved by stirring on a heated plate and then stored at room temperature. 3% triton X-100 was prepared by dilution of this stock.

Anode buffer stock.

75.6g tris base

to 1000ml with d.H_2O

pH was adjusted to 7.51 with concentrated HCl and the buffer stock was stored at 4°C. Immediately prior to use, the stock was diluted 1:10 with d.H_2O resulting in 0.0625M tris-HCl, pH 7.51.

Cathode buffer stock.

28.5g tris base

17.3g glycine

to 500ml with d.H_2O

pH was adjusted to 8.90 with concentrated HCl and the buffer stock stored at 4°C. For 1l of cathode buffer, the stock was diluted as follows:

100ml cathode buffer stock

10ml 20% triton X-100

890ml d.H_2O, resulting in 0.0426M tris-HCl, 0.0461M glycine, 0.2% triton X-100, pH 8.90.

Stacking gel buffer stock.

2.96g tris base

to 100ml with d.H_2O

pH was adjusted to 6.67 using concentrated $H_3 PO_4$. The stock was stored at 4°C.

Resolving gel buffer stock.

18.16g tris base

to 100ml with d.H_2O

pH was adjusted to 8.83 using concentrated HCl and the solution stored at 4°C.

Acrylamide stock: resolving gel, (40%T, 2%C).

19.6g acrylamide

0.4g N,N`methylenebisacrylamide

to 50ml with d.H_2O.

The solution was filtered through a 0.2µM Millipore filter and stored at 4°C in a brown bottle.

Acrylamide stock: stacking gel, (8%T, 20%C).

3.2g acrylamide

0.8g N,N`methylenebisacrylamide

to 50ml with d.H_2O

The solution was filtered through a 0.2µM Millipore filter and stored at 4°C in a brown bottle.

1:10 TEMED.

1ml TEMED was diluted to 10ml with d.H_2O and stored at 4°C.

0.01% riboflavin solution.

10mg riboflavin

to 100ml with d.H_2O

The solution was filtered through a 0.2µM Millipore filter and stored at 4°C.

Potassium persulphate/ riboflavin solution.

30mg potassium persulphate

10ml 0.01% riboflavin

to 50ml with d.H_2O

The solution was prepared immediately prior to use.

Amaranth dye solution.

0.5g Amaranth dye was dissolved in 4ml d.H_2O and stored at 4°C.

1.0M Tris-HCl.

12.114g Tris base

to 100ml with d.H_2O

pH was adjusted to 7.4 using concentrated HCl and the solution stored at 4°C.

0.5M magnesium nitrate.

1.282g magnesium nitrate

to 10ml with d.H_2O

The solution was stored at 4°C.

0.1M Calcium chloride.

1ml analar 1M calcium chloride was diluted to 10ml with d.H_2O and stored at 4°C. Further calcium chloride dilutions were made from this stock.

100mM Na_2EDTA.

0.744g Na_2EDTA

to 20ml with d.H_2O

pH was adjusted to 7.4 using 8M NaOH and the solution stored at 4°C.

100mM EGTA.

0.761g EGTA

to 20ml d.H_2O

pH was adjusted to 7.4 using 8M NaOH. 10mM EGTA stock was prepared by 1:10 dilution of this solution with d.H_2O. Solutions were stored at 4°C.

Leupeptin stock.(10mg/ml)

100mg leupeptin was dissolved in 10ml d.H_2O. 200µl aliquots were frozen at -20°C.

Aprotinin stock.(10mg/ml)

100mg aprotinin was dissolved in 10ml d.H_2O and 100µl aliquots were frozen at -20°C.

Wash buffer W3 stock.

Used for washing cell monolayers before scraping cells.

12.114g Tris base

43.830g sodium chloride

4.505g glucose

to 500ml with d.H_2O

pH was adjusted to 7.4 using concentrated HCl and the solution stored at 4°C.

The stock was diluted 1:10 immediately prior to use and the proteolysis inhibitors leupeptin and aprotinin added.

Diluted W3, final concentrations:

20mM Tris-HCl, pH 7.4
150mM sodium chloride
5mM glucose
10µg/ml leupeptin
2mg/ml aprotinin

Homogenization buffer H8.

Used in the preparation of cell extracts for loading onto gels and prepared immediately prior to use.

Addition	Final concentration
2ml 1.0M Tris-HCl, pH 7.4	20mM
2ml 100mM EDTA, pH 7.4	2mM
2ml 100mM EGTA, pH 7.4	2mM
40µl ß-mercaptoethanol	6mM
100µl leupeptin stock	10µg/ml
20µl aprotinin stock	2µg/ml
to 100ml with d. H_2O	

Extraction buffer H8-1%NP-40.

Extraction buffer was prepared as described for H8, with the addition of 1g nonidet (NP-40) per 100ml buffer.

Elution buffer.

Used for eluting protein from gel slices overnight.

Addition	Final concentration
2ml 1.0M Tris-HCl, pH 7.4	20mM
1ml 10mM EGTA, pH 7.4	0.1mM
5ml 1.0mM sodium chloride	50mM
350µl ß-mercaptoethanol	50mM
100µl leupeptin stock	10µg/ml
20µl aprotinin stock	2µg/ml
to 100ml with d. H_2O	

The buffer was prepared on the day of use.

10% TCA + phosphate.

1kg trichloroacetic acid (TCA)
100-200g sodium dihydrogen orthophosphate
to 10 litres with d.H_2O

The solution was cooled to 4°C before use.

300mM ATP.

0.165g ATP (sodium salt)
to 1ml with d.H_2O

50µl aliquots were frozen at -20°C. When required, an aliquot was thawed rapidly and diluted to 60mM before addition to the assay mix.

Assay mixture.

For one assay, the following components were mixed:

4µl 1M Tris-HCl, pH 7.4

4µl 0.5M magnesium nitrate

0.08µl 60mM ATP

100µg protamine sulphate

0.5µCi [$-^{32}P$]-ATP

to 50µl with d.H_2O

Assay mix sufficient for 200-300 assays was usually required and prepared immediately before use.

2.2.3 Stock solutions for the mixed micellar binding assay.

200mM Tris-HCl.

2.423g Tris base

to 100ml with d.H_2O

pH was adjusted to 7.5 with concentrated HCl and the buffer stored at 4°C.

22mM calcium chloride.

Analar 1M calcium chloride solution was diluted 1:45.45 with d.H_2O and stored at 4°C.

3% (w/v) Triton X-100.

20% (w/v) Triton X-100 was diluted 1:6.67 with d.H_2O and stored at room temperature.

Buffer A.

Addition	Final concentration
50ml 200mM Tris-HCl, pH 7.5	20mM
100µl 1M calcium chloride	200µM
375µl 20% (w/v) Triton X-100	0.015%

to 500ml with d.H_2O

The buffer was stored at 4°C.

Buffer B.

Addition	Final concentration
50ml 200mM Tris-HCl, pH 7.5	20mM
0.1g sodium azide	0.02%

to 500ml with d.H_2O

The buffer was stored at 4°C.

Bovine brain PKC.

Comprising α,β_I, β_{II} and γ isoforms, stored at -20°C in the following buffer at 500U/ml:

20mM Tris-HCl, pH 7.5

2mM EDTA

50mM ß-mercaptoethanol
50% (v/v) glycerol
PKC was diluted to 3U/ml in the above buffer immediately prior to use.

2.2.4 Stock reagents for assay of PKC by Western Blot analysis.

30% acrylamide 0.8% bisacrylamide stock.

15g electrophoresis pure acrylamide
0.4g N,N` electrophoresis pure methylenebisacrylamide
to 50ml with d.H_2O
The solution was filtered through a 0.2µM Millipore filter and stored at 4°C.

10% SDS.

10g SDS
to100ml with d.H_2O and stored at room temperature.

1.5M Tris-HCl.

45.43g Tris base
to 250ml with d.H_2O
pH was adjusted to 8.8M using concentrated HCl and the solution stored at 4°C.

0.5M Tris-HCl.

15.14g Tris base
to 250ml with d.H_2O
pH was adjusted to 6.8 using concentrated HCl and the solution stored at 4°C.

0.125M Tris-HCl + 0.1% SDS.

25ml 0.5M Tris-HCl, pH6.8
1ml 10% SDS
74ml d.H_2O
the solution was stored at 4°C.

Bromophenol Blue.

5mg bromophenol blue
to 10ml with d.H_2O.

Running buffer.

15.15g Tris base
72g glycine
5g SDS
to 5 litres with d.H_2O
pH (approximately 8.3) was not adjusted as introduction of Cl^- ions may prevent stacking of proteins. The buffer was stored at 4°C overnight.

Ammonium persulphate.

0.1g ammonium persulphate
to 1ml with d.H_2O
The solution was prepared immediately prior to use.

Sample buffer.

Addition	Final concentration
1.0ml 0.5M Tris-HCl, pH 6.8	0.125M
0.8ml glycerol	20%
1.6ml 10% SDS	4%
123mg dithiothreitol (DTT)	0.2M
0.2ml 0.5mg/ml bromophenol blue	0.025mg/ml
to 4ml with d.H_2O	

Sample buffer, minus DTT was stored at 4°C. DTT was added immediately prior to use.

Electrode solution.

Addition	Final concentration
2.93g glycine	39mM
5.81g Tris base	48mM
0.375g SDS	0.0375% (w/v)
200ml methanol	20%
to 1 litre with d.H_2O	

The solution was stored at 4°C.

Tris-buffered saline (TBS).

Addition	Final concentration
2.42g Tris base	20mM
8g NaCl	137mM
to 1 litre with d.H_2O.	

pH was adjusted to 7.6 using concentrated HCl and the buffer stored at 4°C.

TBS-Tween (TBS-T).

1ml Tween 20 (0.1%) in 999ml TBS and stored at 4°C.

Blotting detection kit (for the detection of mouse antibodies bound to antigens on nitrocellulose).

Kit components: mouse immunoglobulin (IgG positive control), dried milk (membrane blocking agent), anti-mouse immunoglobulin, biotinylated species-specific whole antibody (from sheep), streptavidin-biotinylated alkaline phosphatase preformed complex, nitro-blue tetrazolium (NBT) in dimethyl formamide, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in dimethyl formamide and diethanolamine.

The complete kit was stored at 4°C, under which conditions reagents were stable for 3 months. Contents were allowed to equilibrate to room temperature directly before use.

Diethanolamine buffer.

Addition	Final concentration
1.05g diethanolamine	100mM
100mg $MgCl_2.6H_2O$	5mM
to 100ml with d.H_2O	

pH was adjusted to 9.5 with 5M HCl and the buffer stored at 4°C.

2.2.5 Electron microscopy solutions.

0.18M sodium cacodylate.

19.26g sodium cacodylate

to 500ml with d.H_2O

The solution was stored at 4°C.

0.05M sodium cacodylate + 0.2% glutaraldehyde.

5.35g sodium cacodylate

1g glutaraldehyde

to 500ml with d.H_2O

The solution was stored at 4°C.

2.2.6 Flow cytometry reagents.

Propidium iodide solutions.

5mg PI

to 1ml with d.H_2O

the solution was prepared in an eppendorf tube immediately prior to use, wrapped in silver foil and held on ice.

2mg PI

to 20ml with d.H_2O

the solution was prepared the day before use and stored at 4°C.

2µM Bodipy-3-propionyl-13-acetate (Bod-3-PE) solution.

20µl Bod-3-PE (0.1mM stock)

to 1ml with DMSO

the solution was prepared immediately prior to use and held on ice in the dark for the duration of the experiment.

Section 3: METHODS

3.1 Cell culture procedures.

Sterile techniques were adopted. All culture procedures were carried out within the laminar flow sterile safety cabinet, which had been treated for 15 min with UV light and swabbed down with 70% industrial methylated spirits (IMS) prior to use.

3.1.1 Maintenance of cells in culture.

A549 cells were obtained from the American Type Culture Collection, Rockville, Maryland, USA. Cultures were maintained in Nutrient Hams F-12 media supplemented with 100 U/ml penicillin, 10pg/ml streptomycin, 20mM L-glutamine and either 10% FCS, 2% US, or 1% ITS plus 2mg/ml fetuin. Medium was routinely replenished every 2-3 days. Cultures were maintained in the logarithmic phase of growth at 37°C and passaged every 5-7 days, when confluent, by trypsinization.

3.1.2 Detachment of cells by trypsinization.

Medium was aspirated from the cultures and cells were washed briefly with versene solution to remove any debris. Versene was removed by aspiration and trypsin-versene solution added to the cells which were then incubated at 37°C for 2-4 minutes until detached. An equal volume of medium was then added to inactivate the trypsin. For routine subculturing, approximately 5% of the original culture was reseeded into a fresh tissue culture flask.

3.1.3 Storage of cells in liquid nitrogen.

Cells in logarithmic phase of growth were trypsinized as described and pelleted using an Heraes Christ labofuge 6000 centrifuge at a speed of 1000 rpm for 5 min. The supernatant was removed by aspiration and the cell pellet gently washed in maintenance medium and resuspended at a density of 10^6 cells/ml in medium containing 10% DMSO. Aliquots (1ml) of the cell suspension were transferred to cryogenic vials and cooled by degrees to -80°C before immersion into liquid nitrogen.

Upon removal from the cell bank, vials were rapidly thawed and the cells seeded immediately into culture flasks. After 24h, the original medium was aspirated and fresh medium introduced into the culture flasks. Cells were maintained in culture for no longer than 6 months. A bank of low passage A549 cells were stored in nitrogen and stocks were replenished annually.

3.1.4 Counting of cells.

A model ZM coulter counter (Coulter Electronics Ltd., Luton, Bedfordshire, England) was used routinely to count cells. Aliquots (200µl) from a single cell suspension were diluted with 9.8ml isoton (Coulter Electronics Ltd.) and counted on the settings of current: 130, attenuation: 16, lower threshold: 12.0, upper threshold: 99.9. Occasionally cell suspensions were counted using a haemocytometer (Weber Scientific International Ltd.).

3.1.5 Growth curves.

Cells ($2x10^4$) were seeded into 35mm diameter six well multidishes in 3ml medium and allowed 4h to attach to the plastic. Compounds dissolved in DMSO were then added such

that the concentration of DMSO did not exceed 0.33%. Control cultures received an equivalent volume of DMSO. Media was replenished and compound under investigation added every 48h. Cell numbers were counted daily, in triplicate, following trypsinization, usually over a 6 day period. Reusable plastic boxes, previously sprayed with fungicide and 70% IMS, were utilized for enclosing culture dishes before introduction into the incubator.

3.1.6 Measurement of incorporation of [^{3}H]TdR into cells.

Cells (10^5 or $2x10^5$) were seeded into 35mm six well multidishes and compounds added 4h later. Following the required treatment period, cells were briefly washed with media and incubated for 1h in 1ml medium containing 1μCi [^{3}H]TdR. Cultures were then washed 6 times with ice-cold PBS, pH 7.4, fixed with 50% (v/v) methanol, 10% (v/v) acetic acid and stored at 4°C for > 30 min. Wells were washed twice more with ice-cold PBS and the remaining insoluble fraction was solubilized in 2 x 0.5ml aliquots of 1% SDS. Optiphase MP scintillation fluid (10ml) was added to each sample and radioactivity was counted in a Packard Tricarb CA2000 scintillation counter. Experiments were conducted in triplicate with concurrent cell counts in duplicate, results were expressed as % control [^{3}H]TdR incorporation/10^5 cells.

3.1.7 Measurement of IC_{50} by cell number.

Cells ($2x10^4$) were seeded into 35mm six well multidishes in 3ml medium and allowed 4h to attach to the plastic. The synthetic compounds under investigation were added in DMSO vehicle to give final concentrations ranging from 10^{-4} to 10^{-9}M. Controls received DMSO only. Treatment was repeated 24 and 48h later. Cultures were allowed a further 48h growth period before cell counts were performed in triplicate. Deducting $2x10^4$ from final cell number, growth was expressed as % control.

3.1.8 Estimation of cytotoxicity by assay of release of LDH from cells.

Lactate dehydrogenase (LDH) catalyses the reaction:

$$\text{Pyruvate} + \text{NADH} + H^+ \longrightarrow \text{Lactate} + NAD^+$$

The assay measures leakage of LDH from cells damaged by toxic insult by following spectrophotometrically the decrease in absorption at wavelength 340nm caused by the oxidation of NADH to NAD^+. The method was adapted from that described by Leathwood and Plummer (1969).

Cells (10^5) were seeded into 35mm six well multidishes and allowed to attach for 4h. Medium was then replaced by 1.5ml medium supplemented with 1% FCS, antibiotics and glutamine. Reduction in serum levels was necessary as endogenous NADH oxidising enzymes present in FCS significantly interfered with the assay. The test compound was added in DMSO vehicle to give a final concentration range of 10^{-9} to 10^{-4}M, controls received vehicle alone. Multidish wells were incubated overnight before the media was transferred to centrifuge tubes and stored at 4°C. Wells were further treated with the test compound as described to mimic multiple drug additions and incubated for a further 5h. The two aliquots of media were combined, centrifuged for 5min at 1000 rpm to remove cell debris and assayed for LDH activity.

PBS (2.4ml), pH 7.4, 100μl NADH stock solution (final assay concentration: 0.12mM)

and 400µl medium sample were added to a quartz cuvette, (1cm path length). The assay cocktail was allowed to equilibrate at 37°C for 5min before initiating the reaction by the addition of 100µl stock sodium pyruvate solution (final concentration: 1.0mM). The rate of change of absorbance over 5min was measured using a Beckman DU-7 spectrophotometer. Maximal release of LDH was determined by lysing cells in 1% Triton X-100. Immediately prior to the assay, 160µl of 20% Triton X-100 was added to previously untreated cells in 3ml medium supplemented with 1% FCS. After 5min incubation, the supernatant was centrifuged and 400µl assayed for LDH activity. Thus the rate was obtained to represent 100% cell death. LDH release was measured in untreated cells to obtain a figure representing natural death during normal cell turnover. Experiments were conducted in triplicate and in order to correct for cell growth at non or marginally toxic concentrations, cell counts were performed. Toxicity due to compound was expressed as % Triton-releasable LDH activity/10^5cells.

3.1.9 Electron microscopy.

Cells were seeded into 35mm six well multidishes and treated as required. Medium was aspirated and cells briefly washed with PBS, pH 7.4, before being fixed for 1h in 0.05M sodium cacodylate containing 0.2% (w/v) glutaraldehyde. The fixative was aspirated then samples were rinsed and stored in 0.18M sodium cacodylate. Dr. S. Townsend at the MRC Radiobiology Unit, Harwell processed samples. Briefly, samples were dehydrated through graded alcohols to propylene oxide and embedded in Spurr`s resin. Ultra-thin sections were cut onto M50 copper grids and examined under a CORA transmission electron microscope after staining with uranyl acetate and lead citrate.

3.2 Quantitative assay of PKC by non-denaturing PAGE.

Quantitation and subcellular distribution of PKC was determined using the method of Fabbro *et al.* (1985). Briefly, crude cytosolic and membrane subfractions were partially purified by non-denaturing PAGE. Gels were frozen then sliced into 1mm sections before eluting protein from the slices overnight. Eluates were assayed for PKC activity by measuring the incorporation of ^{32}P from [-^{32}P]ATP into protamine sulphate.

3.2.1 Preparation of polyacrylamide gels.

Gels were prepared on the day before sample application, a maximum of twelve gels could be run in a single experiment. All gels were cast in borosilicate glass rods of length 14cm, outer diameter 0.7cm, inner diameter 0.5cm at 4°C. A double layer of parafilm sealed the rods during gel polymerization.

The lower, resolving gels were prepared as a total monomer concentration of 10% acrylamide with 2% bisacrylamide crosslinks. For twelve 10% polyacrylamide gels, 6ml resolving gel acrylamide stock, 6ml resolving gel buffer stock, 6ml potassium persulphate/riboflavin (KP/RF) solution and 6ml d.H_2O were mixed in a brown bottle on ice. The gel mix was deaerated while stirring on ice for 5 min by passing nitrogen through the bottle. The addition of 240µl 20% Triton X-100 and 240µl TEMED solution initiated polymerization. Gel mix (1.6ml) was then immediately introduced into each glass rod and

gently overlaid with 200µl d.H_2O. Resolving gels of 6cm length were photopolymerized by illumination with fluorescent light for 30 min at 4°C. After this process was completed the gel surface was rinsed 3 times with d.H_2O.

The upper, stacking gels were prepared as a total monomer concentration of 3.5% acrylamide with 20% bisacrylamide crosslinks. For twelve gels, 6ml stacking gel acrylamide stock, 3ml stacking gel buffer stock and 3ml KP/RF solution were mixed in a brown bottle on ice and whilst constantly being stirred, the stacking gel mixture was deaerated for 5 min by a stream of nitrogen. The addition of 120µl 20% Triton X-100 and 120µl TEMED solution initiated polymerization. 700µl of the gel mix was immediately poured into the rods above the polymerized resolving gels. An overlay of d.H_2O was introduced and photopolymerization performed as described previously. It was essential that the volume of stacking gel (700µl) was at least twice that of sample volume loaded onto the gel (200µl).

3.2.2 Preparation of subcellular fractions.

Cells were cultured in 140mm diameter petri dishes, maintained at 37°C in a humidified atmosphere of 95% air with 5% CO_2 and used in experiments when 75-95% confluent. When required, compounds were dissolved in DMSO and added to the cultures such that the final DMSO concentration was between 0.05 and 0.1%. Using a maximum of six culture dishes per experiment; 12 gel samples were prepared on the day before partial purification by PAGE.

Media was discarded and the cell monolayer washed three times with 10ml ice cold wash buffer W3. All W3 was removed by aspiration and 0.5ml homogenization buffer, H8 was introduced into dishes. The cell monolayer was gently scraped off the dish and transferred to a 10ml thick-walled centrifuge tube held on ice. Remaining cells within the dish were rinsed and scraped with 0.5ml H8, and transferred to the same centrifuge tube. Cells were then disrupted by sonication (3 x 10s bursts, setting 26, MSE sonicator) and centrifuged at 100,000g for 30min at 4°C in an AP Pegasus ultracentrifuge, yielding the membrane pellet and supernatant cytosol. The latter was supplemented with 10% glycerol and immediately frozen at -20°C in two aliquots; one of which was for the determination of protein.

The membrane pellet was resuspended in 1.5ml extraction buffer, H8 + 1% (w/v) NP-40, sonicated and centrifuged as above. The resulting supernatant, designated as the particulate fraction was supplemented with 10% glycerol and frozen at -20°C overnight in two aliquots.

3.2.3 Electrophoresis and protein elution.

Parafilm was removed from the ends of the rods and the stacking gel surface washed 3 times with d.H_2O. Cytosolic and particulate fractions were rapidly thawed and aliquots of 270µl were pipetted into a 1.5ml Eppendorf tube. Cytosol samples were mixed with 30µl cathode buffer containing 10% glycerol, 2% Triton X-100 (final concentration 0.2%) and Amaranth front dye (sufficient for the dye to be visible when loading onto the gel). Membrane samples received 30µl cathode buffer containing 10% glycerol and Amaranth front dye only.

200µl of sample was loaded onto the stacking gel and overlaid to the top of the rod with cathode buffer containing 0.2% Triton X-100.

Electrophoresis was performed using a Bio-Rad Model 175 tube cell gel apparatus. Anode and cathode buffers had previously been cooled to 4°C overnight. The temperature of the system was maintained at 4°C by circulating ethyleneglycol antifreeze (precooled to -20°C using a Haake DK12 refrigeration unit) through the central core of the gel apparatus.

Conditions of electrophoresis were as follows: the voltage was fixed at 100V until the dye boundary had moved through the stacking gel, then increased to 170V while the boundary moved through the resolving gel. Throughout the process, current in excess of 0.8mA per gel was avoided if possible. Running time was approximately 12h and electrophoresis was complete when the dye boundary had reached the end of the resolving gel.

Gels were expelled from the rods by injecting d.H_2O between their inner rim and the gel within, until it became free. The stacking gel was discarded, the resolving gel was placed in a polystyrene tube and laid on a block of dry ice until frozen. Frozen gels were sliced into 1mm sections using a Mickle gel slicer (Mickle Laboratory Engineering Co. Ltd.,UK) and the gel discs transferred to 3.5ml centrifuge tubes containing 300µl elution buffer. Enzyme activity was eluted from slices overnight by gentle shaking at 4°C.

3.2.4 Protein kinase C assay.

PKC activity was assayed in gel eluates using a microtitre assay technique adapted from the method of Kikkawa *et al.* (1983).

96-well U-form microtitre plates were securely taped to the top of a perspex box designed such that d.H_2O, heated and circulated by a thermostirrer, maintained solutions in the microtitre wells at 32°C. Gel eluates (150µl) were pipetted into the wells and allowed to equilibrate to 32°C. The enzyme reaction was initiated by the addition of 50µl protamine-assay mix (0.5µCi [-^{32}P]ATP/assay) to the wells using a multichannel pipette (Flow Laboratories Ltd.). Final concentrations of assay components in the incubation mixture were: 20mM Tris-HCl, pH 7.4, 10mM $Mg(NO_3)_2$, 24µM ATP and 100µg protamine. Following a 10 min incubation period, 150µl reaction mix was removed and immediately spotted onto Whatman 17Chr filter paper (30mm x 5mm), numbered for identification purposes and arranged in 96-well microtitre plates. The soaked papers were then plunged into ice cold 10% (w/v) TCA to quench the reaction. Papers were then gently shaken in four, 15 min fresh washes of 10% (w/v) TCA, before being drenched briefly in methanol (to aid drying) and placed in a hot oven until dry. Dried papers were transferred to 10ml scintillation vials where 6ml Optiphase MP scintillation fluid was added. Radioactivity was counted using a Packard Tricarb CA2000 scintillation counter. One unit PKC activity is expressed as the amount of enzyme which incorporates 1pmol ^{32}P into protamine sulphate substrate per min at 32°C.

3.2.5 Determination of protein.

Protein was determined by the method of Bradford (1976) using Bio-Rad dye reagent. 0.8ml d.H_2O and 0.2ml dye reagent were vortexed with 5 or 10µl protein sample, giving dilutions of 1:200 and 1:100. Samples were allowed to stand for 5 min before their absorbance was read at 595nM using a Beckman DU-7 spectrophotometer. Blank readings

were obtained using d.H_2O and dye reagent only. A standard curve was constructed with bovine serum albumin and used to estimate protein concentrations.

3.3 Detection of PKC by Western Blot.

Immunoblotting was performed according to the method of Laemmli (1970). Briefly, proteins were resolved according to their molecular weight on 8% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. The membrane then underwent successive incubations in primary (1°), and secondary (2°) antibodies (Abs), a streptavidin-biotinylated alkaline phosphate complex and finally alkaline phosphatase substrates leading to the visualisation of intact α and ß isozymes of PKC.

3.3.1 Preparation of gels.

Gels were prepared one day before sample application, all gel associated equipment had been swabbed with 70% IMS before use. Glass plates (16cm x 16cm) were used to contain the gel, separated by 1mm plastic dividers on the right and left extremes and sealed at the base. Gel casting was conducted at room temperature, the following ingredients comprising the separating gel:

Addition	Final concentration
5ml 1.5M Tris, pH 8.8	0.375M
0.2ml 10% SDS	0.1%
5.336ml acryl/bisacryl stock	8%
0.1ml ammonium persulphate	0.5mg/ml
9.364ml d.H_2O.	

The above ingredients, sufficient for one gel, were mixed gently using a magnetic stirrer. TEMED (7.5µl) was added and allowed to mix before the solution was taken up into a 20ml syringe. With the aid of fine tubing, the gel mix was introduced in between the two plates, and ceased a distance of 12cm from the bottom of the gel plate. D.H_2O (1cm) was introduced above the gel providing protection against oxygen and the gel was allowed to polymerize for 30 min, after which time the overlay was removed and the gel surface dried. The stacking gel, comprising the following solutions, was then poured:

Addition	Final concentration
2.5ml 0.5M Tris, pH 6.8	0.125M
0.1ml 10% SDS	0.1%
1.0ml acryl/bisacryl stock	3%
0.05ml ammonium persulphate	0.5mg/ml
6.35ml d.H_2O	

The ingredients were mixed, 5µl TEMED added and the stacking gel solution was poured above the separating gel. A comb was inserted at an angle to the gel solution for the purpose of channel formation within the stacking gel. After ensuring that air bubbles were not trapped beneath the comb, d.H_2O was layered over the mixture and the gel allowed to set. After 30 min, polymerization was complete, the comb was removed, d.H_2O was removed from the channels and replaced with 0.125M Tris, pH 6.8 containing 0.1% SDS.

The gel was then covered with parafilm overnight to prevent evaporation.

3.3.2 Sample preparation.

Intact PKC within material from whole cell homogenates or cytosolic and particulate fractions can be detected by this technique.

Cells were trypsinized, pelleted and washed in 0.5M Tris, pH 6.8. After being pelleted once more, cells were resuspended in sample buffer diluted 1:1 with d.H_2O at a density of 8 x 10^6/ml. Samples were sonicated (3 x 10s bursts, setting 26) and boiled for 5 min before loading onto gel. Alternatively, samples were stored at -20°C for no longer than two weeks.

Cytosolic and particulate material from 1.6 x 10^7 cells was prepared as described in section 3.4.2 in 1 ml aqueous extraction buffer. An equal volume of sample buffer was added to the samples which were briefly sonicated (3 x 5s bursts, setting 26), then boiled for 5 min before being stored at -20°C.

Adjacent to the cell samples, a positive control of mouse IgG (1:100 dilution) was run. The diluent was the 1:1 diluted sample buffer containing 1% ß-mercaptoethanol. The positive control was boiled for 2 min before loading onto gel. Rainbow Markers of molecular weight range between 14300 and 200000 provided a specific indication of electrophoretic progress. After being boiled for 1 min, 50µl diluted rainbow marker solution (1:10 dilution in 1:1 sample buffer) was loaded onto the gel.

3.3.3 Electrophoresis.

Emptied wells were filled with precooled running buffer prior to loading the samples. Following brief centrifugation to pellet the debris, 50µl of sample containing material from 4 x 10^5 cells, was loaded into each well. Gel plates were introduced into a tank containing running buffer, at an angle, care being taken to ensure that no air bubbles were trapped at the base of the plates. Running buffer was introduced into the upper reservoir, sufficient to cover the electrode. Electrophoresis was performed using an LKB macrodrive power supply, the current was set to remain constant at 25mA, with maximum voltage of 500V. Temperature was maintained at 10°C by an LKB multitemp II thermostatic water circulator and gel runs were completed after 3-4 h.

3.3.4 Transfer of proteins onto nitrocellulose.

Transfer of proteins from polyacrylamide gels to immobilizing membranes was performed electrophoretically using an LKB Novablot kit with Multiphore II base and safety lid. Construction of trans units enabled simultaneous transfer from more than one gel. To ensure that the current passed only through the gel, filter papers, immobilizing and dialysis membranes were cut to the same size as the gel. The anode plate was soaked with d.H_2O and 9 filter papers presoaked in electrode solution were placed onto the anode electrode. The nitrocellulose, soaked in the same solution was placed on top of the papers, beneath the gel. Any trapped air bubbles between nitrocellulose and gel were expelled with the aid of electrode solution. Three more filter papers were immersed in electrode solution and placed above the gel, completing the trans unit. When more than one gel was to be transferred, a dialysis membrane, rinsed in d.H_2O was inserted at this point, before the continued assembly of another trans unit. 6 filter papers, presoaked in electrode buffer

were placed above the units before the cathode electrode plate, saturated with d.H_2O was placed on top of the trans unit stack. Current was set constant at 0.8 mA/cm^2 throughout transfer which was completed in 1h.

3.3.5 Immunodetection.

The nitrocellulose membrane was blocked by overnight immersion, at 4°C in a solution of TBS-T (0.1%) containing 5% dried milk. All further stages were performed at room temperature under constant shaking.The membrane was further blocked for 1h in a solution of TBS-T (0.5%) containing 5% dried milk before being briefly rinsed twice in TBS-T (0.3%) and washed 3 times in TBS-T (0.3%) for 5 min each wash. The nitrocellulose was then incubated for 1h in 1° Ab solution; either the mouse anti PKC Ab, recognizing the intact cleavage region of the α and ß isoforms or the α or ß isozyme-specific Abs. The final dilution was 1:500 in TBS-T. Subsequent incubations, for 20 min each were conducted with: i) the 2° Ab, diluted 1:500. The affinity-purified biotinylated Ab was prepared from polyclonal antisera and thus binds with high affinity and specificity to mouse 1° Abs. ii) the streptavidin-biotinylated alkaline phosphatase preformed complex, diluted 1:3000. Dilutions during these initial detection stages were in TBS-T (0.1%) containing 1% dried milk. iii) NBT plus BCIP, one drop of each in diethanolamine buffer.

Between each incubation, the membrane was washed in TBS-T as detailed previously. Following the final incubation with alkaline phosphatase substrates, during which time, visualisation of PKC occurs, the membrane was rinsed twice then washed 3 times (5 min each) in d.H_2O and dried between filter paper sheets.

3.4 Mixed micellar assay for phorbol ester binding.

Phorbol ester binding to specific receptors within crude cytosolic preparations or to pure PKC was assayed by separating receptor bound [^{3}H]PDBu in the presence of Triton X-100/phospholipid mixed micelles from free [^{3}H]PDBu by molecular sieve chromatography (Hannun and Bell, 1987).

3.4.1 Column preparation.

Silanized` glass Pasteur pipettes were packed with equal volumes of Ultrogel AcA 202 (approximately 2ml) and washed with 20mM Tris-HCl, pH7.5 containing 200µM $CaCl_2$ and 0.015% (w/v) Triton X-100 (buffer A). When not in use, columns were stored in 20mM Tris-HCl, pH7.5 containing 0.02% sodium azide (buffer B), at 4°C for no longer than 2 months.

3.4.2 Preparation of the mixed micelles.

Phosphatidylserine (PS) in 95% chloroform/5% methanol, calculated to give a final micellar concentration of 20 mol% as a mole fraction of Triton X-100, where 1 mol% PS is equivalent to 43µM, was transferred to a glass centrifuge tube. Under circumstances where competition for the phorbol ester binding site of PKC was being investigated, the compound of interest, dissolved in chloroform,was also introduced into the glass tube. The solvent was evaporated under a stream of nitrogen and the PS/compound combination solubilized in 3% (w/v) Triton X-100 by vortexing vigorously for 1 min and incubating

for 10 min at 27°C.

3.4.3 Assay procedure.

For one assay, for the determination of total binding, the following reagents were mixed: 5µl (3% (w/v) Triton X-100 plus 20 mol% PS), 5µl 200mM Tris-HCl, pH 7.5, 5µl $CaCl_2$ and 25µl 100nM [^{3}H]PDBu. The final concentrations of assay components were: 20mM Tris-HCl, pH 7.5, 0.3% Triton X-100, 50nM [^{3}H]PDBu and 200µM $CaCl_2$ (the concentration of free Ca^{2+} in the assay was determined by the amount of EGTA in the protein sample),. For the determination of non specific binding, cold PDBu (final concentration 50µM) was added to the mixture. The addition of 10µl protein sample initiated the binding reaction, which was carried out at room temperature. Steady state binding was reached in less than 1 min, but a period of 10 min was allowed for equilibration, whereupon samples were transferred to ice. All further procedures were conducted at 4°C.

The protein sample was either a crude cytosolic fraction prepared from A549 cells as described in section 3.4.2 or purified bovine brain PKC.

The reaction mixture (50µl) was applied to a Pasteur containing 2ml AcA 202 gel previously equilibrated with 3ml buffer A, and washed through the column with 0.95ml buffer A. The extruded 1ml fraction containing PKC/receptors, mixed micelles and bound [^{3}H]PDBu was designated as the total binding fraction and collected directly into scintillation vials. Non specific binding was determined by collecting the first 1ml fraction when the binding reaction was performed in the presence of 50µM unlabelled PDBu. This value was subtracted from the total binding to give specific phorbol ester binding.

The gel was then washed with a further 2ml buffer A and the eluate collected in different scintillation vials. This fraction was designated as the free [^{3}H]PDBu fraction. Scintillation fluid (20ml) was added to each vial and radioactivity was counted using a Packard Tricarb CA2000 scintillation counter.

Gels were washed by eluting the columns with 20ml buffer A at the end of each separation, they were then stored in buffer B until further use.

3.5 Assay of phorbol ester binding in cell monolayers.

The procedure is adapted from the method of Gescher and Reed (1985). Cells ($5x10^5$) were seeded into 35mm six well multidishes in 3ml media and allowed to attach to the plastic for a minimum of 4h before being treated with compound in DMSO vehicle. Control wells received DMSO alone. Cells were incubated for 24h at 37°C in a humidified atmosphere of 95% air, 5% CO_2. At the time of assay, cells were confluent. Medium was aspirated and cells quickly washed 3-6 times with medium supplemented with either 1% FCS or 0.2% US. For the determination of total binding, wells (in quadruplicate) received 0.5ml medium again supplemented with either 1% FCS or 0.2% US containing 50nM [^{3}H]PDBu. Non specific binding was determined by carrying out the binding reaction in duplicate in the presence of 50µM unlabelled PDBu. Compounds, whose affinity to the phorbol ester receptor was to be investigated,were added to the incubate in DMSO vehicle (DMSO concentration was 0.5%). Cells were incubated for 30 min at 37°C, after which

time medium was aspirated and cultures washed six times with ice cold PBS, pH 7.4. Cells were solubilized in two 0.5ml aliquots of 0.1M NaOH and added to 10ml Optiphase MP scintillation fluid. Radioactivity was counted in a Packard Tricarb CA2000 scintillation counter. Specific binding was calculated as the difference between cell associated radioactivity in the presence and absence of unlabelled PDBu. Concurrent cell counts were performed, when required, such that results could be expressed as % control binding/10^5 cells.

3.6 Assay of phorbol ester binding in cell suspensions.

Cells were trypsinized as described in section 3.1.2 and pelleted by centrifugation at 1000 rpm for 5 min. Cells were washed 3 times in medium supplemented with 1% FCS or 0.2% US before being resuspended at a density of 10^6/ml in the above medium. Determination of total binding was performed in quadruplicate in the presence of 50nM [^{3}H]PDBu. For the determination of non specific binding, which was conducted in duplicate for each experimental condition, medium contained 50nM [^{3}H]PDBu plus 50μM unlabelled PDBu. Incubations were carried out at 37°C for 30 min under constant shaking, in the absence or presence of TPA, dissolved in DMSO, such that vehicle concentration did not exceed 0.5%.

At the end of this period, Eppendorf tubes, within which the binding reaction had occurred were placed on ice and all further procedures were carried out at 4°C. Samples were pelleted using a Heraeus microfuge, medium was carefully aspirated and cells were washed 4 times in ice cold PBS, pH 7.4. Cells were finally microfuged and the pellets transferred individually to scintillation vials whereupon 10ml Optiphase MP scintillation fluid was added. Sample radioactivity was counted in a Packard Tricarb CA2000 scintillation counter. Specific binding was calculated as the difference between total binding, and non specific binding.

3.7 Multiparameter flow cytometry (FCM).

The fluorescence activated cell sorter (FACS) is able to detect particle information based on fluorescence and the degree of light scattered. The suspended stream of cells are excited individually during their passage through a focused high power argon laser beam (excitation 488nM) and the fluorescence emitted is collected through selective filters to 2 photomultiplier tubes and quantified to allow measurement of dye bound to each cell.

3.7.1 Cell cycle analysis.

Between 5 x 10^5 and 10^6 cells were seeded into 50cm^2 flasks and allowed a minimum of 4h to attach to the plastic. Following the required treatment, in triplicate, cells were trypsinized and washed 3 times in ice cold PBS (pH 7.4). The pelleted cells were fixed in 70% ethanol at 4°C under constant agitation, to prevent cell clumping. In this fixed state cells could be stored at 4°C for several days. Immediately prior to flow cytometric analysis, the fixative was removed and the cells washed in PBS (pH 7.4) before being resuspended at a concentration of 10^6/ml in PI/ RNA-ase solution. Analysis of 20 000 events per sample was performed on a Beckton Dickson FACS 440.

3.7.2 Analysis of phorbol ester binding using Bodipy-3-propionyl-13-acetate (Bod-3-PE).

Cells in 250ml flasks, 75-90% confluent were trypsinized, pelleted and washed in ice cold media. After centrifugation once more, cells were suspended in media supplemented with either 1% FCS or 0.2% US at a density of 10^6/ml and held on ice. Cells having undergone treatment with TPA were seeded into 50ml flasks and allowed 4h to attach before the phorbol ester was introduced into the flask. Following trypsinization and washing these cells were also suspended at 10^6/ml in the above mentioned media and held on ice.

Aliquots, 1ml per sample were incubated at 37°C for 10 min in the presence of 10nM Bod-3-PE. Incubations were performed in triplicate for each experimental condition. Unlabelled PDBu (5μM) was included in duplicate incubates to determine non specific binding. In competitive binding studies, cells were coincubated with increasing concentrations of PKC activators (final concentration range 10^{-10} - 10^{-7}M). Propidium iodide (PI) (32μM final concentration) was added immediately prior to analysis allowing exclusion of non-viable cells. 10 000 cells were analysed per sample with respect to 90° scatter (a measure of particle size), red fluorescence (PI > 630nM) and green fluorescence (Bod-3-PE: 515-560 nM). Specific binding was calculated as the difference in green fluorescence in the presence and absence of PDBu and represented as % of control.

3.8 *In vivo* studies.

In vivo investigations were performed in collaboration with Dr. Simon Langdon, ICRF laboratories, Edinburgh. Early passage A549 cells were resurrected from the cell bank and subcultured in Hams F12 media supplemented with 10% FCS, glutamine and antibiotics for 2 passages before being transferred to the ICRF laboratories and injected into mice. Thereon, A549 tumour xenografts began routinely to be passaged. Tumour portions of approximately $1mm^3$ were transplanted subcutaneously into the flanks of athymic mice. Palpable tumours formed in 70-80% of animals. Experiments were performed following an incubation period of 6-8 weeks when tumour size was approximately $64mm^3$. In each protocol, in a single experiment there were 7 tumours in 5 animals, per group. Each animal was implanted with a tumour in each flank, but not all tumours were used as some were too small.

Mice were injected intraperitoneally (i.p.) with PDBu or bryo 1 in vehicle containing 10% DMSO and 90% arachis oil to give less than 20μl DMSO per mouse and final concentrations of 10μg/kg or 50μg/kg. Control animals received vehicle alone. Three injections were administered allowing a 48h interval between each injection. To assess the rate of tumour growth, mice were sacrificed 6, 8, 11, 20, 25 and 28 days after the first injection and tumour diameter was measured with calipers and the volume evaluated according to the formula 0.5 x length x $width^2$. Alternatively, mice were sacrificed 72h after the final injection and the following tissues: tumour, lung, brain, and liver were excised quickly from the body and placed into 2ml homogenization buffer (H8) on ice. Tissues were homogenized and dispatched on cardice to the Aston laboratories. In a second protocol, animals were sacrificed 6h after receiving a single i.p. injection of PDBu. Tissues were excised and homogenized as above. Following transportation to Aston, tissues were

stored at -70°C. Mixed micelle assays were performed on these isolated tissues following the preparation of crude cytosolic extracts (as described in section 3.4.2). Concurrent assays were conducted to determine total protein content, and cytosolic phorbol ester binding was represented as pMoles PDBu bound per mg protein.

4.1 Examination of the antiproliferative properties of biologically active tumour promoting phorbol esters in A549 human lung carcinoma cells.

4.1.1 Investigation of the cytostatic effect of TPA on cells maintained in medium supplemented with 10% FCS.

Introduction.

It has been established that TPA arrests the growth of the A549 human lung carcinoma cell line at nM concentrations for 5-6 days (Gescher and Reed, 1985). Treated cells undergo dramatic morphological alteration, normally epithelioid in form, they become highly condensed, rounded and protruding. After this period, in the continued presence of TPA, cultures resume growth at a rate similar to that of control cells and normal morphology is regained. It has been established that the ability to overcome growth inhibition is a property of all cells and not due to the emergence of TPA-resistant clones (Dale, 1989). Reseeding treated cultures appears to render the population once again sensitive to TPA. However, from cultures continually subcultured in the presence of 10nM TPA, gradually arise cells which become refractory to the growth inhibitory properties of TPA. Such acquired phorbol ester resistance is reversible, as its removal from culture conditions leads, in 14 days, to a TPA-sensitive population (Dale, 1989). Experiments described in this section were designed to investigate further the antiproliferative properties of tumour promoting phorbol esters in human A549 lung carcinoma cells.

Results and discussion.

We wished to determine whether the presence of phorbol ester was required throughout the 6 day period to maintain arrest of cell growth. To this end, cells were treated with 100nM PDBu. The use of PDBu was adopted because of its decreased lipophilicity compared with TPA (Blumberg, 1988). Following 24h incubation, one group of cells was washed free of phorbol ester. Their growth was monitored and compared with the growth of untreated cells and cells in the continued presence of PDBu. Figure 4 demonstrates recovery of proliferative capacity upon removal of phorbol ester during the growth arresting phase, such that by day 3, the rate of cell growth has returned to the rate of growth of untreated cells. Such reversibility of the phorbol ester induced response was not observed in HL-60 cells. These cells became committed to terminal growth arrest and cellular differentiation following exposure to TPA (Fibach *et al.*, 1982).

As noted, when cells, which have regained proliferative capacity following the period of growth arrest, are reseeded into fresh culture plates they are rendered once again sensitive to growth inhibition by TPA. Any cellular mechanism conferring resistance is reversed.

The following experiment was designed to test the hypothesis that reacquired sensitivity to phorbol esters is a consequence of cellular exposure to trypsin. Certain proteins are digested by trypsin, for example components of the basement membrane (Kolega and Manabe, 1990). It is conceivable that during trypsinization of cells, digestion of certain

membrane bound proteins occurs which may be responsible for the mediation of resistance to phorbol esters. Cells were seeded into 140mm diameter petri dishes. One group were treated with 10nM TPA for 6 days. On the seventh day of exposure, cells were either scraped or trypsinized and reseeded in order to examine their sensitivity to TPA. To this end, growth curves, in the presence or absence of 10nM TPA, were set up. Additionally, cells were reseeded in order to conduct [^{3}H]TdR incorporation assays following exposure to 10nM TPA for 24 and 48h. It was speculated that TPA-treated, scraped cells, released from growth inhibition should not display such sensitivity to TPA upon re-exposure. However, the results appear to indicate that cell scraping introduced such a degree of stress, that cells failed to re-attach to plastic. Cellular debris was observed in the wells, hence the assay to assess DNA synthesis could not be conducted. The formation of colonies, in the absence of TPA only, could be detected following 14 days culture of scraped cells; indicating that few cells remained intact with subsequent colony forming efficiency.These cells had been scraped and reseeded to monitor daily growth. Prior to cell scraping, the growth of one group had been arrested by 10nM TPA. Negligible colonies had arisen in all wells containing TPA (figure 5). No apparent difference in sensitivity to TPA could be detected between cells previously exposed to TPA for 6 days and untreated cells. These experiments were unable to establish firmly whether untrypsinized cells maintained any resistance to TPA. The scraping procedure was traumatic to the cells and cytotoxic. The introduction of TPA at nM concentrations to so few viable cells may provide added cytotoxic stress, prevent cellular attachment and render these cells uncharacteristically responsive to TPA.

It was possible to compare directly the growth of untreated cells and cells pre-exposed to 10nM TPA for 6 days prior to trypsinization. Comparable growth curves were obtained (figure 6a). A slight lag in the growth rate was observed in pretreated cultures when compared with previously untreated cells. The introduction of 10nM TPA resulted in similar growth arrest within both populations at low cell densities. Assays conducted to measure DNA synthesis demonstrated a high degree of sensitivity to TPA (figure 6b), irrespective of the cells` pre-exposure to TPA. At higher cell densities, pre-treated cells displayed slightly more rapid recovery from growth arrest, possibly indicative of the cells` ability to switch to a more `resistant` phenotype. This may be a consequence of cellular secretion of a factor, stimulated in the presence of TPA, able to confer resistance to TPA.

Evidence indicates that many tumour types are capable of synthesizing and secreting growth factors which may be important for tumour growth *in vivo* (Anzano *et al.*, 1989; Partridge *et al.*, 1989). A549 cells have been found to secrete factors *in vitro* with the properties of both transforming growth factor-α (TGF-α) and insulin-like growth factor-1 (IGF-1) (Siegfried 1989). Moreover, medium conditioned by this cell line was able to stimulate the growth of both monolayer colony formation and growth in semi-solid medium of cells cultured from primary solid tumours. An exogenously added monoclonal antibody (Ab) against recombinant human TGF-α has been reported to evoke A549 cell growth inhibition indicating that TGF-α may function as an autocrine growth factor in this cell line

(Imanishi *et al.*, 1989). TGF-ß, discussed in detail in section 4.1.5.1, is also secreted by A549 cells, in a latent form (Wakefield *et al.*, 1987). TGF-ß is inhibitory with respect to cell growth (Roberts *et al.*, 1985).

We wished to investigate the hypothesis that cells secrete a factor or factors which may act as a positive stimulus for growth in the presence of TPA. Medium was conditioned over confluent A549 cultures for 72h, briefly centrifuged, filtered and supplemented at a ratio of 60:40 with fresh, serum-supplemented medium. The conditioned medium was introduced to untreated A549 cells and their sensitivity to TPA was examined. Figure 7a) and b) demonstrate clearly that factors present in the conditioned medium were not sufficient to maintain A549 growth in the presence of 10nM TPA.

Medium fortified with 10nM TPA was conditioned for 72h over cells no longer sensitive to growth inhibitory properties of TPA. This was briefly centrifuged, filtered and supplemented with fresh media. The ability of this medium to arrest the growth of naïve A549 cells is demonstrated in figure 7c). It was thought that in the presence of TPA, cells may secrete (a) factor(s) responsible, in part for resumption of growth. Control cultures comprised of either cells exposed to fresh medium in the presence and absence of 6nM TPA, or cells grown in medium conditioned over naïve A549 cells for 72h and supplemented with fresh medium, in order to observe the extent of nutrient depletion on proliferative potential. The incorporation of [^{3}H]TdR was dramatically reduced in naïve cells subjected to the conditioned medium (figure 7c). It may be concluded that insensitivity to growth arrest induced by TPA is not conferred by factors released by proliferating cells in the presence of TPA.

This data combined indicates that the cells` ability to grow in the presence of TPA does not result from loss of TPA activity or the accumulation of a pro-proliferative factor secreted into the medium. The ability to overcome growth arrest is a property each cell itself is able to acquire after a fixed exposure time to TPA even following the addition of fresh medium and TPA; but lost however following the disruption of culture conditions.

In contrast, Aguanno *et al.* (1990), have provided evidence to indicate that the growth arrest of a human rhabdosarcoma cell line, in the presence of TPA, occurs as a direct consequence of factors secreted by treated cells (discussed in section 4.1.5).

Section 4.1.2 Derivation of a serum-free environment for cell culture.

Introduction.

Foetal serum contains many substances which would never come into contact with most cell types *in vivo*. Components of FCS are essential for the developing embryo whose aggressive rate of growth resembles that of malignant neoplasia. Thus it can be appreciated why FCS is a favoured medium to support the growth of immortal cells in culture. Serum provides cells with nutrients, such as amino acids and growth factors, some of which are capable of initiating the transduction of cellular signals which ultimately lead to processes such as cellular differentiation and proliferation. Hence, its mere presence in growth medium *in vitro* may modify the phenotypic behaviour of cells. For example an

Figure 4

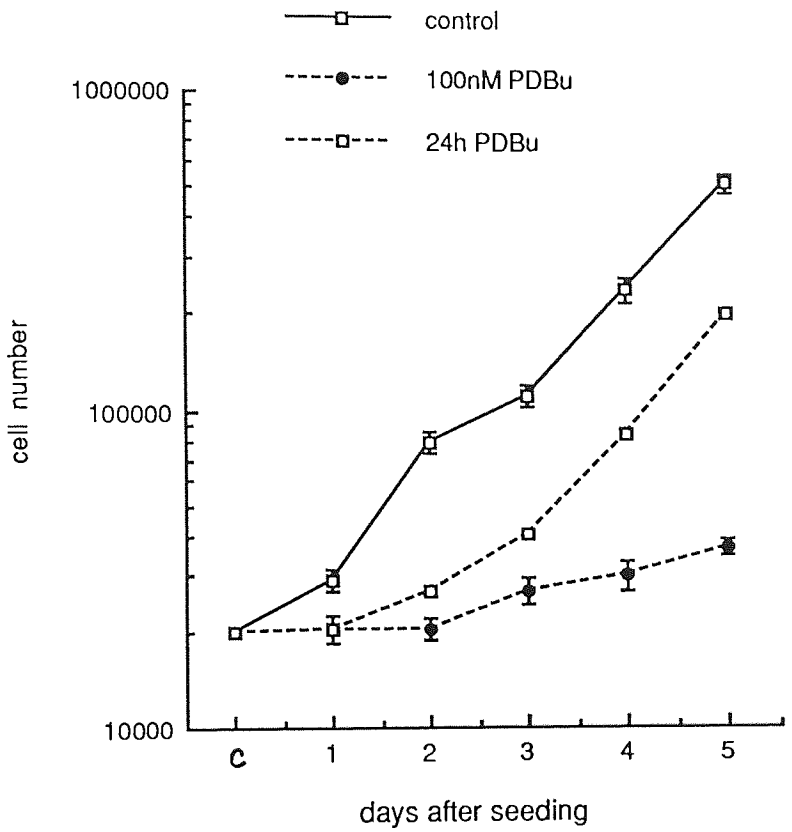


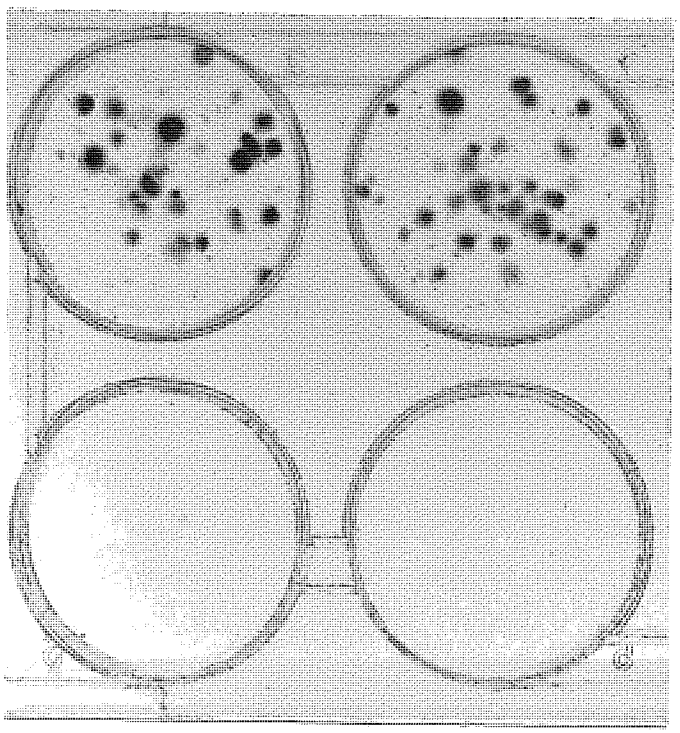
Figure 4. Effect of PDBu on A549 cell growth.
Cells (2×10^4) were seeded and allowed to attach for 4h before treatment with 100nM PDBu commenced.Following 24h exposure, phorbol ester was washed from one group of cells. Growth of a second group was monitored in the continued presence of PDBu. Cell counts were performed at the times indicated (mean $\bar{+}$ SD, n=9).

Figure 5. Effect of TPA on scraped cells pre-treated with TPA.
Cultured cells, i) previously untreated or ii-iv) following prior exposure to 10nM TPA for 6d were scraped and re-seeded at a concentration of 2 x 10^4 per well
a) cells only
b) 10nM TPA included in the incubation. Colonies were fixed and stained in methylene blue following 14d culture.

i

a

b



ii

a

b

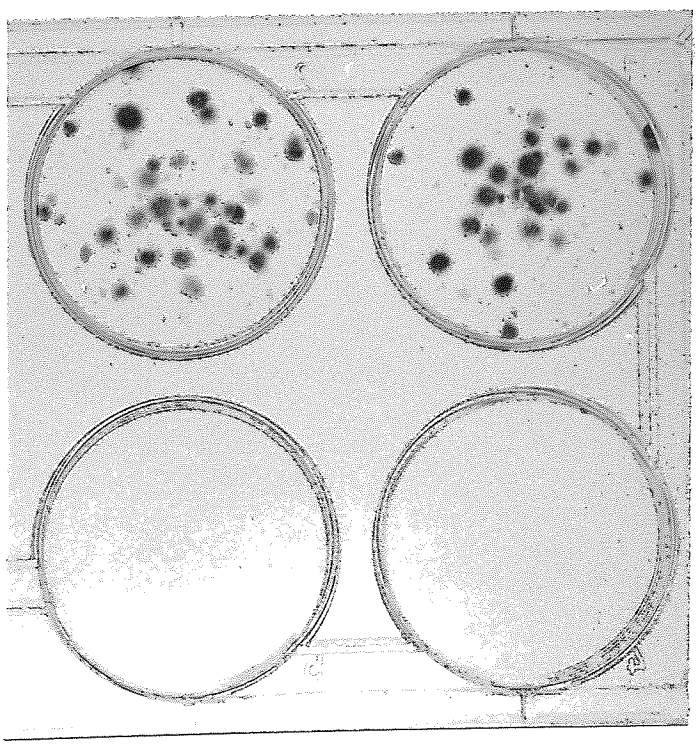
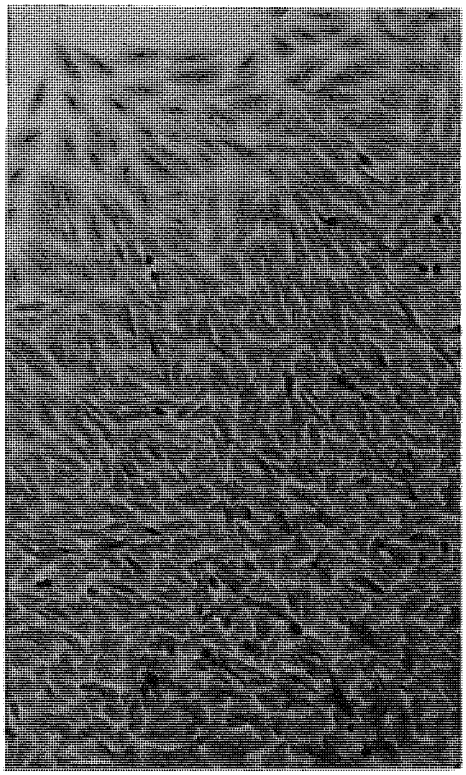


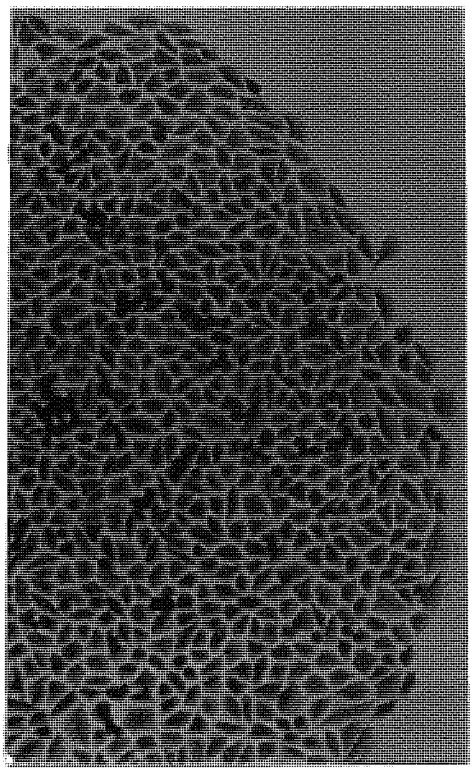
Figure 5 continued.
iii a) and iv a) magnification 10x
iii b) and iv b) magnification 25x.

a

b

iii

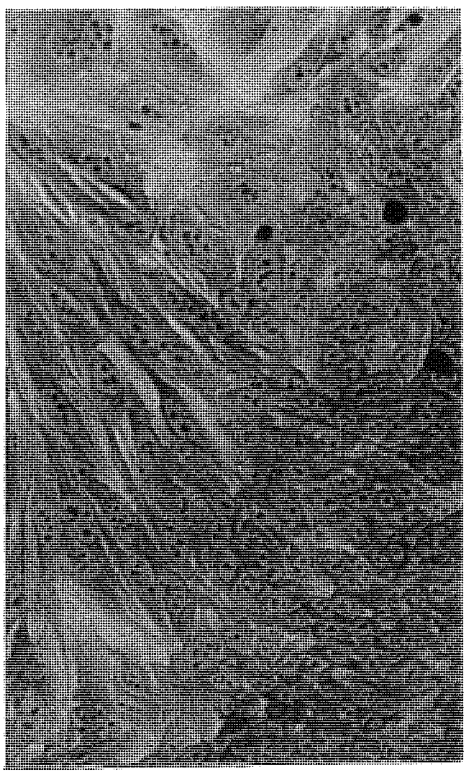




a

b

iv



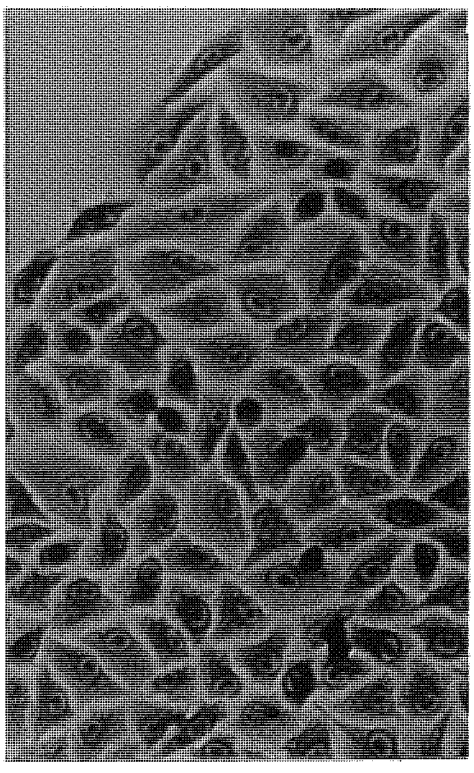
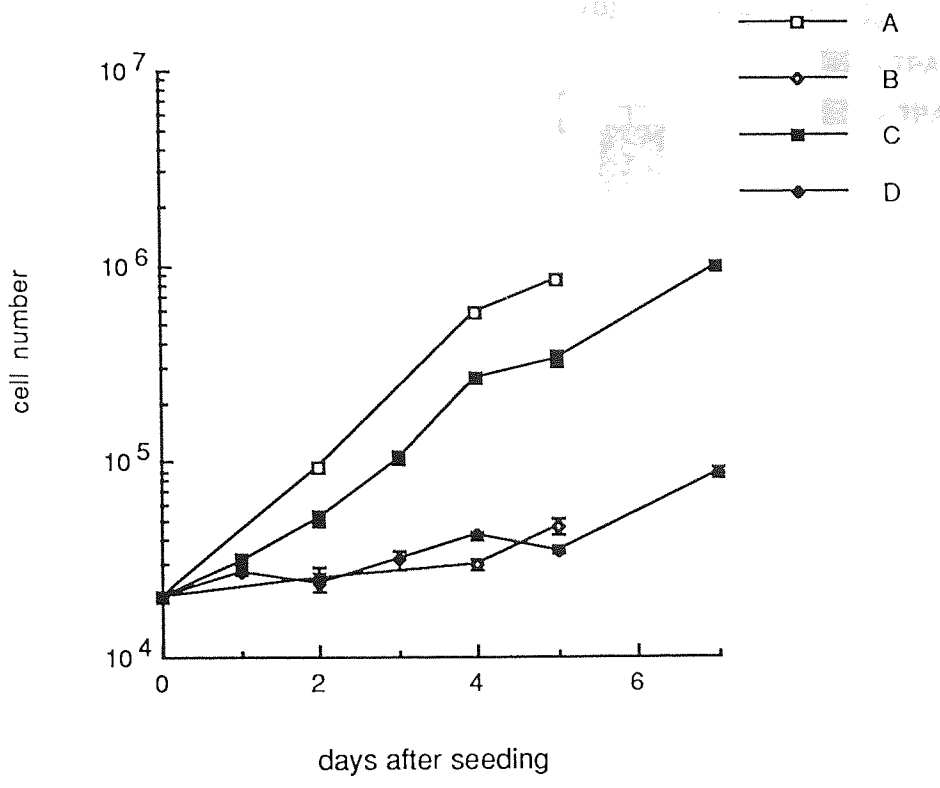


Figure 6a)



6b)

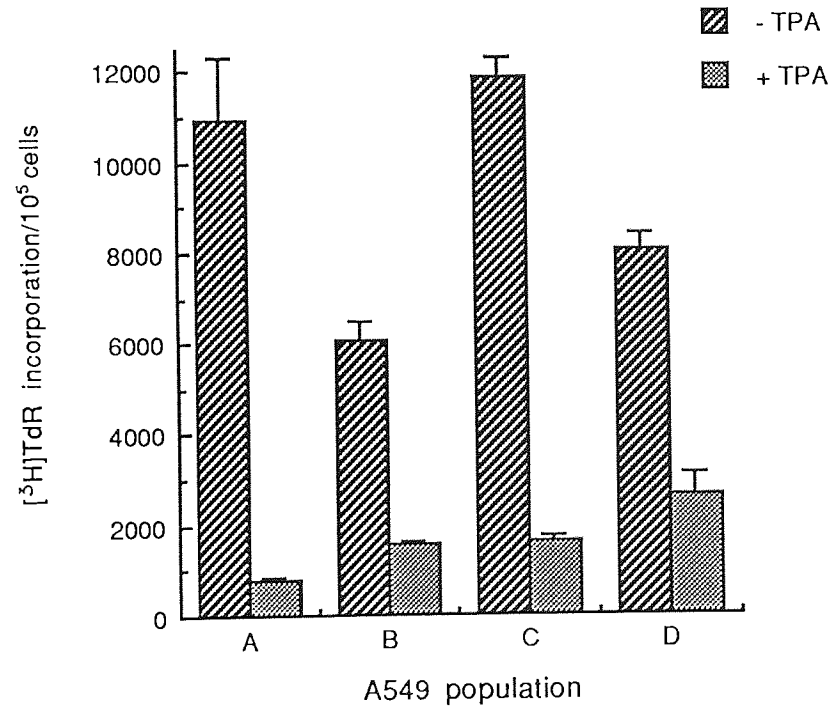


Figure 6. Effect of TPA on trypsinized cells pre-treated with TPA.
a) Cells were treated for 7 days with 10nM TPA, trypsinized and reseeded at a density of 2 x 10^4 per well. Growth was monitored in the absence and presence of 10nM TPA (mean ∓ SD, n=9).
b) Cells were treated for 7 days with 10nM TPA, trypsinized and reseeded at densities of 2 x 10^5 or 10^5 per well.[^{3}H]TdR incorporation was assessed following 24 and 48h in the presence and absence of 10nM TPA (mean ∓ SD, n=9).
Identicle experiments were performed in parallel using presviously untreated cells.

6a) A, B previously untreated cells
C, D cells pre-exposed to TPA
A, C -10nM TPA
B, D +10nM TPA

6b) A, B 24h
C, D 48h
A, C previously untreated cells
B, D cells pre-exposed to TPA

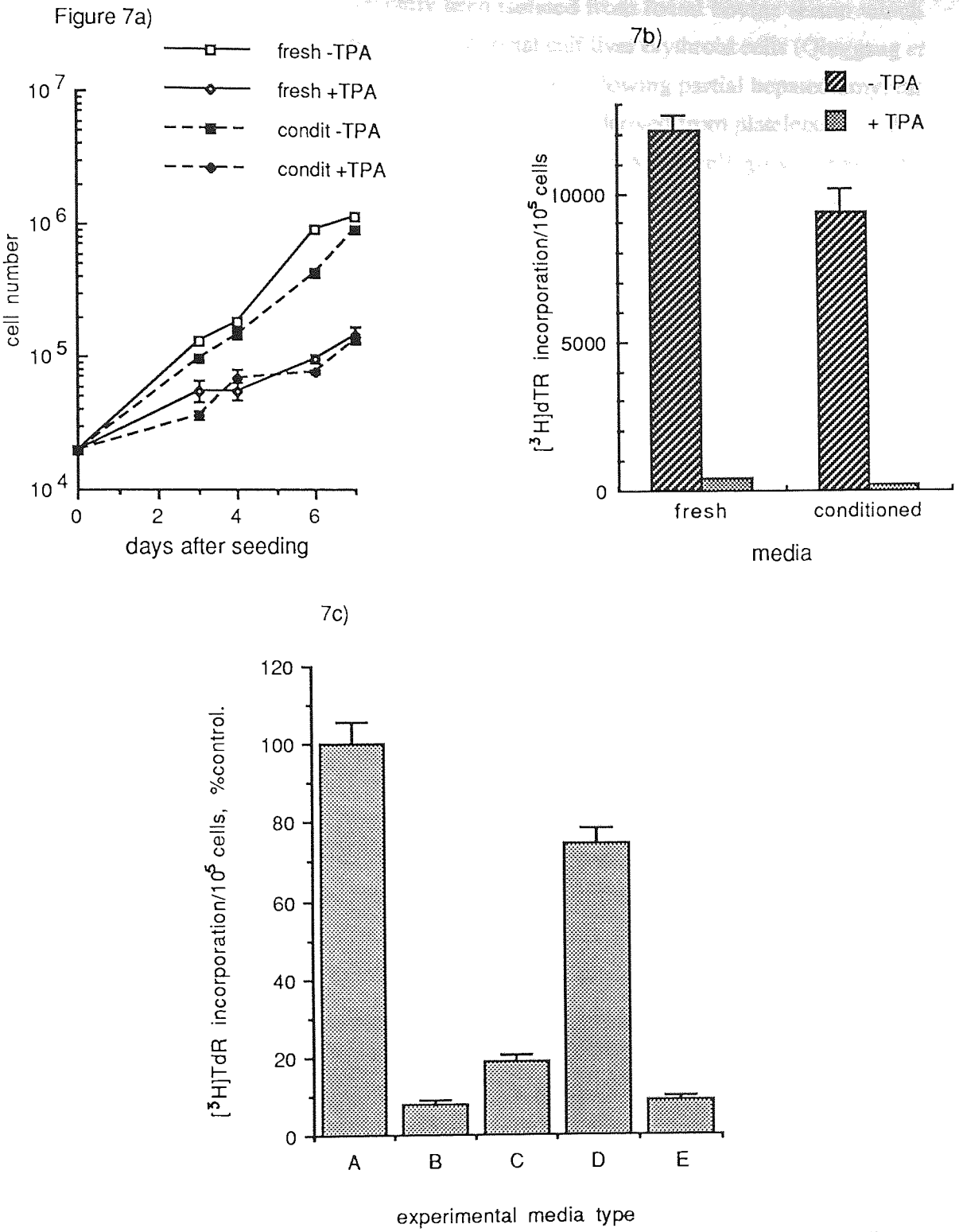


Figure 7. Effect of TPA on the growth of A549 cells cultured in conditioned medium.
a) Cells (2×10^4) were seeded in either medium conditioned for 72h over confluent A549 cultures or fresh medium in the presence and absence of 10nM TPA. Growth was monitored by means of cell counts (mean $\pm$ SD, n=9).
b) Cells (2×10^5) were seeded in either medium conditioned for 72h over confluent A549 cultures or fresh medium in the presence and absence of 10nM TPA. [^{3}H]TdR was assessed following 24h exposure to TPA (mean $\pm$ SD, n=9).
c) Cells (2×10^5) were seeded in A: fresh medium
B: fresh medium plus 6nM TPA
C: medium plus TPA conditioned over proliferating cells.
D: medium only conditioned over proliferating cells.
E: medium conditioned over proliferating cells plus 6nM TPA. [^{3}H]TdR incorporation was assessed following 24h incubation (mean $\pm$ SD, n=6-9).

apolipoprotein-H-like protein has recently been isolated from foetal bovine serum which inhibits thymidine incorporation into cultures of foetal calf liver erythroid cells (Qinggang *et al.*, 1990). Also, Ichihara (1989), has demonstrated that following partial hepatectomy, rat serum contains a growth factor for hepatocytes, apparently derived from platelets.

Thus far, examination of the effects of phorbol esters on A549 cell growth has been conducted in medium supplemented with 10% FCS. The complex and undefined nature of FCS complicates the design of experiments aimed at clarifying the interaction of specific ligands with cellular receptors and interpretation of the ensuing cellular response. The purpose of the experiments described herein was to develop a serum-free, chemically defined medium for continuous culture of human A549 lung carcinoma cells.

Results and discussion.

A549 cells were cultured in two types of serum-free media. Hams F-12 was supplemented with 1% ITS premix, which contains insulin, transferrin and selenium, but not linoleic acid or bovine serum albumin, together with 2mg/ml fetuin, prepared from foetal bovine serum. In a second protocol, serum was replaced by 2% ultroser G (2% US), the contents of which are listed in appendix 1. It is suggested by Gibco that ultroser G should be used at a final concentration of 2%, equivalent to 10% FCS. However, higher or lower concentrations may be used to supplement medium, though the effect on cell growth has been reported as not always proportional to US concentration. A549 cells were therefore cultured in media containing various strengths of US to evaluate optimal growth conditions.

Cell growth was monitored in the chemically defined media, following routine culture for at least 4 passages in media supplemented with the advised concentration of serum substitute. For the duration of the experiments, supplement concentration was varied. The initial seeding density was $2x10^4$ and after a period of 96h cells were counted. Growth curves were constructed and compared to curves obtained with medium supplemented with 10% FCS. The results, illustrated in figure 8a) and b), demonstrate that cell growth proceeds more rapidly in serum-supplemented medium. Growth of cells in the ITS supplement increased proportionally with the amount of fetuin present. ITS alone was unable to support A549 growth, many cells clumped and failed to attach to the plastic substratum. Mean doubling times were crudely calculated as 21.8h, 26.6h and 36.0h for cells grown in media supplemented with 10% FCS (A549-FCS), 1% ITS plus 2mg/ml fetuin and 2% US (A549-US) respectively. Morphologically, cells cultured in medium containing ITS plus fetuin were indistinguishable from serum-supplemented cells. However, A549-US cells underwent significant morphological alterations, demonstrated in figure 12. Cells were perceived to be larger, possibly because of a flatter nature. They appeared more linearly spread-eagled rather than polygonal. They reached confluence at a density which was 64% of the cell number of confluent A549-FCS cells. Judging by cell numbers presented in figure 8b, the growth of A549-US cells appeared to be significantly retarded, when compared directly with A549-FCS cell numbers. However, experiments measuring cellular ability to incorporate labelled thymidine suggested that the rate of growth of A549-US cells was density dependent. A lag phase, during the initial period after seeding, throughout

which time DNA synthesis was minimal is demonstrated in figure 18. When cells reached a density of approximately 1.4 x $10^4/cm^2$, their proliferative capacity increased significantly such that the growth rate (figure 8a) and ability to incorporate [^{3}H]TdR (figure 18) approached levels comparable to cells maintained in 10% FCS. Moreover, density dependence is apparent immediately, following introduction of A549-FCS cells into the US supplemented environment (Figure 23). It may be speculated that the immediate growth retardation is due to the removal of paracrine or endocrine factors present in FCS, yet not present in sufficient quantities in the US serum substitute to maintain an equivalent proliferative capacity. Thus, cells cultured in medium supplemented with 2% US may have to switch to, or rely more heavily upon the autocrine production of factors to maintain growth. This theory is supported by the observation that at higher cell densities DNA synthesis and hence growth rate increased. Indeed, it has been established that A549 cell cultures synthesize and secrete factors possessing properties of both transforming growth factor α (TGF-α) and insulin-like growth factor-1 (IGF-1) (Siegfried 1989). As noted, the addition of these factors to medium stimulated the growth and colony formation of lung tumour cells.

The culture of A549 cells under serum-free conditions has been described previously (Zirvi *et al.*, 1987). Chee`s essential medium (CEM) plus transferrin, insulin, triiodothyronine, EGF and selenium, a cocktail which was able to maintain growth of HT29 colon carcinoma cells, was unable to support the growth of A549 cells. The additional factors fetuin, oleic acid, and linoleic acid included in the medium conferred the ability to maintain continuous cell growth and proliferation. The authors described a slightly increased doubling time by these cells compared to the parent serum-supplemented cells, with comparable densities at confluence.

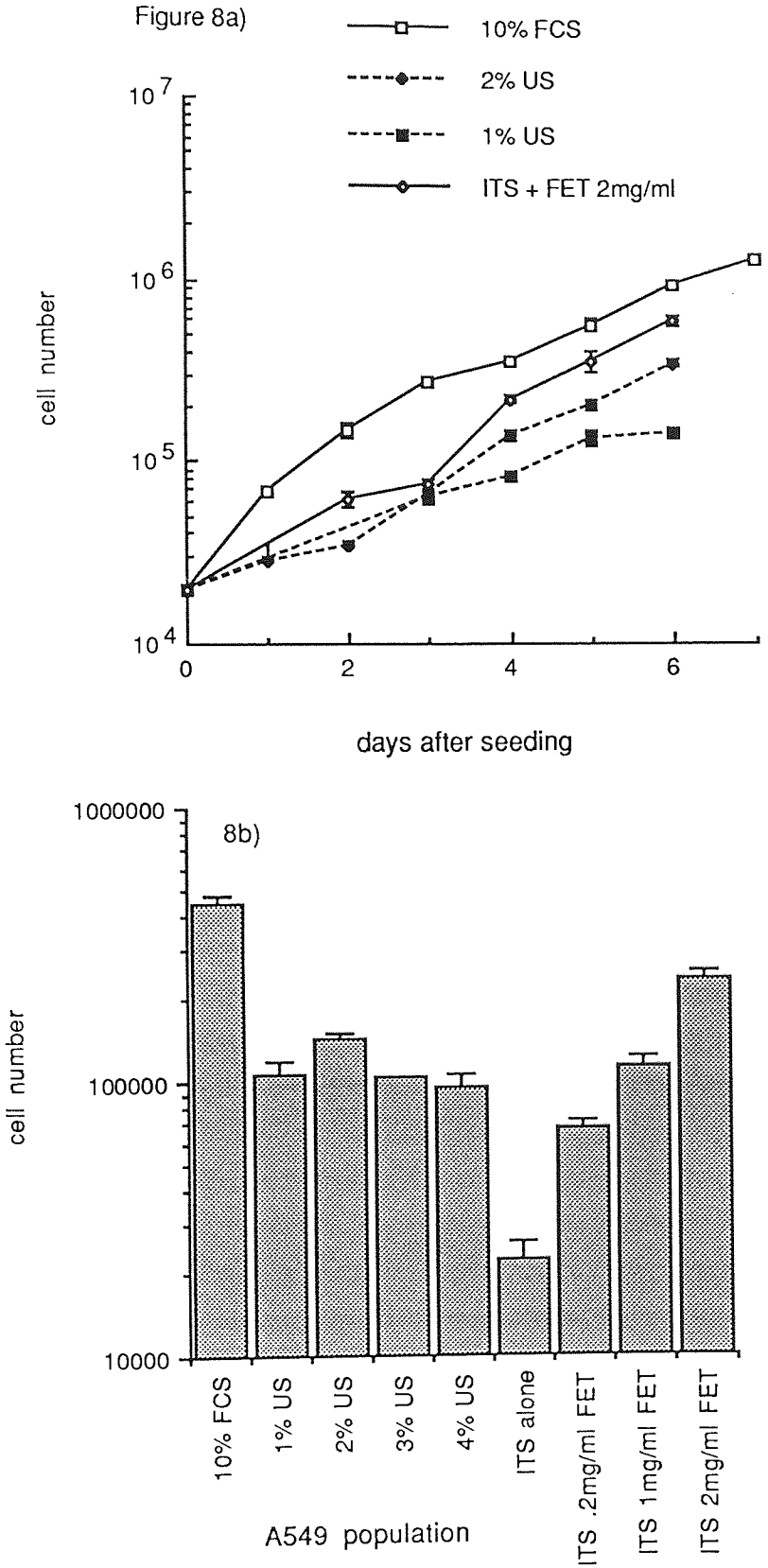
The growth of certain other tumour cell lines under serum-free conditions has been described. Serum-deprived MCF-7 cultures supplemented with insulin only, possessed populations demonstrating a faster rate of growth and a more differentiated phenotype than cells maintained in medium supplemented with 10% FCS (Medrano *et al.*, 1990). Moreover, cells growing in the presence of serum were only loosely attached to the plastic substratum whereas cultures in the chemically defined environment were firmly attached to the plastic and required a mixture of trypsin and collagenase for detachment. These authors conclude that the growth and differentiated properties of tumour cells may depend on their cellular environment.

4.1.3 Investigation of the growth inhibitory properties of phorbol esters in FCS-deprived A549 cultures.

Introduction.

The complex mixture of hormones, growth-promoting and growth-inhibiting factors contained within serum may interact with the receptor-effector system based on phosphoinositide hydrolysis. In this section, the hypothesis under examination was that

Figure 8. A549 cell growth in media supplemented with 1% ITS plus fetuin, US or 10% FCS. a) Cells were seeded at a density of 2 x 10^4 per well. Counts were conducted following the incubation periods indicated (mean ∓ SD, n=9-12).
b) Cells were seeded at a density of 2 x 10^4 per well in the media indicated and counted following an incubation period of 96h (mean ∓ SD, n=9-12).



constituents of serum may affect the cellular response to tumour promoting phorbol esters. Components of serum may induce or influence cellular responses that are carried over a number of generations. Hence, unless specified otherwise, cells were cultured in the chemically defined media for at least 9 weeks before experiments were performed.

Results and discussion.

Figure 9a demonstrates the growth of cells in medium supplemented with 1% ITS premix plus fetuin (2mg/ml). As observed in medium supplemented with 10% FCS, growth of cells and DNA synthetic activity (figure 10) was arrested by 10nM TPA for 5-6 days. They also underwent temporary morphological transformation. Cells took on rounded and protruding characteristics, accompanying growth arrest. On the 6th day, recovery of proliferative potential and more normal morphology could be detected. In stark contrast, the growth of cells in medium supplemented with the serum substitute 2% US did not appear to be arrested by 10nM TPA as assessed by cell counts (figure 9b). An initial lag in cell numbers was observed in the presence of 10nM TPA. However recovery was swift and by the 6th day, cell numbers in the presence of TPA slightly exceeded control cell numbers. That the apparent insensitivity of A549-US cells to the growth inhibitory properties of TPA extended to other tumour promoting phorbol esters has been explored. Growth of A549-US cells was monitored in the presence of 100nM and 1nM PDBu. The results are illustrated in figure 11. It can be concluded that the cells demonstrate a similar resistance to growth inhibition by PDBu. Phorbol esters induced only very short term morphological changes, during the initial period of exposure. Observations performed 48h after initial treatment revealed cell morphologies indistinguishable from control cultures (figure 12).

Such morphological alterations upon exposure to TPA are not peculiar to A549 cells. Nicks *et al.*, (1989) have described the response of 2 cell lines to treatment with TPA. Cells of the non-tumourigenic cell line, derived from normal mouse lung epithelium were contact inhibited. TPA induced these normally flattened cells to become rounded and more highly condensed. The normally bipolar, rounded cells derived from a mouse lung adenoma were caused to flatten out, upon treatment with TPA. A549 cells display certain features characteristic of a differentiated phenotype, they are subject to contact inhibition which appears to be enhanced in A549-US cultures. Moreover, A549 tumour cells are reported to contain multilamellar bodies and synthesize disaturated lecithin utilizing the cytidine diphosphocholine pathway, typical of cells responsible for pulmonary surfactant synthesis. This finding indicates retention of differentiated functions even after approximately 1000 cell generations *in vitro* (Lieber *et al.*, 1976). However, by traditional growth criteria, A549 cells behave as a transformed cell line, displaying the ability to form colonies in soft agar and proliferate on a fibroblast monolayer. Moreover, progressively growing tumours result when A549 cells are injected into athymic mice (section 4.7).

The effects of TPA on colony forming efficiency and growth of normal human bronchial epithelial cells (NHBE) and 5 lung squamous carcinoma cell lines has been compared in medium containing 1% foetal bovine serum (FBS) and in serum-free medium (Sanchez *et al.*, 1987). It was observed in this study that the growth of 2 carcinoma cell lines which was retarded by TPA in the presence of 1% serum was stimulated by TPA in the absence of

serum. In contrast, the absence of FCS was found to be inconsequential for the inhibition of proliferation by TPA in MCF-7 cells (Osborne *et al.*, 1981).

The activation of PKC by TPA does not elevate intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in several cell types (Miyashita *et al.*, 1989); for example during the induction of squamous differentiation of normal and transformed human bronchial epithelial cells by TPA. In contrast, the induction of differentiation in these cells by 8% FCS was accompanied by an elevation of $[Ca^{2+}]_i$. In serum-supplemented A549 cells it was demonstrated by Dale (1989) that exposure to TPA was accompanied by an increased $[Ca^{2+}]_i$ signal. It is possible that in A549 cells, elevation of $[Ca^{2+}]_i$ is an important prerequisite for maintained growth arrest and that the presence of certain serum components are required to elicit this response. It has been hypothesized that (a) serum factor(s), through receptor agonist coupling, may activate PLC, thus generating $I1,4,5P_3$ and DAG; the former triggering a pulse increase of Ca^{2+} by release from intracellular compartments. Consistent with this hypothesis is the observation that exogenously introduced PLC may induce squamous differentiation (Miyashita *et al.*, 1989). Additionally, Peres *et al.*, (1988) have demonstrated the immediate opening of Ca^{2+}-activated channels in quiescent human fibroblasts upon application of FCS. It may be postulated that the induction of the observed cellular response, for example arrest of A549 cell growth for 6 days, is a consequence of a synergistic interaction between activation of PKC by TPA and the elevation of $[Ca^{2+}]_i$ by serum components. Indeed, it has been shown that the calcium ionophore A23187 was able to potentiate monocytic differentiation of HL-60 cells by TPA (Masui *et al.*, 1986).

Arteaga and Osborne (1989) report that the type I somatomedin receptor mediates the mitogenic effects of insulin and the IGFs. A monoclonal antibody to this receptor inhibited the anchorage dependent growth of 6/7 breast cancer lines in the presence of 10% FCS. In contrast, the antibody was unable to inhibit the basal growth of the breast cancer lines under serum-free conditions. Thus it may be suggested that an altered environment may lead to the altered phenotypic behaviour of cells.

Incorporation of $[^3H]$TdR was assessed following 24h exposure of A549-US cells to increasing concentrations of TPA. A biphasic dose response, illustrated in figure 13, was obtained. Maximum inhibition of DNA synthesis after this period of time occurred upon exposure to 1nM TPA. At concentrations exceeding 1nM, the potential to arrest DNA synthesis was diminished. At a concentration of 1μM, TPA evoked approximately 28% decrease in proliferative capacity compared to untreated controls.

Little *et al.* (1985) examined the inhibition of colony formation by TPA in various human cell types. A prostate cancer cell line demonstrated no response to TPA. Increasing inhibition of colony formation corresponding to increased concentrations of TPA (up to 100ng/ml) was observed using a melanoma cell line. Normal fibroblasts displayed a biphasic dose response with maximum inhibition of colony formation following treatment of cells with 1ng/ml TPA. Minimal effects were observed using 100ng/ml, the maximum concentration of TPA examined. The possibility was raised that cells may contain two classes of specific phorbol ester receptors mediating antagonistic responses. However, in

this study, linear Scatchard plots were obtained for PDBu binding in all 3 cell lines, consistent with one major homogeneous class of binding sites. A study of the effect of tumour promoters on human skin fibroblasts from individuals genetically predisposed to cancer with adenomatosis of the colon and rectum (ACR cells) has been performed (Kopelovich and Bias, 1980). Cultured ACR cells displayed a biphasic dose response to TPA. The cloning efficiency was significantly lower at concentrations of TPA 1-2 ng/ml than at high TPA concentrations; 10-500 ng/ml. Following cell growth in agar, the biphasic pattern was still evident, though less pronounced. These authors speculated that TPA may affect at least two distinct processes of cell growth, one of which is inhibitory and can be saturated at relatively low concentrations of TPA, the other which stimulates cell proliferation until concentrations are reached that evoke cell kill. This hypothesis is consistent with the observations demonstrated in figure 13. Interestingly, members of another class of potent PKC activators, the bryostatins (bryos), induced a biphasic cytostatic effect on A549 cells, hence this hypothesis may be extended to encompass the bryo mode of action (Dale and Gescher, 1989; discussed in detail in section 4.6). The hypothesis, however is inconsistent with Scatchard analysis of receptor binding; which implies a homogeneous phorbol ester receptor. It is now known that PKC comprises a family of at least 7 isozymes differentially expressed to unique permutations and combinations in each cell type. It may be postulated therefore that the cellular growth response obtained may be a consequence of a combination of the following two factors: cell type-specific isozyme content and different affinities between isozyme and activator. It should be noted however, that recent evidence favours the existence of another cytosolic protein specifically recognizing TPA, which upon interaction translocates to the nuclear envelope (Hashimoto and Shudo, 1990). Indeed, one of the hypotheses forwarded to explain the biphasic nature of bryo-induced growth arrest is the presence of a secondary receptor site recognizing phorbol ester with low affinity (Dell`Aquila *et al.*, 1988). Interaction at this secondary site, according to the hypothesis mediates the pro-replicative response. Factors present in serum may potentiate the growth-inhibitory response by inhibiting interaction at this secondary site, thus reducing its sensitivity to phorbol ester. It has been demonstrated by Dale (1989) that following 24h exposure to 1μM TPA, serum-supplemented cells are able to incorporate more [^{3}H]TdR than cells exposed to 10nM TPA. One may speculate that non-specific cytotoxicity at concentrations above 2μM TPA prevents the detection of the specific pro-replicative response in A549-FCS cells.

Growth curves performed using A549-US cells revealed negligible inhibition of cell growth in the presence of 1nM and 100nM PDBu. Assays were conducted to determine the response of cells to increasing concentrations of PDBu for 24h. Biphasic dose response curves following treatment of A549-US and A549-FCS cells were not evident (figure 14). Inhibition of [^{3}H]TdR incorporation increased proportionally with PDBu concentration. Pro-replicative responses were not detected in either cell population at the time point examined. Consistent with reports that PDBu is a weaker tumour promoter than TPA, the concentration of PDBu calculated to inhibit 50% [^{3}H]TdR incorporation in A549-FCS cells following 24h exposure 18.9nM, compared with 0.2nM TPA (Dale, 1989). It could be

speculated therefore that the saturation or occupation of specific receptors, mediating inhibitory responses or triggering a pro-replicative response may require higher concentrations of PDBu. An extended incubation period may have revealed a possible biphasic nature in the response of serum-deprived cells to PDBu, this has not been examined.

Cell counts were performed following 96h exposure of A549-US cells to increasing concentrations of TPA. A weak biphasic response was obtained. The Maximum cytostatic effect occurred in incubates containing 1nM TPA. (figure 15). Subsequent examination of the growth of A549-US cells incubated with 1nM TPA revealed an ability to retard growth to a greater degree than 10nM TPA (figure 16).

The ability of A549-US cells to incorporate [^{3}H]TdR was monitored over time, in the presence of various concentrations of TPA. The results are illustrated in figure 17. Each concentration of TPA tested induced a very transient, but potent arrest in DNA synthesis. The onset of inhibition of labelled thymidine uptake was immediate and maximal following incubation periods of between 12 and 22h with each concentration of TPA (1, 10, 100nM and 1μM). It is noteworthy that the higher concentrations of TPA were able to evoke a proreplicative response more rapidly than lower TPA concentrations, this is particularly evident upon consideration of [^{3}H]TdR incorporation following 30h exposure. Recovery from growth arrest was complete, indeed, after 48h exposure proliferative capacity exceeded that of control cultures. The transient nature of growth arrest induced by TPA in A549-US populations is also demonstrated in figure 18 and contrasted with the DNA synthetic potential of A549-FCS cells treated with 10nM TPA for 24, 72 and 144h.

An alternative argument may be considered, consistent with the theory of Miyashita *et al.* (1989) discussed above. The maintenance of TPA-induced cell growth inhibition in A549-FCS cell populations may be Ca^{2+} dependent. It is possible that the Ca^{2+} dependent arm of the bifurcating pathway is only activated in the presence of TPA and serum factors. Thus only in the presence of serum is growth inhibition by TPA maintained. We have observed transient arrest of DNA synthesis in cells following treatment with bryo. Interestingly, it has been noted by Gschwendt *et al.* (1988) that those effects of TPA which are Ca^{2+}-dependent are not mimicked by bryo. One may speculate that bryo blocks the ability of serum to elevate [$Ca^{2+}]_i$. It would be interesting to determine whether co-application of TPA with a Ca^{2+} ionophore to serum-deprived cells would result in the maintenance of growth inhibition, similar to that observed in A549-FCS cells when exposed to TPA alone.

The dependence of A549 cell growth inhibition by TPA on the presence of FCS is illustrated in fig 19. Cells, routinely subcultured in medium supplemented with 10% FCS were introduced experimentally into medium containing 0, 1, 5 and 10% FCS. Cells were incubated for 48h in the presence or absence of 10nM TPA, after which time, the ability to incorporate labelled thymidine was assessed. An inverse relationship between serum content and ability to synthesize DNA in the presence of 10nM TPA was observed. These results appear to support the hypothesis suggesting that the maintenance of TPA-induced growth arrest is consequential to the synergistic interaction between TPA and serum

factors.

We wished to investigate the ability of TPA to arrest growth of cells maintained in medium supplemented with serum other than foetal calf serum. To this end, cells were cultured for 4 passages in medium supplemented with 10% new born calf serum (NBCS) before experiments were instigated to examine whether maintained A549 growth arrest was peculiar to an environment containing foetal serum components.

The growth rate of cells maintained in medium supplemented with 10% NBCS was indistinguishable from the parent A549-FCS cell population. This fact was established from assessment of ability to incorporate [^{3}H]TdR and by cell counts performed for the purpose of a growth curve. However, as demonstrated in figure 20, although growth of this cell population is significantly retarded in the presence of 10nM TPA, arrest of growth was not so potent as observed in cells maintained in medium supplemented with 10% FCS. Investigation of the time course of inhibition by 10nM TPA (figure 21) revealed a transient, potent arrest in DNA synthesis, followed by significant recovery of proliferative capacity beginning 18h after treatment. Following an exposure period of 72h, incorporation of [^{3}H]TdR in the presence of 10nM TPA was 78% of control values. We wished to determine whether a biphasic dose response curve was observed following 24h exposure to increasing concentrations of TPA. The results are represented in figure 22. Maximum inhibition of [^{3}H]TdR incorporation after this time occurred with 10nM and 100nM TPA. At a concentration of 1μM, the ability of TPA to inhibit DNA synthesis was attenuated to a small degree. Hence, it may be that at concentrations at and above 1μM TPA, the proreplicative response in cells maintained in medium fortified with 10% NBCS may begin to take effect. The results obtained above, particularly demonstrated in figures 20 and 21, appear to indicate that specifically foetal serum possessed the ability to potentiate arrest of growth of A549 cells for 6 days induced by TPA.

We have tested the hypothesis that introduction of medium containing 10% FCS into A549-US cultures would be sufficient to restore sensitivity to TPA upon these cells. Thus, for the duration of the experiment, cells were exposed to an environment containing 10% FCS in the presence or absence of 10nM TPA. Additionally, A549-FCS cells were seeded in serum-free medium and 4h later TPA or DMSO vehicle was added. Cells were either exposed to these conditions for 48h, after which time, incorporation of [^{3}H]TdR was determined (figure 23a). Alternatively, cell counts were performed after 96h exposure (figure 23b). It can be seen that the presence of serum led to an increase in the proliferative capacity of A549-US cells and immediate partial restoration of TPA-sensitivity. After 48h, DNA synthesis in A549-US cells, in an environment containing 10% FCS was reduced from 106.3% to 47.7% of control A549-US [^{3}H]TdR incorporation by 10nM TPA. However, it was observed by means of growth curve that the introduction of FCS to A549-US cells cultured in 2% US-supplemented medium and 10nM TPA for 48h, was unable to provide any sensitivity to TPA (result not shown). Thus it appears that FCS may only restore sensitivity if introduced prior to exposure to TPA, and cells which have acquired resistance to TPA, maintain resistance in the presence of FCS, prior to trypsinization. Moreover, the exposure of A549-FCS cells to 2% US-supplemented,

serum-free environment resulted in an immediate decrease in proliferative capacity and rendered cells significantly less sensitive to growth inhibition by TPA. After an exposure period of 48h, inhibition of [^{3}H]TdR incorporation by 10nM TPA was reduced from 87% to 37% in the absence of serum (figure 23). It may be proposed that certain components of foetal calf serum are required for the maintained growth arrest of A549 cells by tumour promoting phorbol esters.

4.1.4 Examination of the cytostatic effect of TPA on cells in conditioned media.

Introduction.

Whether TPA alters the cellular response to factors either produced in an autocrine fashion or present as components of serum, or whether these factor(s) are able to modify the cells` response to TPA is matter for speculation. Indeed, TPA has been shown to alter the response to growth promoting factors, including EGF, insulin, transferrin, and somatomedin C through receptor phosphorylation as well as influence the regulation of cognitive processes *via* phosphorylation of GTP-binding proteins (Germolec *et al.*, 1988; Ashendel 1985).

The following experiments were performed to examine whether autocrine factors are involved in A549 growth arrest induced by TPA.

Results and discussion.

Media was conditioned alone or in the presence of 10nM TPA for 72h over A549-FCS or A549-US cells, centrifuged briefly and introduced at a ratio of 60:40 with fresh media, into cultures of A549-US cells. We wished to examine the effect of TPA in the presence of conditioned media on cell growth. Growth inhibition did not exceed the growth inhibitory effect of 10nM TPA on A549-US cultures in fresh media. These experiments were instigated by the following observations. It has been demonstrated that proliferation of a human rhabdomyosarcoma cell line was dramatically and reversibly inhibited by 100nM TPA (Aguanno *et al.*, 1990). Growth inhibition was accompanied by increased expression of differentiative characters. However, these effects were not observed if the medium and TPA were replaced daily. Hence growth arrest may be a consequence of factors secreted by treated cells. It has been suggested that TPA might affect the activity or secretion of TGF-ß or fibroblast growth factor in these cells. This hypothesis remains to be tested. Autocrine factors which inhibit tumour cell growth have been isolated from human tumours, including a malignant melanoma cell line (Bogdahn *et al.*, 1989). Interestingly, the inhibitory properties were partially antagonized by a serum component. The polypeptide growth inhibitor oncostatin M is produced by U-937 cells upon treatment with TPA (Zarling *et al.*, 1986). It has been established that A549 cells possess cellular receptors for oncostatin M and are potently growth arrested by this agent, with 50% growth arrest occurring between 50 and 100pM (Linsley *et al.*, 1989). Moreover, Brown *et al.* (1987) have reported a synergistic antiproliferative response with suboptimal concentrations of oncostatin M in combination with TGF-ß in A375 human melanoma cells. Oncostatin M and its interaction with other cytostatic polypeptide growth inhibitors may play a role in the

Figure 9a)

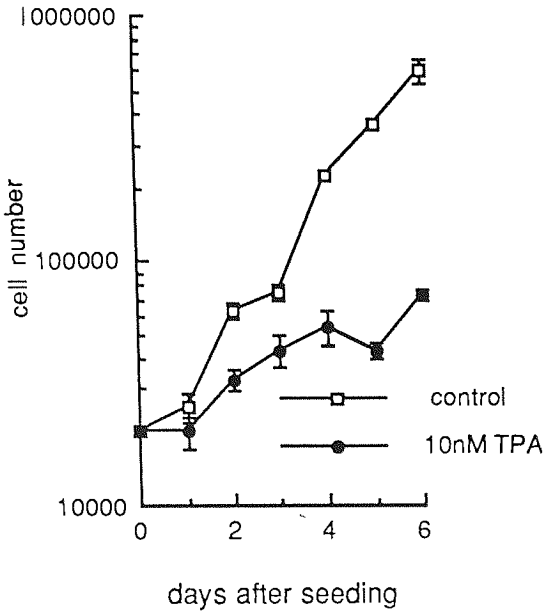


Figure 9b)

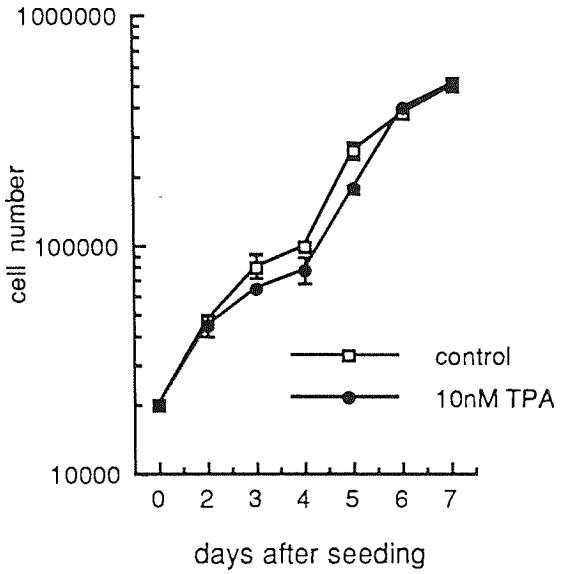


Figure 9. Growth of serum-deprived cells in the presence or absence of 10nM TPA. Cells maintained in media supplemented with a) 1% ITS plus 2mg/ml fetuin or b) 2% US were seeded at a density of 2 x 10^4 per well and treated with 10nM TPA 4h later. Cell counts were performed daily (mean ∓ SD, n=9-12).

Figure 10

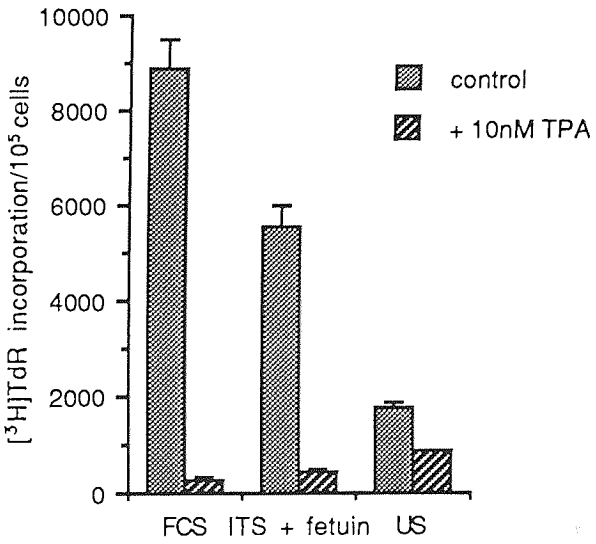


Figure 10. Effect of TPA on DNA synthesis within A549 cell populations. Cells, maintained in media supplementedwith 1% ITS plus 2mg/ml fetuin, 10% FCS or 2% US were seeded at a density of 2 x 10^5 per well and allowed 4h to attach. Following 24h exposure to 10nM TPA or vehicle alone, [^{3}H]TdR incorporation was assessed (mean ∓ SD, n=9).

Figure 11

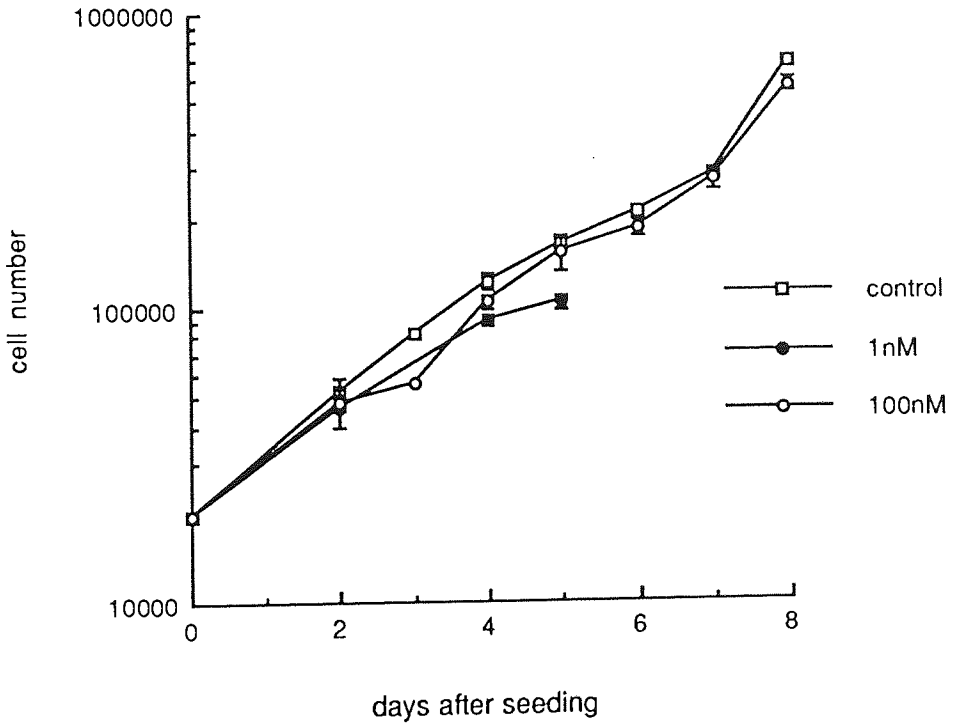
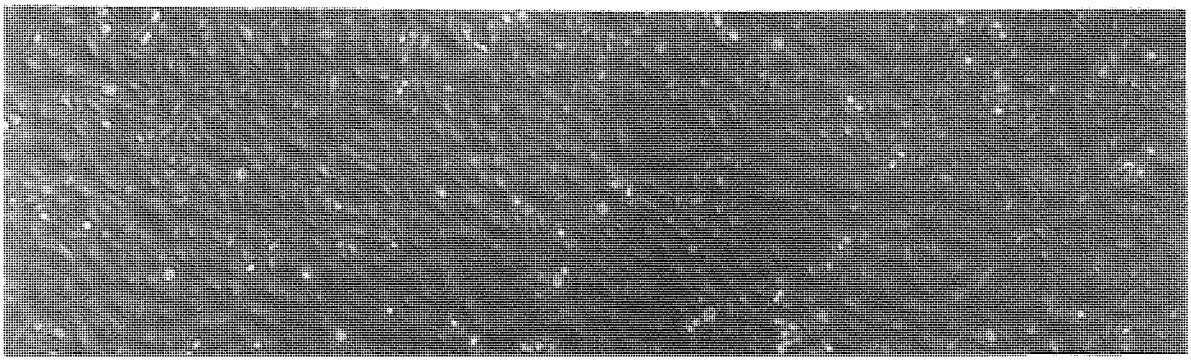


Figure 11. Growth of A549-US cells in the presence or absence of PDBu.
Cells (2 x 10^4) were seeded and treated with 1nM or 100nM PDBu 4h later. Cell counts were performed following the indicated incubation periods (mean $\pm$ SD, n=6-9).

Figure 12. Phase contrast micrographs of cultured A549 human lung carcinoma cells.
i) A549-FCS
ii) A549-FCS following 48h exposure to 10nM TPA
iii) A549-US
iv) A549-US following 48h exposure to 10nM TPA.

i



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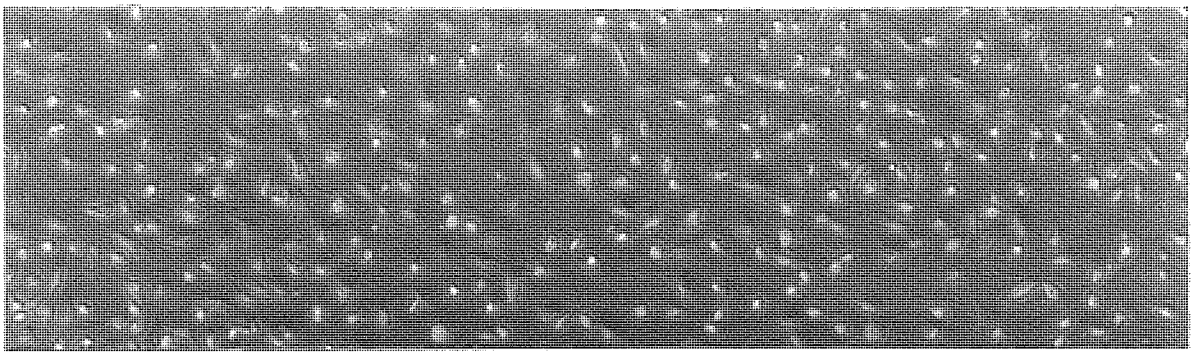

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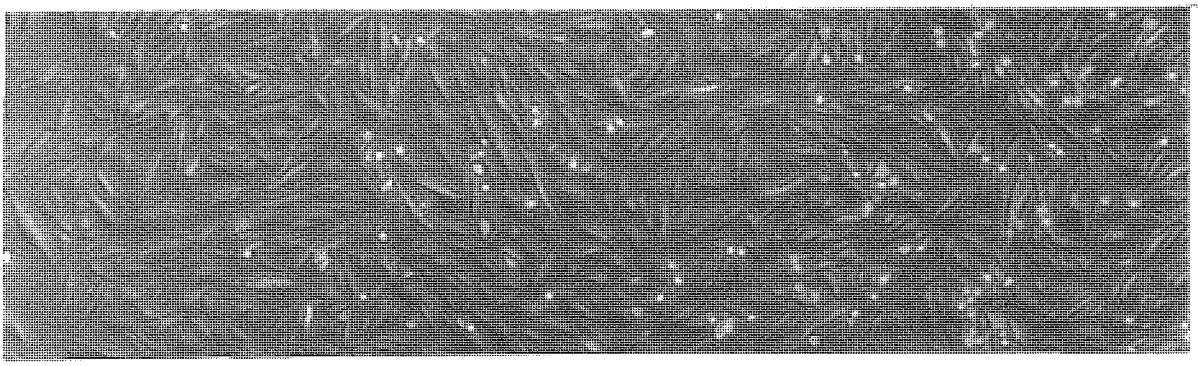

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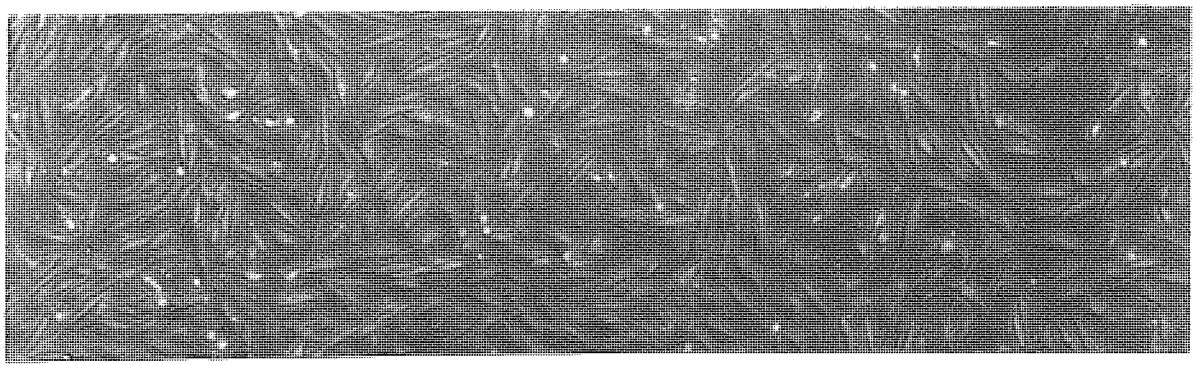


Figure 13

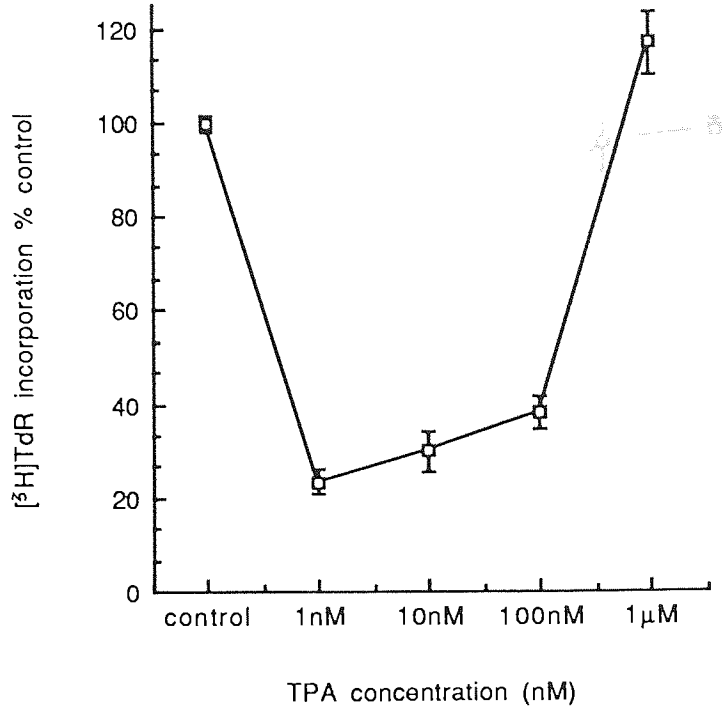


Figure 14

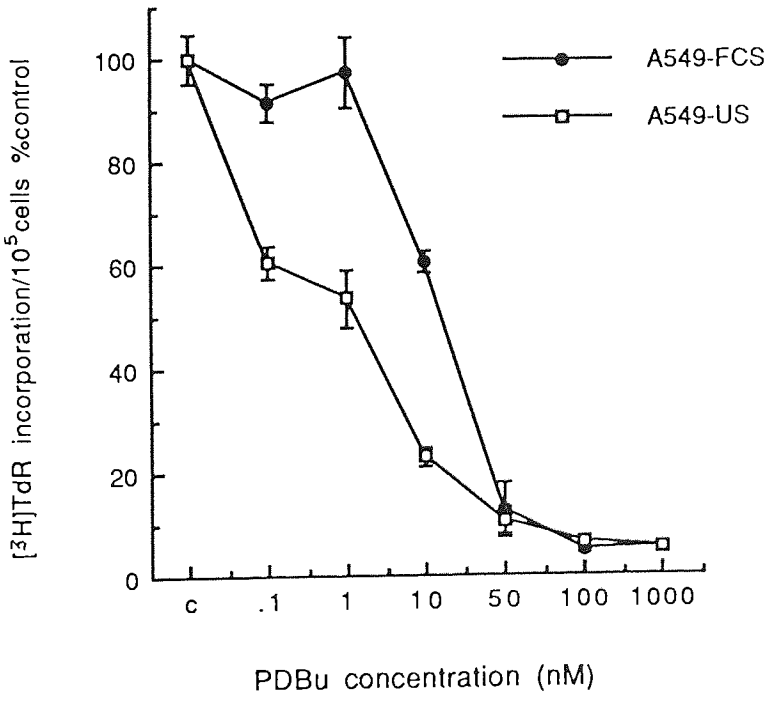


Figure 13. Effect of increasing concentrations of TPA on A549-US cell proliferation.
Cells (2 x 10^5) were seeded and allowed 4h to attach. They were exposed to varying concentrations of TPA for 24h before [^{3}H]TdR incorporation was assessed (mean + SD, n=9-12).

Figure 14. Effect of increasing concentrations of PDBu on A549-FCS and A549-US cell proliferation.
Cells (2 x 10^5) were seeded and allowed 4h to attach. They were exposed to varying concentrations of PDBu for 24h before [^{3}H]TdR incorporation was assessed (mean + SD, n=6).

Figure 15

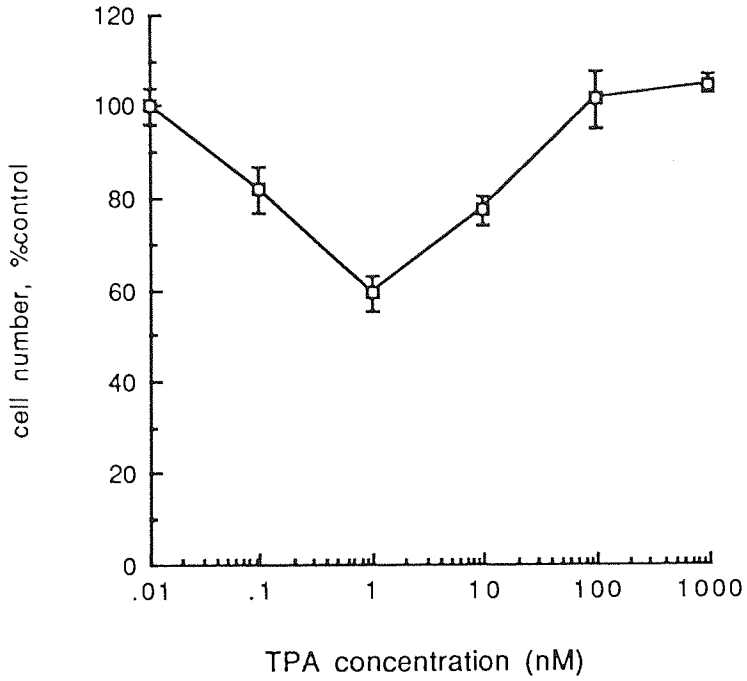


Figure 16

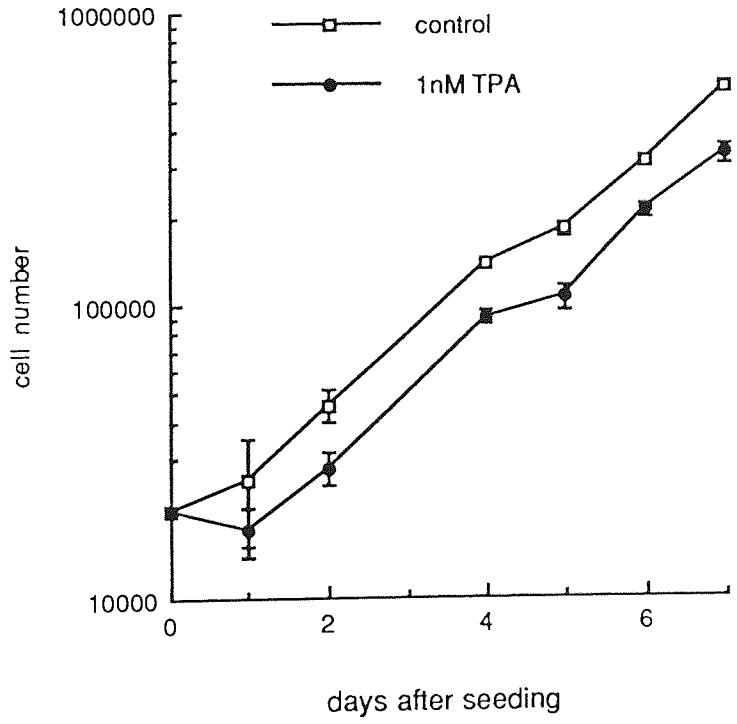


Figure 15. Effect of increasing concentrations of TPA on A549-US cell number.
Cells (2 x 10^4) were seeded and allowed 4h to attach. They were exposed to varying concentrations of TPA for 96h before cell counts were conducted (mean $\bar{+}$ SD, n=9).

Figure 16. Growth of A549-US cells in the presence or absence of 1nM TPA.
Cells (2 x 10^4) were seeded and treated with TPA 4h later. Counts were performed daily. (mean $\bar{+}$ SD, n=9).

Figure 17

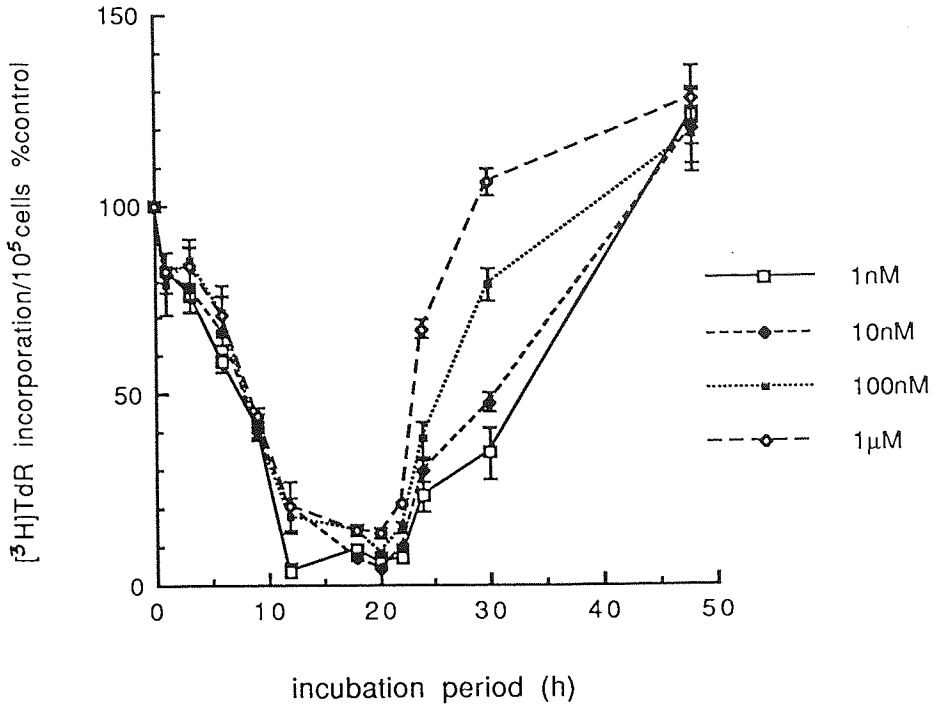


Figure 18

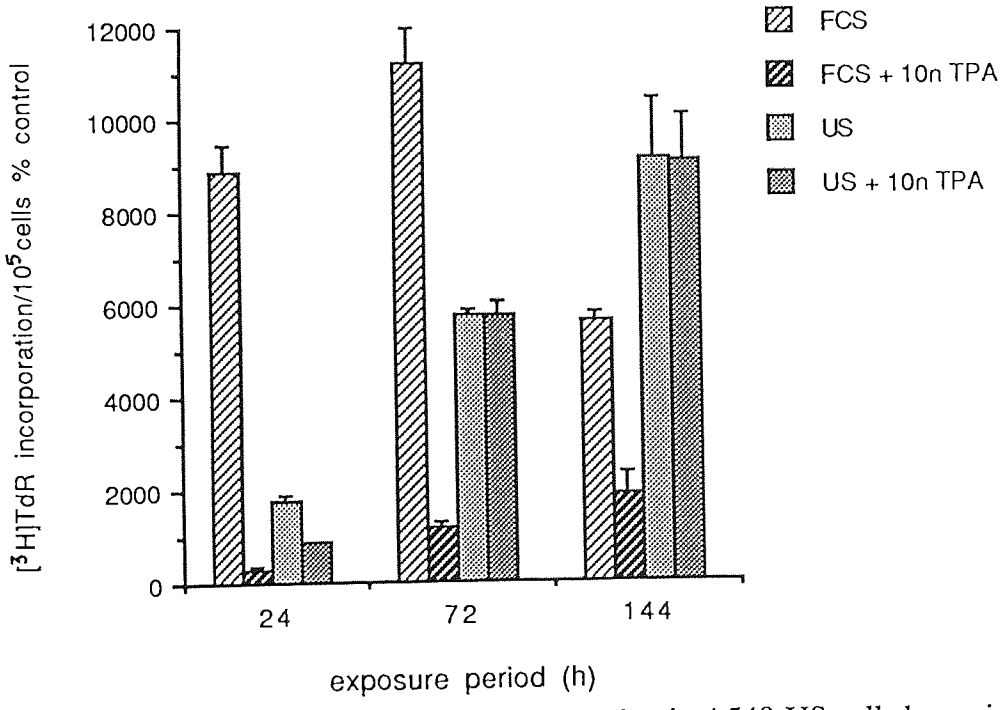


Figure 17. Time course inhibition of [^{3}H]TdR incorporation in A549-US cells by various concentrations of TPA.
Cells were seeded at 10^5 or 2 x 10^5 and incubated for increasing time intervals with various concentrations of TPA before incorporation of [3H]TdR was determined. (mean ∓ SD, n=3, 1 expt. representative of 3).

Figure 18. Measurement of incorporation of [^{3}H]TdR in A549-US and A549-FCS cells exposed to 10nM TPA.
Cells (5 x 10^4) were seeded in maintainance media containing either 2% US or 10% FCS supplement. Treatments commenced 4h later and following exposure periods of 1, 3 and 6 days DNA synthesis was determined. (mean ∓ SD, n=3, 1 expt. representative of 3).

Figure 19. Effect of FCS on inhibition of DNA synthesis evoked by TPA in A549-FCS cells. Cells (10^5) routinely cultured in medium supplemented with 10% FCS were seeded in medium fortified with decreasing serum concentrations and allowed 4h to attach. Following 48h exposure to 10nM TPA, the ability to incorporate [H]TdR was determined (mean ∓ SD, n=9).

Figure 19

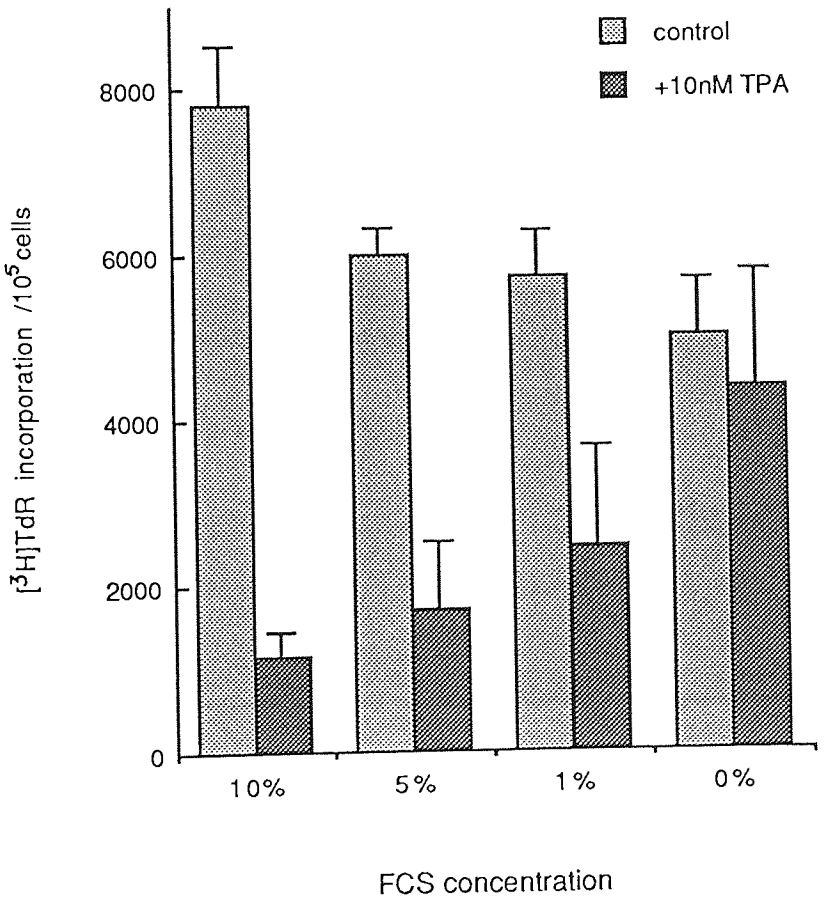


Figure 20

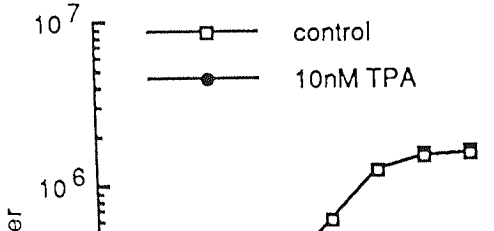


Figure 20. Growth of cells maintained in medium supplemented with 10% NBCS in the presence or absence of 10nM TPA. Cells (2 x 10^4) were seeded and allowed 4h to attach before treatment with TPA began. Cells were counted following the indicated incubation periods (mean ∓ SD, n=6).

Figure 21

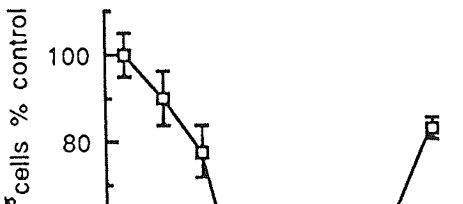


Figure 21. Time course inhibition of [^{3}H]TdR incorporation induced by 10nM TPA in cells maintained in medium fortified with 10% NBCS. Cells (10^5 or 2 x 10^5) were seeded and exposed to 10nM TPA for varying lengths of time before [^{3}H]TdR incorporation was measured (mean ∓ SD, n=6).

Figure 22

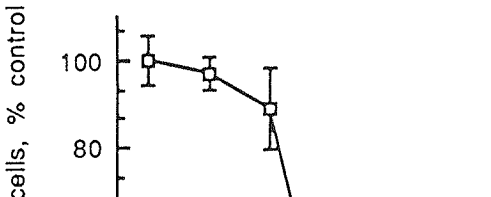
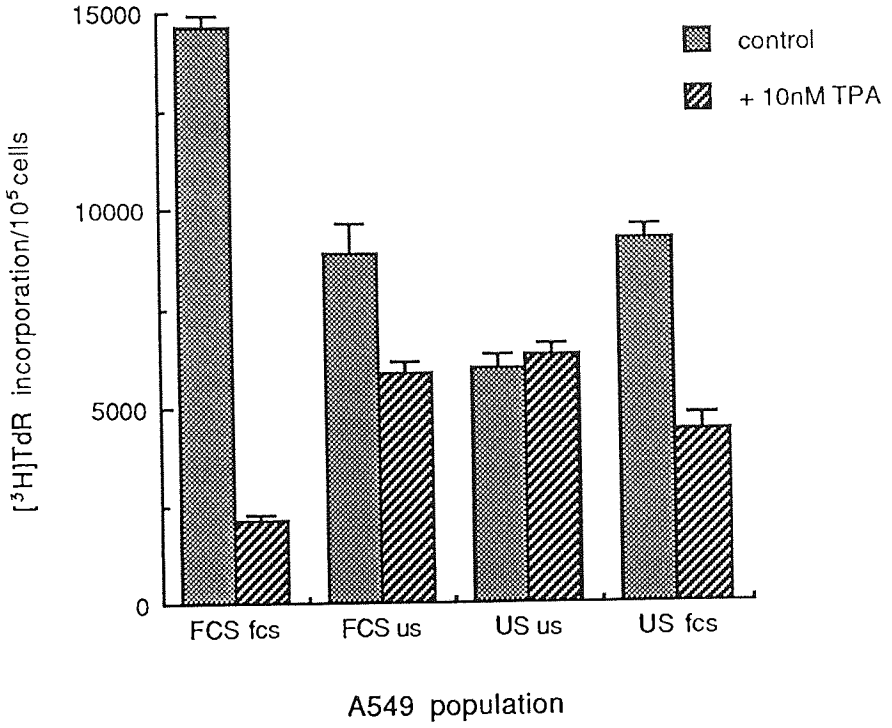


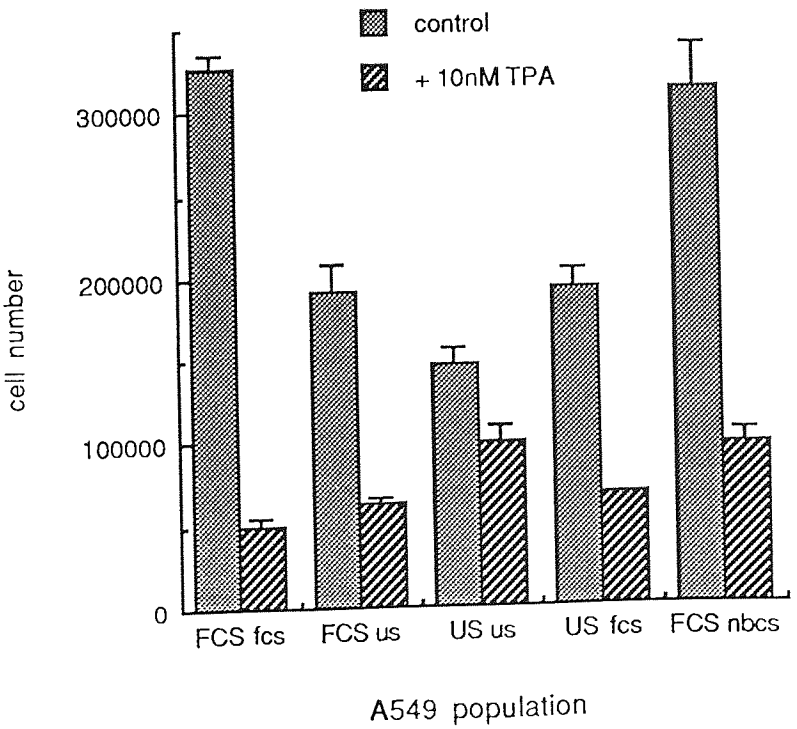
Figure 22. Effect of increasing concentrations of TPA on the proliferation of cells maintained in medium supplemented with 10% NBCS. Cells (2 x 10^5) were seeded and allowed 4h to attach before being treated with increasing concentrations of TPA. [^{3}H]TdR incorporation was assessed following 24h exposure (mean ∓ SD, n=6).

Figure 23. Effect of 10nM TPA on the inhibition of A549 cell growth following a change in culture conditions. A549-FCS cells were seeded in medium supplemented with 2% US for the duration of the experiment, in the presence or absence of 10nM TPA. Similarly, A549-US cells were seeded in medium supplemented with 10% FCS.
a) Cells (10^5) were seeded and allowed 4h to attach. Following 48h exposure to TPA, [H]TdR was assessed (mean ∓ SD, n=6).
b) Cells (2 x 10^4) were seeded and allowed 4h to attach. Following 96h exposure, cell counts were performed (mean ∓ SD, n=6-9).

Figure 23a)



23b)



regulation of tumour cell growth. There is no evidence to suggest that oncostatin M is secreted by A549 cells although they possess receptors and, as is the case with many tumour cell lines, their growth is inhibited by this polypeptide. A549 cells do secrete other positive (Siegfried, 1989) and negative regulators of growth such as TGF-ß. Thus the examination of cell growth in the presence of TPA plus conditioned media was a crude attempt to define whether TPA may act in consort with autocrine factors to arrest the growth of A549-US cells. We obtained no evidence to support this hypothesis.

4.1.5 Examination of factors present in FCS which may augment growth inhibition by TPA.

Introduction.

Evidence has suggested that agents which inhibit DNA synthesis, including TPA, may sensitize cells to respond to differentiation inducing factors contained within serum (Anderson *et al.*, 1989). Concentration-dependent responses were evident with respect to both drug and FCS concentration. Certain cytokines including TGF-ß and tumour necrosis factor (TNF) have demonstrated a similar effect. Interestingly, these agents were found to evoke PKC translocation from cytosol to plasma membrane.

We wished to investigate the hypothesis that factors present in FCS, absent from US supplement are critical for the maintained growth arrest of A549-FCS cells by TPA. To this end, the ability of TGF-ß, platelet-derived growth factor (PDGF), retinoic acid (RA), epidermal growth factor (EGF) and fetuin, in the presence or absence of 10nM TPA, to retard the growth of serum-deprived cells has been examined. These factors are discussed briefly in turn.

Results and discussion.

4.1.5.1 TGF-ß

TGF-ß is recognized as being a multifunctional regulator of cell growth and differentiation during such processes as embryogenesis, wound repair and carcinogenesis (Fürstenberger *et al.*, 1989; reviewed by Roberts *et al.*, 1988). Three highly homologous members of the human TGF-ß gene family have been identified (Arrick *et al.*, 1990). TGF-ß is reported to increase the rate of proliferation of cells of mesenchymal origin, for example osteoblasts; but is a potent inhibitor of normal epithelial-derived cells and a number of tumour cell lines. TGF-ß inhibited growth and induced differentiation in normal human bronchial epithelial cells, thus it is considered to be an important serum factor responsible for growth inhibition and induction of differentiation (Miyashita *et al.*, 1989; Masui *et al.*, 1986). Indeed, an anti TGF-ß antibody abolished the ability of serum to induce squamous differentiation. It has previously been reported that pM concentrations of TGF-ß may cause almost complete A549 growth inhibition in soft agar. In monolayer cultures a dose dependent increase in cell cycle time was obtained (Roberts *et al.*, 1985). Twardzik and coworkers (1989) have described how TGF-ß inhibited, in a concentration dependent fashion, the growth of A549 xenografts in male athymic BALB-C mice and that histologically, tumours appeared to be more differentiated. Such data raises the possibility that the growth of certain normal cells

may be regulated in a negative autocrine manner by TGF-ß, and loss of this regulation may confer a growth advantage upon cells, and aid genesis and maintenance of the malignant state. A549 cells possess 10 000 TGF-ß receptors per cell and have been shown to secrete the latent non-biologically active form of TGF-ß at a rate of 625pg/10^6 cells/24h (Wakefield *et al.*, 1987). However, this cell line has lost the ability to activate latent TGF-ß and thus is incapable of responding to endogenous TGF-ß.

It may be speculated that TPA can restore the cells` ability to activate TGF-ß, which would result in the observed maintained growth arrest evoked by TPA. Alternatively, TGF-ß may act in consort with TPA to trigger events unattainable to TPA alone, or TGF-ß alone. Thus we have examined whether the introduction of TGF-ß into the serum-free environment may potentiate TPA-induced cell growth inhibition.

The effect of TGF-ß alone on the growth of A549-FCS and A549-US cell cultures was monitored. The results are demonstrated in figure 24c. A concentration-dependent decrease in cell number was observed, following an exposure period of 96h. The growth of A549-FCS cells was retarded to a greater degree than A549-US cultures. The IC_{50} value for the former population was calculated as 18.2pM, whereas in the presence of 25pM, A549-US cell numbers were reduced by 39.8%. Introduction of TPA into incubates of TGF-ß with A549-US or A549-FCS cells led only to an additive inhibitory response (figure 24a, b and figure 27a). Bryostatin 1 also evoked an additive growth inhibitory response upon the two A549 populations when present in conjunction with TGF-ß. Reduced cell numbers resulted when TGF-ß was introduced into the media of A549-FCS and A549-US TPA-resistant cultures (figure 27b). These cultures have been maintained continually in the presence of 10nM TPA in excess of 9 weeks.The presence of both agents in such populations induced an additive inhibitory response, as observed in TPA-sensitive cell populations.

The results of these experiments led to the rejection of the possibility that TGF-ß alone is responsible for enhanced A549 cell growth inhibition by TPA. However, a number of reports have described synergy between responses elicited by TPA and TGF-ß. TPA potentiated the activity of TGF-ß with respect to NRK-49F colony formation in soft agar (Kraft 1986). The effect of TGF-ß on a TPA-resistant MCF-7 breast subline has been examined (Guerrin *et al.*, 1990). In the absence of TPA, cells lost their sensitivity to the growth inhibitory properties of TGF-ß, yet sensitivity was restored by the introduction of TPA into the medium. Moreover, in the presence of a fixed low concentration of TGF-ß, TPA induced a dose-dependent inhibition of proliferation comparable to that observed by TPA in sensitive MCF-7 cells. It has been concluded from this work that TGF-ß may restore sensitivity to TPA by interfering with a step in the PKC pathway subsequent to its activation. It has been established that transcription of the TGF-ß1 gene may be autoregulated and regulated by TPA, via activation of the first promoter region. Kim and coworkers have described two phorbol ester responsive elements in the second promoter region of the TGF-ß1 gene and have illustrated using nuclear extracts of A549 cells that both TGF-ß and TPA act through the same sites. Deletion of these putative phorbol ester responsive elements leads to 70-80% decrease in transcriptional activity (Kim *et al.*, 1989).

The phenotypic changes induced by serum factors, specifically TGF-ß influenced the sensitivity of hamster tracheal epithelial cells to crocidolite asbestos (Sesko and Mossman, 1989). Crocidolite asbestos was able to evoke proliferative and cytotoxic responses similar to those induced by the tumour promoting phorbol esters in these cells and in organ cultures. Upon stimulation with this asbestiform mineral, the activation of phospholipases and accumulation of DAG was demonstrated, implicating PKC activation. These authors have been led to conclude that the environment of tracheal epithelial cells *in vitro* is critical to the expression of biological responses to tumour promoters.

As discussed, TGF-ß, TPA and serum were able to induce the differentiation of human bronchial epithelial cells (Miyashita *et al.*, 1989). Serum, but not TPA induced rise in $[Ca^{2+}]_i$. An anti-TGF-ß Ab abolished the ability of serum to induce differentiation. Thus, it was postulated that TGF-ß, present in serum was responsible for the elevation of $[Ca^{2+}]_i$. However, it was demonstrated that serum factors other than, or in addition to, TGF-ß mediate changes in $[Ca^{2+}]i$, possibly via the activation of PKC.

4.1.5.2 PDGF

The production of PDGF is common to normal and transformed cells (Heldin *et al.*, 1988). This growth factor possesses an autocrine and paracrine function in several physiological and pathological conditions including an established critical role in wound healing processes. Part of the PDGF-stimulated mitogenic pathway includes PLC-mediated hydrolysis of $PI4,5P_2$ with subsequent elevation of $[Ca^{2+}]_i$ (Moolenar *et al.*, 1984) and activation of PKC (Rozengurt *et al.*, 1983). Hata *et al.* (1989) have described the synthesis of 1,2-DAG from monoacylglycerol following stimulation with PDGF. Thus the generation of DAG is augmented *via* several pathways in PDGF-treated cells. Evidence has been presented to suggest that PDGF-induced phospholipid hydrolysis is not subject to negative regulation by PKC (Kawahara *et al.*, 1988). Moreover, it has been suggested that PKC activation is not essential for PDGF-stimulated DNA synthesis (Zagari *et al.*, 1989). We have introduced PDGF into the serum-free environment to test the hypothesis that this factor may augment growth inhibition by TPA.

Interestingly, the presence of 10pM PDGF alone in A549-US cultures led to a small decrease in cell numbers (76.2% of control). However, the presence of TPA eradicated this inhibition, restoring cell numbers to values (mean = 94.6%) significantly no different from controls (Figure 27a). The growth of A549-FCS cell populations was unaffected by the presence of PDGF (0.2-25pM) (result not shown). It was found that 10pM PDGF alone caused modest increases in cell numbers in TPA-resistant A549-US and A549-FCS populations, by 33 and 29% respectively, and that inclusion of 10nM TPA in these incubates reduced dramatically FCS-TPA cell numbers, but decreased only slightly US-TPA cell numbers. These results are illustrated in figure 27b.

4.1.5.3 Retinoic acid

The retinoid class of chemical agents are essential for normal development and differentiation of epithelial tissues (Brockes, 1989). They are agents, moreover, which are able to block the promotion-progression phase of malignant transformation (Ruddon,

1987). Retinoids have been shown to inhibit the transformation of cells in culture by chemicals and the development of chemically induced carcinomas at a variety of organ sites *in vivo*. Indeed, retinoids have reversed the expression of the malignant phenotype in already transformed cells and blocked the promoting effects of tumour promoting phorbol esters (Sporn, 1978).

We wished to investigate the effect of all-trans retinoic acid (RA) on A549 cell growth. It has been demonstrated by Jetten *et al.* (1990) that at concentrations above 0.01μM, RA can inhibit the proliferation in monolayer culture of 6 of 7 cell lines derived from human head and neck squamous carcinoma. In addition, concentration-dependent inhibition of growth by RA has been demonstrated in cultured human epidermal cells (Hashimoto *et al.*, 1985). Concentration-dependent inhibition of A549-FCS cell numbers was obtained (figure 25a) with an IC_{50} value calculated as 857nM RA. The growth of A549-US cultures was not retarded to the same degree, cell numbers in the presence of 1μM RA were 66.6% of control values (figure 27a).

It has been suggested that RA may interact specifically with signal transducing mechanisms responsible for responses induced by phorbol esters; subsequent to the binding of RA to its own specific receptors, classified into subtypes α, ß and γ (Noji *et al.*, 1989). We wished to examine the possibility that RA inhibits growth arrest induced by TPA. Many reports describe the antagonistic effects of retinoic acid on a phorbol ester-induced cellular response (Ruddon, 1987; Vanier *et al.*, 1988; Jetten *et al.*, 1989). Hossain *et al.* (1988) report that TPA inhibited junctional communication and propose that enhanced gap junctional communication by retinoids, to convey growth regulatory signals, may explain much of their chemopreventive action. However, in certain cell types, RA, rather than antagonizing, enhanced phorbol ester-stimulated cellular events. For example, at concentrations <1nM, which were 100-fold less than concentrations able to directly stimulate differentiation, RA potentiated TPA-induced differentiation of the U937 cell as assessed by enhanced adherence to plastic and acquisition of non-specific esterase activity (Ways *et al.*, 1988). Hence, we wished to test the hypotheses that RA may either potentiate the growth inhibitory action of tumour promoting phorbol esters in A549-US cells or antagonise growth inhibition by TPA in A549-FCS cells. The arrest of A549-FCS cell growth by TPA was neither augmented nor attenuated by RA (figure 25b). Moreover, the results, shown in figure 27a demonstrate no amplification of A549-US cell growth inhibition by TPA in the presence of 1μM RA. The response was an additive one.

The effect of 1μM RA on the growth of TPA-resistant A549-FCS and A549-US was examined. It was found that cells of the FCS-TPA population grew more rapidly when TPA was withdrawn from growth medium. RA (1μM) alone was unable to retard further their growth and no significant difference existed between these cell numbers and cell numbers in the presence of TPA alone. However, growth was stimulated by 38% in the presence of both TPA and RA. Thus it appears that in A549-FCS cells, grown continually in the presence of 10nM TPA, RA was able to antagonize the ability of TPA to retard growth. Such an effect was not observed using the A549-US cells grown continually in an

environment containing 10nM TPA (US-TPA). Cell numbers, in the presence of TPA, exceeded cell numbers when growth was monitored following removal of TPA. Moreover, in this population, the growth inhibition caused by RA alone (54.0%), is augmented in the presence of TPA (67.8%). These observations are illustrated in figure 27b.

It has been reported that phorbol ester-induced differentiation of normal human epidermal keratinocytes (Jetten *et al.*, 1989a) and normal human tracheobronchial epithelial cells (Jetten *et al.*, 1989b) was blocked by both RA and bryos 1 and 2 (discussed in section 4.6). However, these agents target different stages in the differentiation process. Bryos 1 and 2 inhibit the induction of terminal growth arrest, a prerequisite to expression of the differentiated phenotype. RA blocks the expression of the differentiated phenotype. This scheme of events does not apply to A549 cells. A549 carcinoma cells have maintained certain characteristics of the differentiated phenotype (Lieber *et al.*, 1976). TPA was able to elicit only temporary growth arrest, which was initially mimicked, then abrogated by bryo 1 (Dale and Gescher, 1989). RA was only able to interact with a TPA-induced effect in cells maintained in a TPA-supplemented environment. We have examined the effect of RA, bryo 1 and TPA on the proliferative capacity of TPA-sensitive and resistant A549-FCS cells. As discussed in section 4.6, bryo 1 was able to abolish the arrest of DNA synthesis caused by TPA in sensitive A549-FCS cells. RA was unable to abrogate growth inhibition by TPA. However, it appears that bryo 1 may attenuate the ability of 1μM RA to inhibit cell proliferation. It was revealed that bryo 1 (1μM > 10nM) could maintain proliferative capacity even in the presence of 10nM TPA and 1μM RA (figure 26a). Figure 26b demonstrates that in A549-FCS TPA-resistant cells, the presence of bryo 1 and TPA evoked behaviour similar to RA and TPA and proliferative capacity was increased.

4.1.5.4 EGF

Epidermal growth factor (EGF) is an important biological regulator in animals, capable of eliciting a myriad cellular and physiologic responses. This growth promoting factor is thought to play a key role in tissue renewal and wound healing. *In vivo* and in culture, EGF may induce accelerated rates of proliferation and differentiation (Reviewed by Ruddon, 1987). Cells grown in the presence of EGF continue to proliferate after cultures become confluent, simulating loss of the density-dependent growth inhibition, observed in transformed cells. Indeed, certain tumour cell lines have been found to secrete EGF or EGF-like molecules in an autocrine fashion (Kurokawa *et al.*, 1989). Many cell types, including epidermal cells, fibroblasts, epidermoid carcinoma cells, and choriocarcinoma cells possess specific, saturable cell surface receptors for EGF, indicating the ubiquitous nature of target cells for this growth factor. Occupation of the receptor by EGF induces receptor autophosphorylation as well as phosphorylation of certain other substrates; among which are two proteins of Mr 41kDa and two proteins of Mr 43kDa whose phosphorylation is induced by serum and phorbol esters as well as by EGF. Many of the actions of EGF in cultured cells have been found to resemble certain responses evoked by the tumour promoting phorbol esters and early suggestions implied that these two agents could act *via* the same receptor-effector system (Osborne *et al.*, 1981). For example, EGF is reported to promote tumour formation after treatment of mouse skin with a tumour initiating

carcinogenic chemical (Rose *et al.*, 1976). Indeed, down-regulation of PKC may abolish certain EGF-induced effects (Susa *et al.*, 1989). *In vitro* experiments have demonstrated that purified EGF receptors may interact with and nick supercoiled double stranded DNA in an ATP-dependent reaction, presumably by enhancing DNA topoisomerase activity (Mroczkowski *et al.*, 1984). Moreover, it has been established that tumour promoting phorbol esters inhibit both binding of EGF to receptors, in a manner paralleling their potency as tumour promoters in mice (Osborne *et al.*, 1981), and EGF-catalyzed receptor autophosphorylation (Hunter *et al.*, 1984). These responses, induced by phorbol esters, *via* activation of PKC, followed phosphorylation of the EGF receptor on threonine residue 654, a key site on the cytoplasmic face of the receptor close to the protein kinase domain. We have hypothesized that EGF, as an important component of foetal serum, may augment the inhibition of A549 growth by phorbol esters.

Indeed, as demonstrated in figure 27a, the presence of 10pM EGF was able to substantially restore sensitivity upon A549-US cells to growth inhibition by 10nM TPA. EGF alone did not significantly interfere with A549-US cell growth: cell numbers were 105.2% of control. Introduction of 10nM TPA reduced cell numbers by 55.0%. In A549-FCS cells, the presence of 10pM EGF alone, following 96h exposure, decreased cell numbers by 26.9% (mean + 2.7, n=8, result not shown); in combination with 10nM TPA an additive growth inhibitory response was obtained. Whether EGF elicits the growth inhibitory response on A549-US cells through mechanisms involving the activation of PKC may only be speculated upon. In certain cases, EGF-induced phosphorylation of an 80kDa cytosolic protein has been reported (Kazlauskas and Cooper, 1988), a phenomenon not observed by others (Rozengurt *et al.*, 1983). However, Wright *et al.* (1988), have demonstrated increases in total DAG following EGF treatment. Thus, evidence is accumulating to suggest that EGF evokes certain responses through activation of PKC. In addition, a recent report has provided evidence to suggest the involvement of PKC-α in the regulation of EGF receptor expression (Eldar *et al.*, 1990). Interestingly, it has been shown that MCF-7 cells exhibited increased amounts of EGF receptor mRNA when grown in serum deficient medium (Medrano *et al.*, 1990).

4.1.5.5 Fetuin

Alpha-fetoprotein (AFP), the predominant constituent of fetuin is a major serum protein of the developing embryo. Its synthesis, by foetal liver and yolk sac is under hormonal regulation (Belanger, 1976). Concentrations of fetuin fall rapidly after parturition, inversely correlated with the rise in concentration of serum albumin, such that negligible levels of the former are present in adult serum (Abelev, 1971). Albumin and AFP have been shown to share structural homology, and it is thought that their genes arise from the same ancestral gene (Alexander *et al.*, 1984). Morphological studies of developing brain have demonstrated developmentally regulated expression of fetuin within neurons of the early cortical plate in neocortex of many species including humans, during a time of intense synaptogenesis (Dziegielewska *et al.*, 1990). However, AFP is also synthesized and secreted by certain tumours. In the newborn, continued elevation of AFP is associated with high incidence of hepatoma. Patients with hepatocellular carcinomas, germinal tumours

containing yolk sac elements and tumours of the gastrointestinal tract possess elevated levels of this oncodevelopmental antigen. AFP is presently monitored as a marker for malignancy in patients with embryonal testicular cancer and hepatocellular carcinoma. Blood levels of fetuin rise during the induction of liver tumours in rodents treated with chemical carcinogens. It has been proposed that intracellular AFP may play a role in controling proliferation of liver cells in response to certain stimuli such as partial hepatectomy.

We wished to test the hypothesis that the presence of this major protein of foetal serum may influence the growth of A549-US cells in response to treatment with TPA.

The introduction of fetuin alone (2mg/ml) into serum-free environment led to enhanced cell growth; following a 96h exposure period, cell numbers increased by 15%. However, in the presence of 10nM TPA, growth was greatly retarded, cell numbers were inhibited by 55% compared to control A549-US cultures and by 61% compared to A549-US cell numbers in the presence of fetuin (figure 27a). Interestingly, upon introduction of 10pM EGF into the medium supplemented with 2% US plus fetuin, cell numbers were reduced by 26.2%. In the presence of 10nM TPA, cell growth was inhibited by 72.64% compared to cells grown in medium supplemented with 2% US and fetuin alone.

The response of cells maintained in serum-free media fortified with fetuin was investigated more thoroughly. In section 4.1.3, it was established that the growth of cells cultured in medium supplemented with ITS premix plus fetuin was arrested by 10nM TPA in a manner identical to A549-FCS cells and that only in the presence of fetuin was ITS able to support long term A549 culture (section 4.1.2). Indeed, fetuin has been reported to promote the *in vitro* growth of many cell lines (Fisher *et al.*, 1958). The ability of A549-FCS cells to incorporate labelled thymidine was determined following 48h exposure to 10nM TPA in experimental media containing 10% FCS, ITS only and ITS plus fetuin. Similarly, DNA synthesis in A549-US cells was examined following 48h exposure to 10nM TPA in the presence and absence of fetuin. The results are demonstrated in figure 28. It was established that the introduction of fetuin into the serum-free environment imparts a dramatic reduction in the ability of cells to synthesize DNA in the presence of 10nM TPA, compared to their control TPA-free counterparts. Incorporation of [^{3}H]TdR in A549-US cells following 48h exposure to 10nM TPA was 105% compared with [^{3}H]TdR incorporation in the absence of TPA. In the presence of fetuin however, DNA synthesis was inhibited by 54.8% by 10nM TPA. The experiments described above suggest that the presence of fetuin is a key factor in determining the inhibitory potential of TPA in A549 cultures. A549-US cells were maintained in medium fortified with 2% US plus fetuin for 4 passages before a growth curve was conducted in the presence or absence of 10nM TPA. The inhibition of [^{3}H]TdR incorporation evoked by TPA was monitored over time and the effect of different concentrations of TPA on the ability of this A549 population to synthesize DNA following 24h exposure was investigated. Figure 30, representing the time course inhibition of [^{3}H]TdR incorporation by cells in the presence of 10nM TPA reveals that reduction in uptake of labelled thymidine was immediate. Potent arrest of DNA synthesis occurred, and was maximal between 9 and 24h exposure. The experiment was

monitored up to a 72h exposure period. Partial recovery of DNA synthetic activity was observed, but complete re-establishment of proliferative potential was not achieved: 40% inhibition of [^{3}H]TdR incorporation was maintained after this time. The growth of cells in serum-free medium fortified with fetuin is demonstrated in figure 29a. In contrast to A549-US cultures, no initial lag in growth rate was obtained, a uniform doubling time of approximately 30h was observed. Moreover, the presence of 10nM TPA, significantly retarded cell growth. This is demonstrated clearly in figure 29b. The effect of 10nM TPA is contrasted in fetuin-fortified and fetuin-free A549-US populations. Cell counts were performed daily and numbers obtained from cultures in the presence of 10nM TPA are presented as a % of cell numbers in the absence of phorbol ester. Fetuin-fortified A549-US cells didnot adopt such distinctive extended morphological alterations, characteristic of A549-FCS cells in the presence of 10nM TPA. Initially, the cells appeared rounded and protruding and following 72h exposure, they were still significantly changed from TPA-free cultures, illustrated in figure 32. After 24h exposure to increasing concentrations of TPA, a biphasic concentration response curve was obtained (figure 31). Maximum inhibition of [^{3}H]TdR incorporation occurred after treatment with 1nM and 10nM TPA. TPA (1μM) diminished DNA synthesis by 41%. The biphasic profile, although not as pronounced as those evoked by TPA in A549-US cells (figure 13) or bryo 1 in A549-FCS cells (section 4.6), supports the conclusions pertaining to the dual action of PKC activators. The ability of TPA to trigger a proreplicative response may possibly occur following occupation of a second receptor site and the presence of fetuin may partially mask such a proreplicative response. Thus, fetuin may prove an important factor of foetal serum which results in the amplification of TPA-induced growth arrest in A549 cells. The observation that FCS, which contains AFP as a major protein, augmented the effect of TPA dramatically, whereas 10% NBCS, whose AFP levels have fallen significantly, was much less effective is consistent with the hypothesis that AFP may act as a key modifier of the effect of TPA on A549 cellular proliferation.

Amplification of growth inhibition by fetuin was selective. Fetuin was unable to augment bryo-induced arrest in DNA synthesis in A549-US cells (section 4.6). Moreover, evidence was obtained which strengthened support for the hypothesis suggesting a role for fetuin in amplification of TPA-induced growth arrest. Bryo 1 was able to eliminate the augmentation of TPA-evoked growth inhibition by fetuin in A549-US cells.

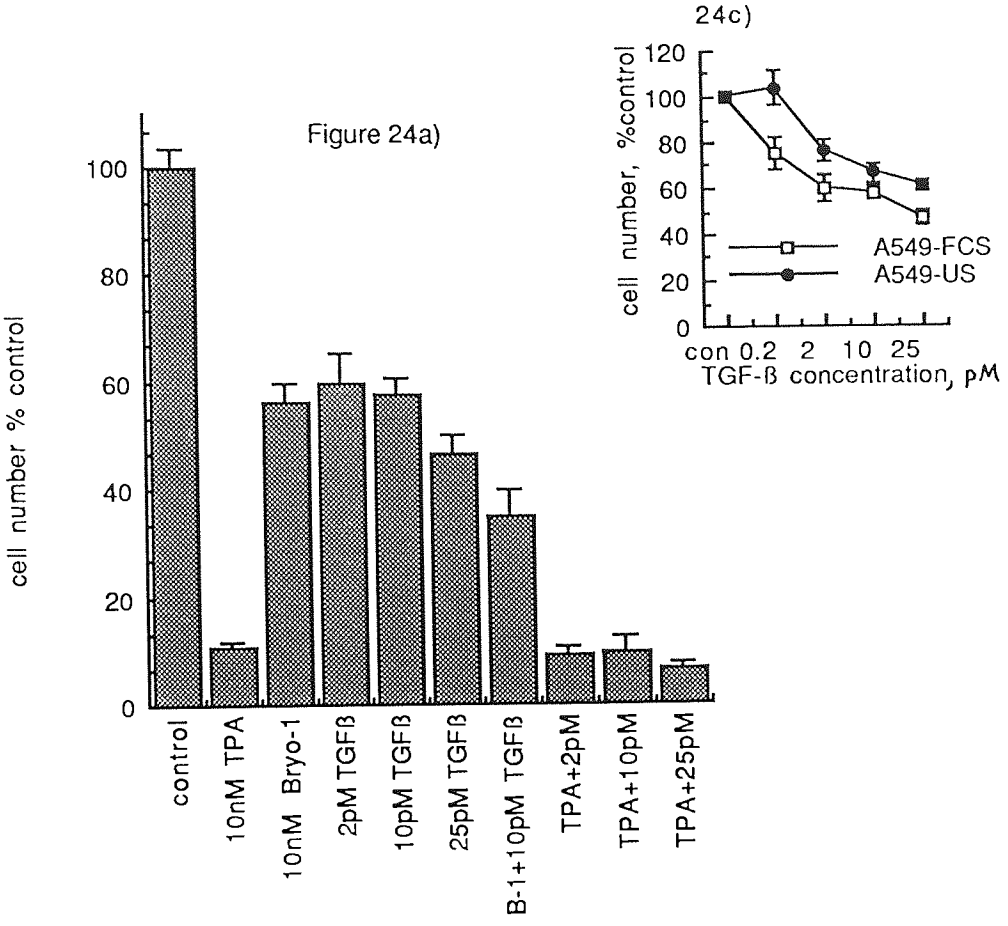
AFP is known to co-operate with growth regulators such as EGF (Leak *et al.*, 1990) and oestrogen (Jacobson *et al.*, 1990). Injection of the reaction product of oestradiol and AFP resulted in cessation of oestrogen dependent breast cancer cell growth and regression of MCF-7 tumour xenografts. Its properties as an antioestrogen have been reported previously (Sonnenschein *et al.*, 1975, 1980; Soto *et al.*, 1980). AFP may prevent oestrogen-sensitive tumour cell growth and control the malignant properties of oestrogen-dependent tumour cells in adult hosts in a dose-dependent fashion. The effect of phorbol esters and hormones on the growth of rat hepatoma cells producing AFP has been examined (Kaneko *et al.*, 1990). In a short term incubation, both TPA and EGF caused a transient 2-fold increase in AFP production and enhanced cellular proliferation.

Interestingly, pulse chase studies have revealed that TPA-sensitive HL-60 cells released phospholipids into the medium whereas TPA-resistant cells released sterols and fatty acids (Malvoisin *et al.*, 1988). Cholesterol and phospholipids play a major role in membrane viscosity and these authors have concluded that sterol composition may regulate specific biochemical processes in the membrane and can be considered as a factor that plays a role in the responsiveness of HL-60 cells to TPA. This would be a valid hypothesis to investigate in A549 cell populations. In the light of the above findings, it may be speculated that the cellular environment, specifically the presence of FCS, may influence membrane lipid composition and that the physical state of membrane lipids may affect the cells` capacity to respond to TPA. Ways *et al.* (1986), have described U937 cells which, in serum-free medium did not differentiate in response to phorbol ester treatment as well as cells cultured in medium supplemented with 5% FCS. It was demonstrated in this study that serum lipoproteins were important modulators of the TPA-induced response.

The cellular growth response observed appears to be governed by many interacting factors and intimates crosstalk between signal transduction mechanisms. It has been established that PKC is a key enzyme in growth factor mediated signal transduction. Serum factors such as EGF and fetuin may elicit recruitment of PKC activity or of a particular PKC isozyme from a cellular compartment which is inaccessible to TPA in the absence of serum, or bryo. The subsequent activation of this isozyme may be important for maximal growth arrest. Alternatively, a synergistic response may arise from the ability of serum factors to precipitate processes sequential and possibly unrelated to PKC activation in the presence of phorbol esters.

The work performed in the following section was designed to aid elucidation of the role of PKC in the induction of A549 growth arrest by TPA.

Figure 24. Effect of TGF-ß on the growth of a) A549-FCS and b) A549-US cell populations in the absence or presence of other modulators of cell growth.
Cells (2 x 10^4) were seeded and allowed 4h to attach. Following 96h exposure to indicated compounds, cell counts were performed (mean ∓ SD, n=9).



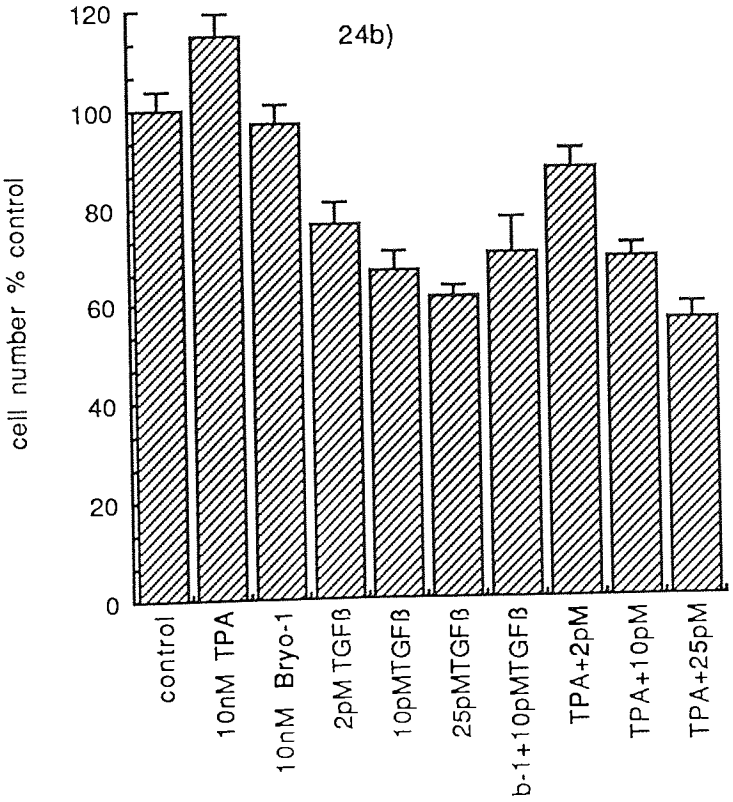
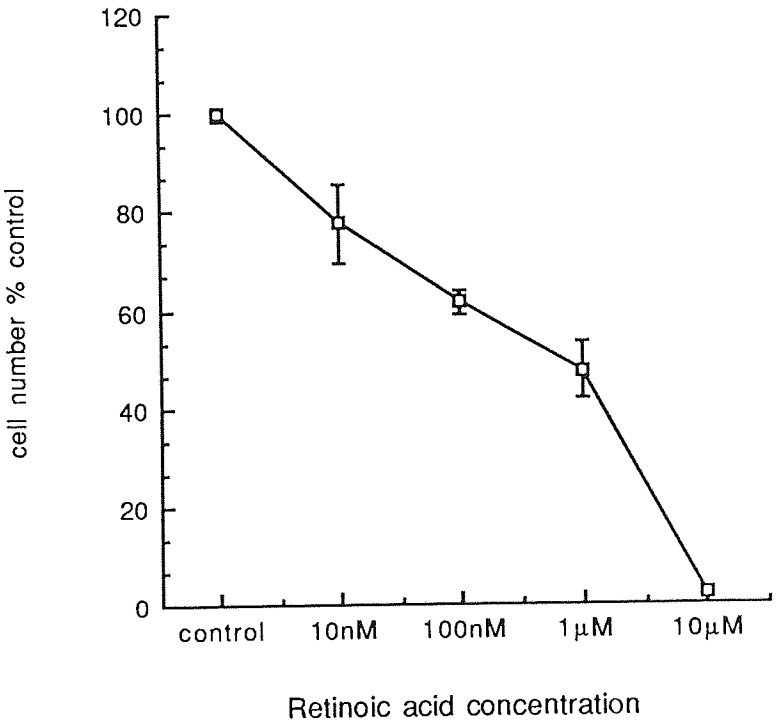
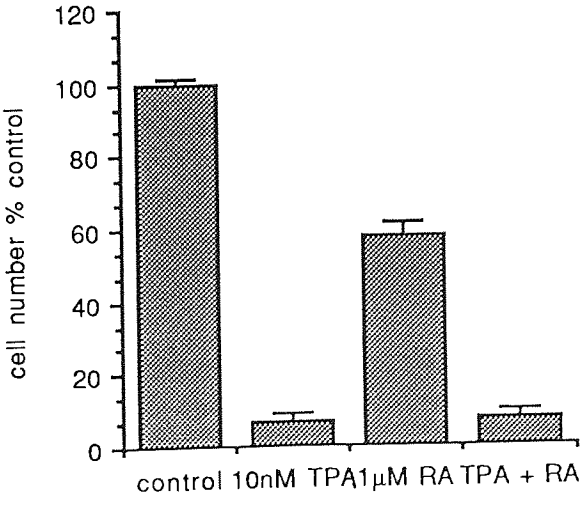


Figure 25. Effect of retinoic acid on A549-FCS cell growth.
Cells (2×10^4) were seeded and treated 4h later with a) increasing concentrations of RA; b) 1μM RA in the presence or absence of 10nM TPA. Cell counts were performed following 96h exposure (mean ∓ SD, n=9).

Figure 25a)



25b)



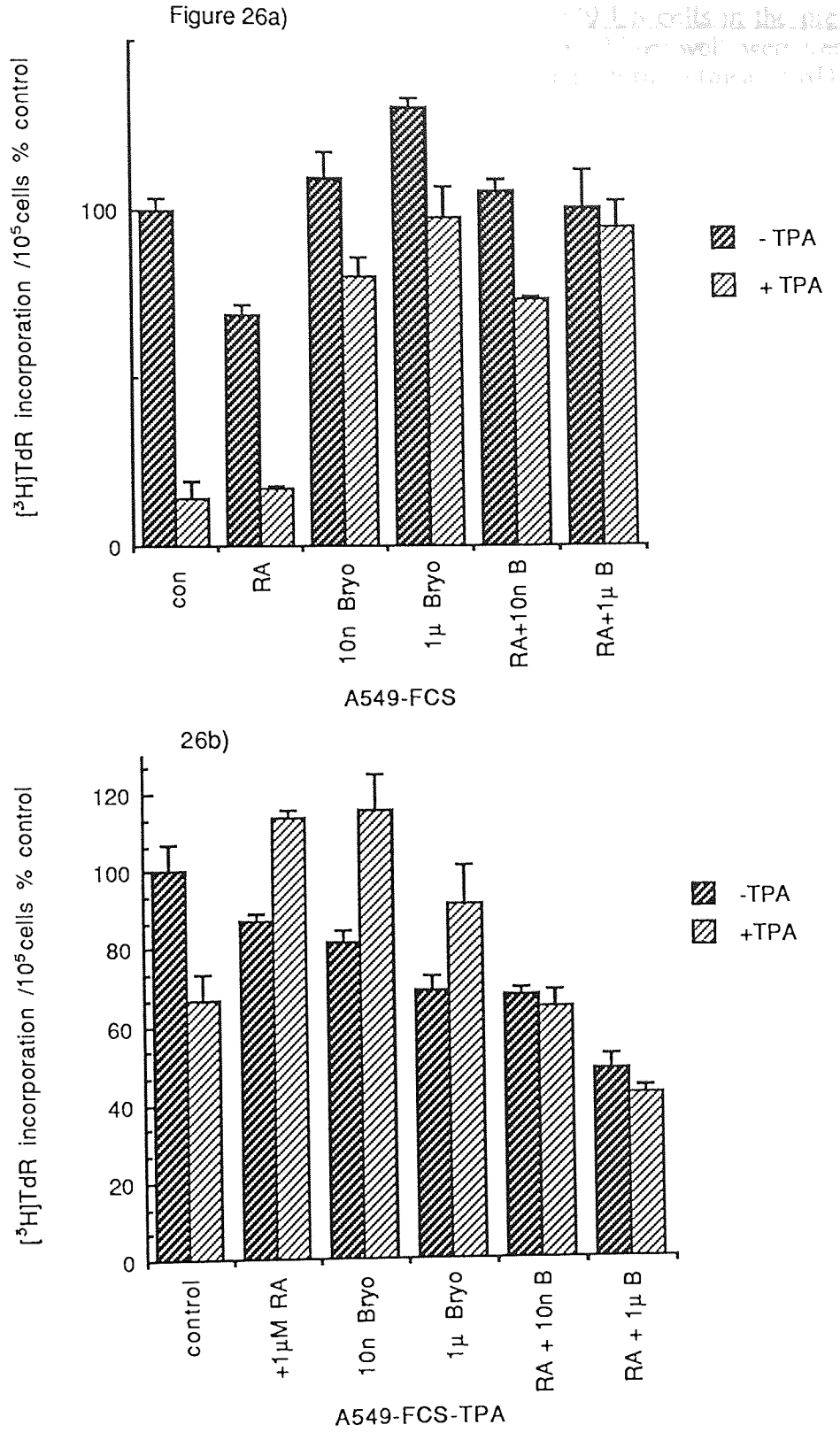


Figure 26. Effect of retinoic acid on A549-FCS TPA-sensitive (a) and `resistant` (b) cell proliferation in the presence or absence of 10nM TPA and/or 10nM bryo 1. Cells (2 x 10^5) were seeded and allowed 4h to attach before treatment began. Following 24h exposure to indicated compounds, [^{3}H]TdR incorporation was assessed (mean $\bar{+}$ SD, n=6-9).

Figure 27a. Effect of 10nM TPA on the growth of A549-US cells in the presence or absence of various factors. Cells, seeded at a density of 2 x 10^4 per well, were treated with agents 4h later. Following 96h exposure, cell counts were performed (mean ∓ SD, n=6).

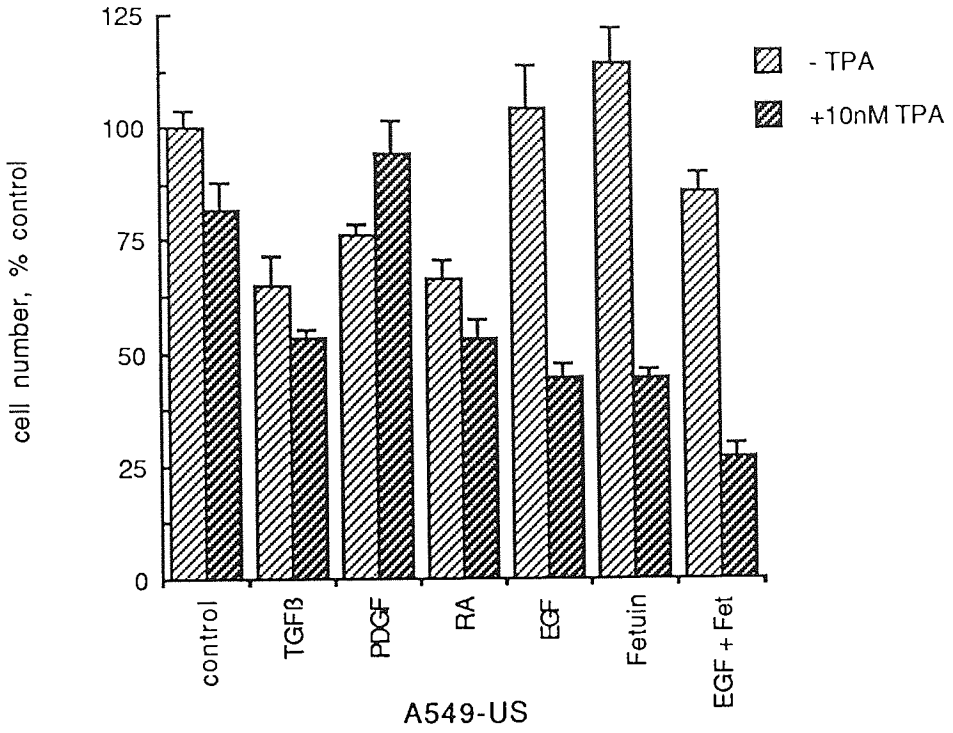


Figure 27b. Effect of factors on the growth of A549-TPA cell populations in the presence or absence of 10nM TPA. Cells were seeded at a density of 2 x 10^4 per well and treated with agents 4h later. Counts were performed following 96h exposure (mean ∓ SD, n=6).

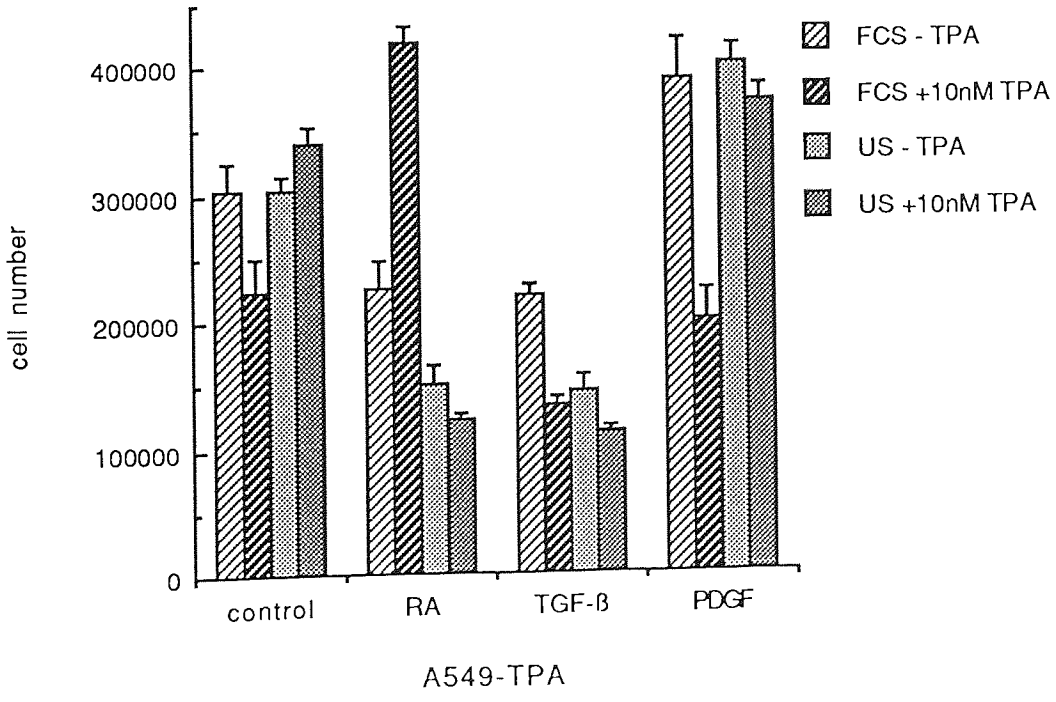


Figure 28

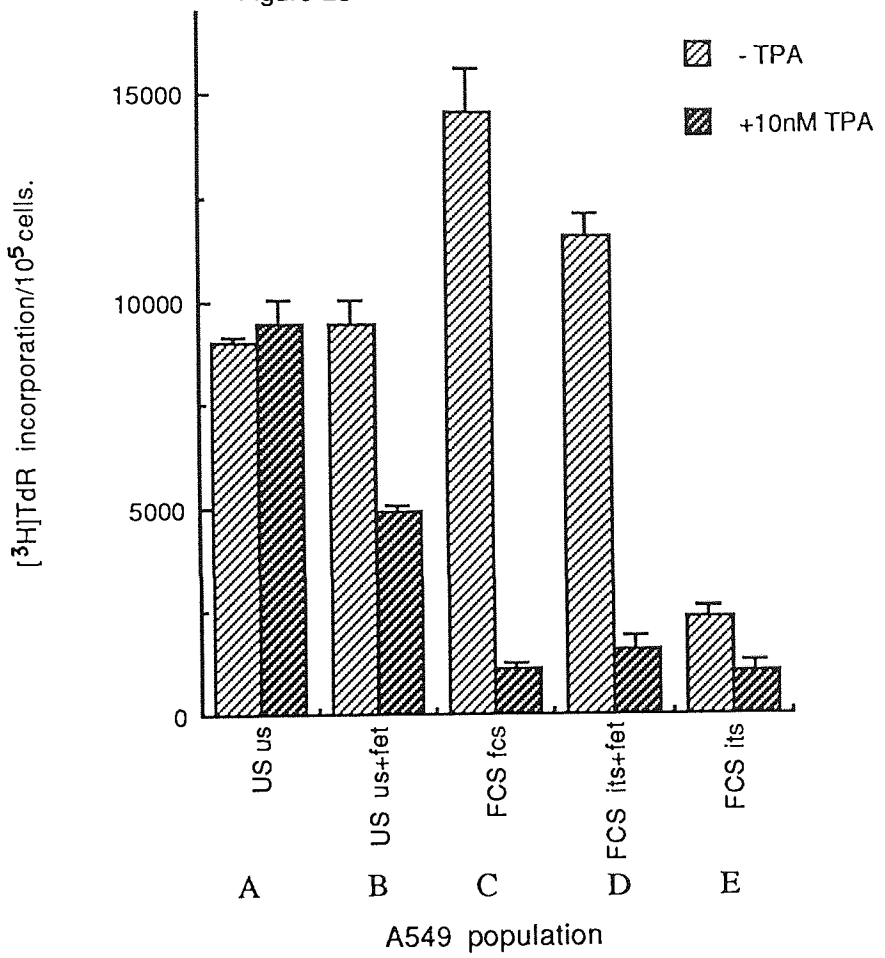
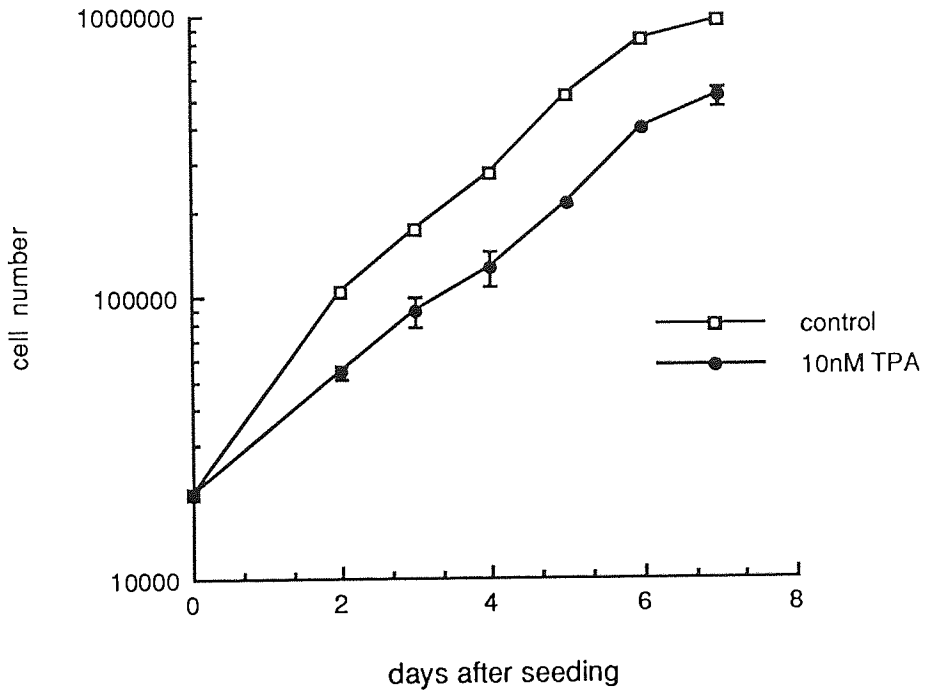


Figure 28. Effect of TPA on the proliferation of cells in different media conditions. A549-US cells (A,B) and A549-FCS cells (C,D,E) (10^5) were seeded in media supplemented with 2%US without (A) and with (B) 2mg/ml fetuin; 10% FCS (C);1% ITS without (D) and with (E) 2mg/ml fetuin. Following 48h exposure to 10nM TPA [^{3}H]TdR incorporation was determined (mean $\pm$ SD, n=9).

Figure 29. Growth of A549-US cells in medium supplemented with 2mg/ml fetuin in the presence or absence of 10nM TPA. Cells (2 x 10^4) were seeded and treated with 10nM TPA 4h later.Counts were performed daily (mean ± SD, n=9).
a) demonstrates the growth profile
b) contrasts A549-US cell numbers in the presence of 10nM TPA, represented as a % age cell number in the absence of TPA in 2%US-supplemented medium lacking (-□-), with medium fortified with 2mg/ml fetuin (-◆-) (mean ± SD, n=9).

Figure 29a)



29b)

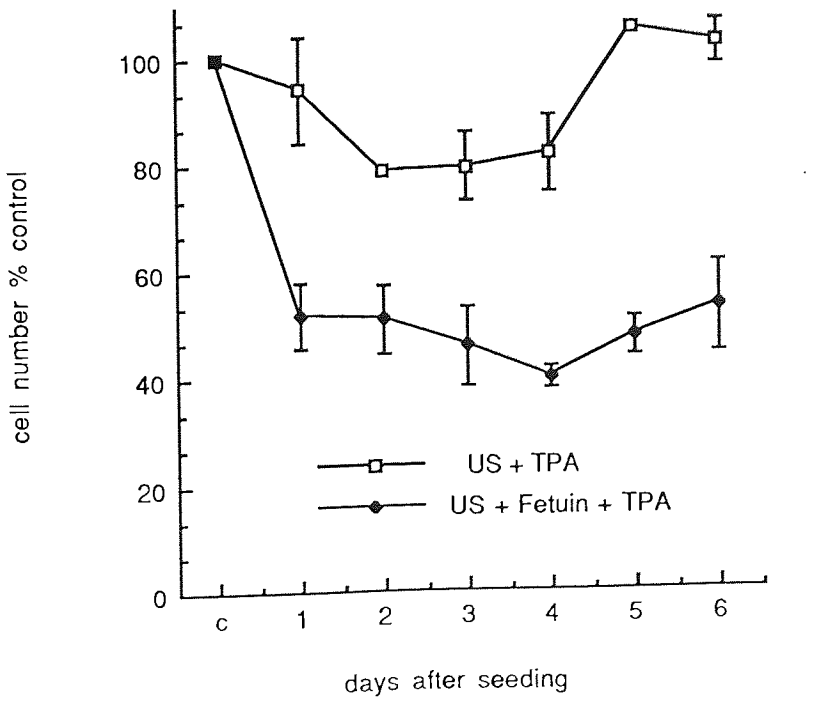


Figure 30. Time course inhibition of DNA synthesis by 10nM TPA in A549-US cells maintained in medium fortified with 2mg/ml fetuin. Cells (10^5 or 2 x 10^5) were seeded and allowed 4h to attach before treatment began. [^{3}H]TdR incorporation was assessed following incubation periods of various lengths (mean ± SD, n=9).

Figure 30

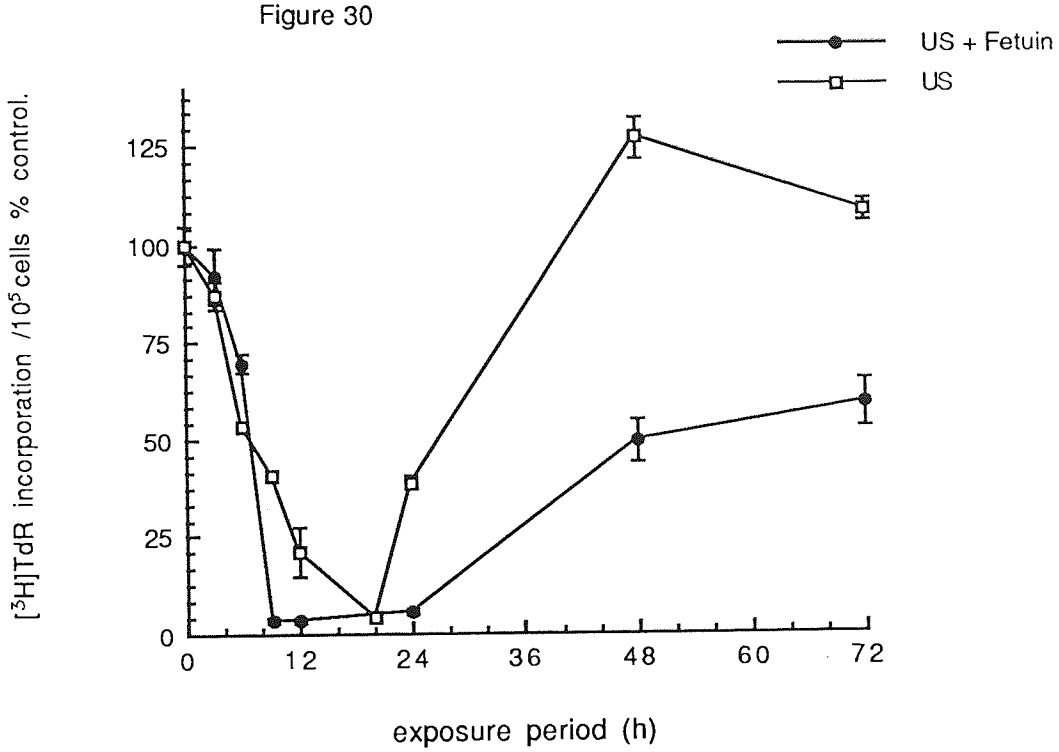


Figure 31. Effect of increasing concentrations of TPA on proliferation of A549-US cells supplemented with 2mg/ml fetuin. Cells (2 x 10^5) were seeded and treated 4h later. Following an incubation period of 24h, [H]TdR was determined (mean ± SD, n=9).

Figure 31

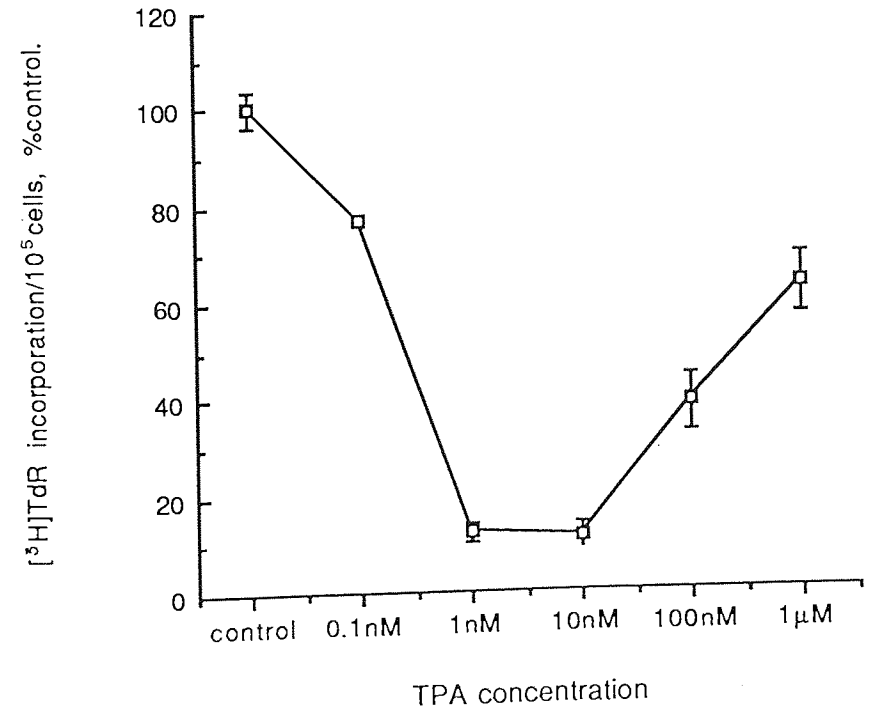
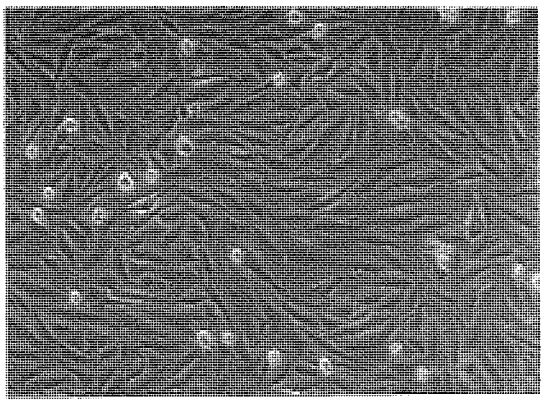


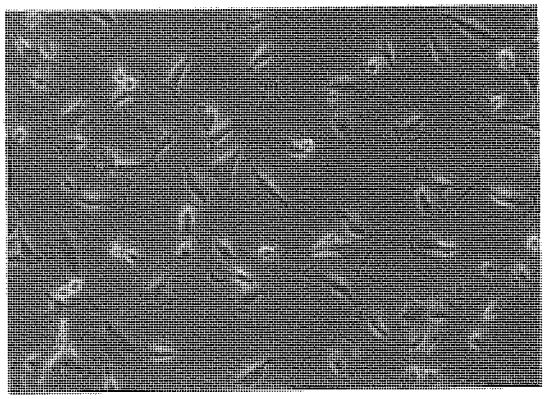
Figure 32. Phase contrast micrographs of A549-US cells maintained in medium fortified with a) 2mg/ml fetuin b) 10pM EGF (magnification x 20).
a) cells (10^5) were seeded and photographs taken following 48h incubation period in the absence (i) or presence (ii) of 10nM TPA.
b) cells (2×10^4) were seeded and photographs taken following 144h incubation period.
i) A549-US, ii) A549-US + TPA, iii) A549-US/EGF, iv) A549-US/EGF + TPA.

A

i



ii



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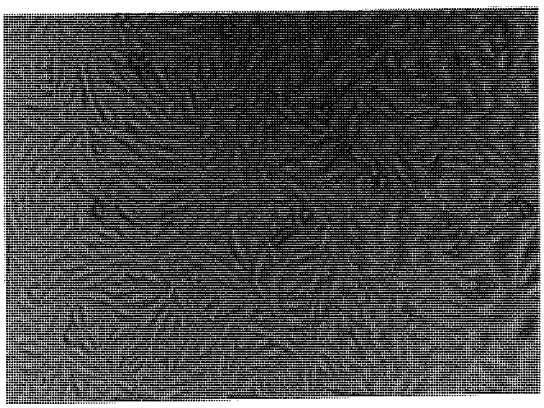

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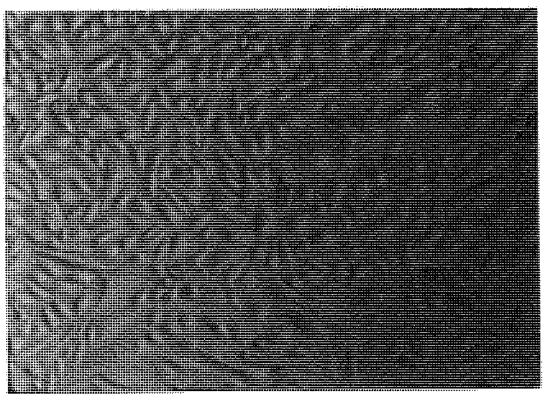
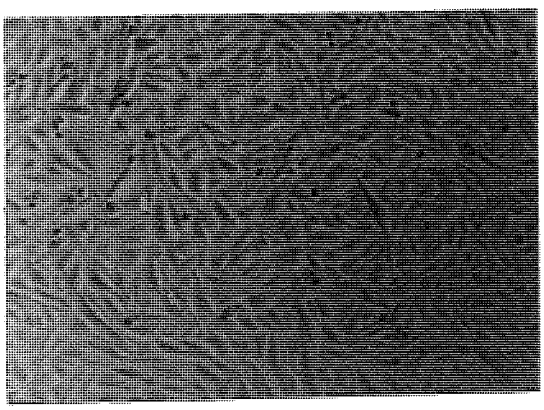


iii



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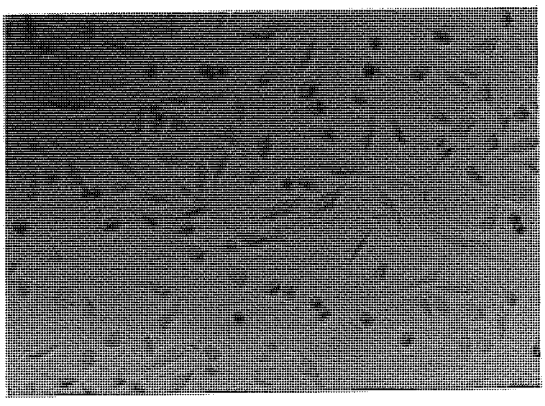


4.2 Investigation of the role of PKC in inhibition of A549 cell growth by phorbol esters.

4.2.1 Examination of the subcellular distribution of PKC activity.

Introduction.

The direct determination of PKC activity in crude cellular extracts is impractical because of the presence of other kinase activities and undefined endogenous PKC inhibitors. Fabbro *et al.* (1985) have developed a method for the quantitative estimation of subcellular PKC activity by polyacrylamide gel electrophoresis under non-denaturing conditions. We have adapted this technique to investigate the ability of phorbol esters to influence the subcellular distribution of PKC activity in A549 cells. Enzyme activity was assayed using protamine sulphate as the exogenous phosphate acceptor (Kikkawa *et al.*, 1983). In the absence of cofactors, protamine stabilizes the active conformation of PKC (Thompson *et al.*, 1988).

Results and discussion.

Several of the PKC assays described in this section were carried out in collaboration with Dr. I.L. Dale. Subcellular fractionation of untreated A549-FCS cells revealed that the specific activity of PKC in cytosol and membrane fractions was 1792.2 and 176.2 units per mg protein respectively. Thus in control populations, only 9% of total PKC activity was recovered in the membrane. In contrast, A549-US cells yielded specific PKC activities of 795.3 and 78.4 units/mg protein in cytosolic and particulate extracts respectively. Thus, these cells possessed only 44.4% of the total enzyme activity measured in the A549-FCS parent population. However, despite the substantial loss of activity in the serum-free environment, 91.0% of the total activity was located within the cytosolic fraction, a distribution comparable to that observed in A549-FCS cells (figure 33). One may speculate that there is a link between PKC activity and cellular response to tumour promoting phorbol esters. Consistent with this theory, the mitogenic response to TPA was enhanced 3-4-fold over controls, in NIH 3T3 cells overexpressing the rat PKC-γ gene and displaying a 3-fold increase in total PKC levels (Cuadrado *et al.*, 1990). It may be postulated that the decreased levels of functional PKC activity in A549-US cells may be related to the slower growth rate of these cells at lower densities. Consistent with this proposal are reports that transfection of PKC subspecies γ and ß into cells may result in enhanced growth rate and tumourigenicity (Housey *et al.*, 1988; Persons *et al.*, 1988; Krauss *et al.*, 1989). It is possible that an environment rich in foetal calf serum may maintain increased levels of PKC. Indeed, re-addition of serum to serum-deprived U937 monoblastic cells led to the resumption of growth associated with a 2-fold increase in particulate PKC activity (Ways *et al.*, 1986). The importance of PKC activity during embryogenesis and the developmentally regulated expression of this enzyme has been documented (Yoshida *et al.*, 1988; Sugden, 1989).

In 1983, it was demonstrated that following treatment of thyoma cells with phorbol esters, PKC adopted a form tightly associated with the particulate fraction (Kraft and Anderson, 1983). It was subsequently proposed that translocation of PKC activity to the membrane

was an early event associated with the specific biological actions of phorbol esters. We wished to examine the subcellular distribution of PKC activity in A549-FCS and A549-US cell cultures following an exposure period of 30 mins to various concentrations of TPA, in order to compare enzyme translocation in these two populations. Figure 34 demonstrates a concentration-dependent shift of PKC activity from cytosol to particulate fractions in both A549 populations. Concentrations of TPA, derived from the values shown in figure 34, calculated as able to induce half maximal translocation of enzyme activity were 41.2nM and 59.0nM for A549-US and A549-FCS cells respectively. Thus, it can be surmised that the resistance to protracted growth inhibition by TPA observed in the A549-US cell population was not associated with a weaker ability of TPA to induce PKC translocation. Initiation of enzyme down-regulation was evident after 30 min exposure at concentrations exceeding 100nM.

A549-FCS cultures were exposed to increasing concentrations of the weaker phorbol ester tumour promoter PDBu and the inactive phorbol ester, 4-αPDD, for 30 mins before enzyme purification and subsequent assay for PKC activity. Reflecting its weaker activity as a tumour promoter (Shoyab and Todaro, 1980) and consistent with its decreased potency in arresting DNA synthesis in A549-FCS cells, PDBu elicited weaker translocation than TPA at equal concentrations (figure 35). The concentration of PDBu calculated as capable of inducing half maximal PKC translocation was 1.8μM. The 4-α analogue of PDD, which is inactive as a tumour promoter and unable to induce A549 growth arrest at non-toxic concentrations, was unable to evoke any shift in enzyme activity even at a concentration of 1μM (figure 36). These observations are in accordance with the hypothesis that enzyme translocation is an important prerequisite to biological activity. Down-regulation of PKC activity was not evident following treatment with either of these two phorbol esters.

The effect of TPA on the subcellular redistribution of PKC in human breast cancer cell lines exhibiting different degrees of sensitivity to growth inhibition by TPA has been examined by PAGE (Regazzi *et al.*, 1986). In this study, no correlation was discovered between cellular PKC content or extent of enzyme translocation in particular cell lines and their sensitivity to TPA. Phorbol esters less potent than TPA induced only partial PKC translocation; a decrease in cytosolic activity was monitored without a rise in particulate activity. Inactive esters induced no subcellular enzyme shift. These authors concluded that tumour promoter-mediated growth arrest of breast cancer cell lines was a consequence of mechanisms occuring after the translocation of PKC.

In experiments described in this thesis, the prolonged exposure of A549 cells to TPA led to protracted PKC activation followed by time and concentration dependent down-regulation of enzyme activity in both A549-FCS and A549-US populations. Recruitment of enzyme activity to the particulate fraction was observed after 5 min treatment with concentrations of TPA equal to or exceeding 100nM. Accumulation of membrane bound PKC was observed until enzyme activity in cells maintained in a serum-supplemented environment declined following 5h, 2h and 30 min exposure to 10nM, 100nM and 300nM TPA respectively

(figure 37). Following 24h exposure to each concentration, negligible PKC activity could be detected. In A549-US cultures, down-regulation of PKC activity commenced after treatment with 10nM TPA for 3h; after 24h exposure, little activity remained (figure 38). PKC activity was also examined in cells desensitized to the growth-inhibitory properties of TPA. Enzyme activity could not be detected in A549-FCS or A549-US cells exposed to 10nM TPA for 3, 6, or 10 days. After 3 days, the growth of A549-FCS cells only was arrested. Following 6 or 10 days exposure to 10nM TPA, both A549 populations had recovered growth potential. Moreover, cell populations maintained in excess of 9 weeks in an environment fortified with 10nM TPA, more permanently resistant to growth inhibition by TPA, demonstrated no detectable PKC activity. Thus, we may tentatively conclude that the phenomenon of PKC down-regulation is not a significant factor leading to inhibition of A549 cell growth. The activation of PKC may prove a more important factor responsible for the initiation of growth inhibition, but appears to play no role in mechanisms leading to recovery of cellular proliferative potential. However, it should be appreciated that this enzyme assay may not be sensitive enough to detect the presence of minute shifts in enzyme activity. It has been reported that activation of a very minor proportion of total cellular PKC content may suffice to elicit PKC-dependent responses (Bosca *et al.*, 1989). During the process of enzyme down-regulation, induced by TPA or bryos (section 4.6) three peaks of protein kinase activity, illustrated in figure 85, were resolved by PAGE. This phenomenon has been described in human breast cancer cells by Fabbro *et al.* (1986). The activity in the major protein kinase peak was phospholipid/Ca^{2+} dependent and corresponded to the PKC holoenzyme. Activities associated with the two minor peaks were the phospholipid/Ca^{2+} independent PKC proteolytic fragments. Loss of these products of enzyme proteolysis was more rapid in cells treated with higher concentrations of agents.

Following the identification of PKC as the phorbol ester receptor, down-regulation of enzyme activity was initially reported after treatment of 3T3 fibroblasts with TPA (Rodriguez-Pena and Rozengurt, 1984). It has since been established that PKC down-regulation is caused by an accelerated rate of degradation of the PKC molecule (Woodgett and Hunter, 1987; Young *et al.*, 1987; Mitzuguchi *et al.*, 1988; Isakov *et al.*, 1990). Such degradation is a consequence of protracted enzyme activation in the presence of TPA, which is poorly metabolized by cells (Welsh and Cabot, 1987). The prolonged incubation of many cell types with tumour promoting phorbol esters results in depletion of cellular PKC activity (Blackshear, *et al.*, 1985; Hovis *et al.*, 1986; Pasti *et al.*, 1986; Stabel *et al.*, 1987; Adams and Gullick, 1989), often accompanied by resistance to many phorbol ester-induced responses (Gainer and Murray, 1985; Glynn *et al.*, 1986). It has been concluded that the inhibition of human skin fibroblast proliferation by phorbol esters is mediated *via* the activation of PKC (Johnson and Johnson, 1990). Down-regulation of PKC by long term treatment with phorbol ester resulted in the elimination of the ability of phorbol ester to arrest DNA synthesis. We have observed that TPA-treated PKC-depleted A549 populations are able to acquire resistance to the growth arresting properties of tumour promoting phorbol esters and display insensitivity to growth inhibition by other activators of PKC (see section 4.6, figure 88). Additionally, cells depleted of PKC by prolonged

exposure to bryo 1 were unresponsive to TPA even at time points when the presence of TPA alone was able to induce growth arrest. This finding may lead to the proposal that the activation of PKC may be responsible for initiation of growth arrest and the extended inhibition of growth observed in A549-FCS cells may be the result of events triggered in the presence of serum, sequential to PKC activation. No detectable PKC activity was found in mutant T lymphocyte cells which failed to respond to the mitogenic influence of TPA seen in wild type T lymphocytes (Mills *et al.*, 1988). Thus in certain cases enzyme activation appears critical for the ensuing phorbol ester-induced response.

However, other studies provide evidence supporting the hypothesis that down-regulation of functional PKC is an essential biochemical event in the mechanism by which tumour promoting phorbol esters elicit their response. For example, conditions promoting the growth of non-tumourigenic murine melanocytes require the constant presence of tumour promoter such as TPA (Bennett *et al.*, 1987). It is inferred therefore that the degradation of PKC is a prerequisite to the phorbol ester-induced response. The induction of enzyme down-regulation and subsequent growth arrest of M5076 tumour cells (Goode and Hart, 1990) was achieved following a single application of TPA or multiple additions of OAG or diC_8. Kischel *et al.* (1989), have proposed that the depletion or inactivation of PKC rather than enzyme activation correlates with the promotion of epidermal JB6 cells to anchorage dependent growth.

Inhibition of MCF-7 growth by TPA has been well documented (Osborne *et al.*, 1981; Regazzi *et al.*, 1986; Regazzi *et al.*, 1988; Issandou and Darbon, 1988; Issandou *et al.*, 1988; Darbon *et al.*, 1990). It has been speculated that since the removal of TPA from cultures led to the resumption of cell growth; growth arrest was a consequence of loss of functional PKC (Fabbro *et al.*, 1988). However, it has been demonstrated that diC_8 may mimic the action of TPA with respect to inhibition of MCF-7 proliferation, cell morphology and phosphorylation of the PKC substrate 28kDa stress protein (Issandou and Darbon, 1988; Issandou *et al.*, 1988). DiC_8 induced partial and transient PKC translocation from cytosol to particulate fraction but was unable to evoke enzyme down-regulation. In contrast, 90% PKC down-regulation followed treatment with TPA. Interestingly, the remaining 10% PKC activity proved sufficient to induce 28kDa protein phosphorylation. Hence, it was concluded that the activation of PKC, not down-regulation was a prerequisite for the biological response.

TPA-resistant variants have been selected in an attempt to clearly define the role of PKC in cellular responses elicited by phorbol esters. Abnormal enzyme translocation and down-regulation have been reported in such resistant populations. Homma *et al.* (1987), demonstrated failure of PKC to translocate following treatment with TPA in HL-60 cell variants resistant to induction of differentiation. A Swiss 3T3 variant cell line has been described which was mitogenically non-responsive to TPA (Bieman and Erikson, 1990). Expression and kinase activity of PKC were identical in variant and parent cells. However, unresponsive cells failed to exhibit PKC down-regulation in response to long term TPA treatment. Moreover, vastly reduced phosphorylation of the 80kDa PKC substrate was observed in the non phorbol ester responsive cells.

Interestingly, the TPA-unresponsive breast cancer cell line T-47-D, exhibited only very low levels of PKC activity (Fabbro *et al.*, 1986). Shoji *et al.* (1987) have investigated mechanisms involved in the induction of differentiation by TPA in the human KG-1 acute myeloid leukaemia cell line and compared it with the subline KG-1a, which displayed resistance to differentiation by TPA. PKC translocation and subsequent enzyme down-regulation was observed in both populations. However, KG-1a cells possessed levels of PKC activity 3 times lower than the parent population. Moreover, enzyme translocation to the plasma membrane occurred in sensitive cells, but primarily to the perinuclear region (Golgi apparatus and endoplasmic reticulum) in KG-1a cells. A 33kDa PKC substrate was predominantly phosphorylated in KG-1 cells, whereas in KG-1a populations, predominant phosphorylation of a 97kDa protein was observed. The treatment of sensitive EL4 thyoma cells with phorbol esters induced growth inhibition (Jenson *et al.*, 1990). It was found that these cells possessed RNA encoding PKC α, ß, γ and ε. Phorbol ester-resistant EL4 cells lacked PKC-ε RNA and expression and induction of jun A protein. The examination of PKC subspecies was undertaken in HL-60 cells selected by Nishikawa *et al.* (1990) for their resistance to induction of differentiation by TPA. Kinase activity in the cytosol of such cells was 30% of that seen in parental HL-60 cells. PKC-ß, a major isoform in the cytosol of HL-60 cells was selectively but not completely lost from cytosol of the resistant cells. These authors suggested that resistance of HL-60 cells toward TPA-induced differentiation may be associated with a decrease in the expression of PKC-ß. A phorbol ester tolerant HL-60 cell line has been selected which displayed resistance to the differentiating effects of TPA, yet retained sensitivity to other differentiating agents such as retinoic acid, DMSO and 1,25-dihydroxyvitamin D_3. Following treatment with phorbol ester, the growth of these cells was arrested for 48h. Thereafter, the original replication rate was resumed (Macfarlane *et al.*, 1988). It was found that these cells demonstrated over-expression of the N-*Ras* oncogene. Interestingly, *ras* transformed C3H $10T^{1}/2$ cells have been shown to possess reduced PKC levels (Haliotis *et al.*, 1990).

Variant lines of M5076, murine macrophage cells have been selected which display resistance to the antiproliferative effects of phorbol esters (Goode *et al.*, 1990). Levels of PKC, enzyme activation and down-regulation, determined by inducible 80kDa protein phosphorylation, were unaltered, as was c-*fos* mRNA expression. However, c-*fos* protein expression was reduced in cells unresponsive to growth inhibition by TPA.

It has been concluded by Guerrin *et al.* (1990), that resistance to TPA is unrelated to PKC. Experiments were performed which demonstrated that TGF-ß was able to restore sensitivity to growth arrest by TPA upon TPA-resistant MCF-7 cells. These authors have suggested that TGF-ß may interfere with the PKC pathway independently of enzyme activation. Kraft *et al.* (1987), Larsson *et al.* (1988), Gailani *et al.* (1989) and Jalava *et al.* (1990), have provided evidence suggesting that the expression, activation or down-regulation of PKC activity alone was insufficient to induce differentiation of HL60, U937 and SH-SY5Y cells following treatment with TPA.

Thus, increasingly, evidence is emerging to suggest a role for factors other than PKC

Figure 33. Subcellular distribution of PKC in A549-FCS and A549-US cell populations. PKC activity was measured following partial purification by non-denaturing PAGE (mean ± SD, n=3-6).

Figure 33

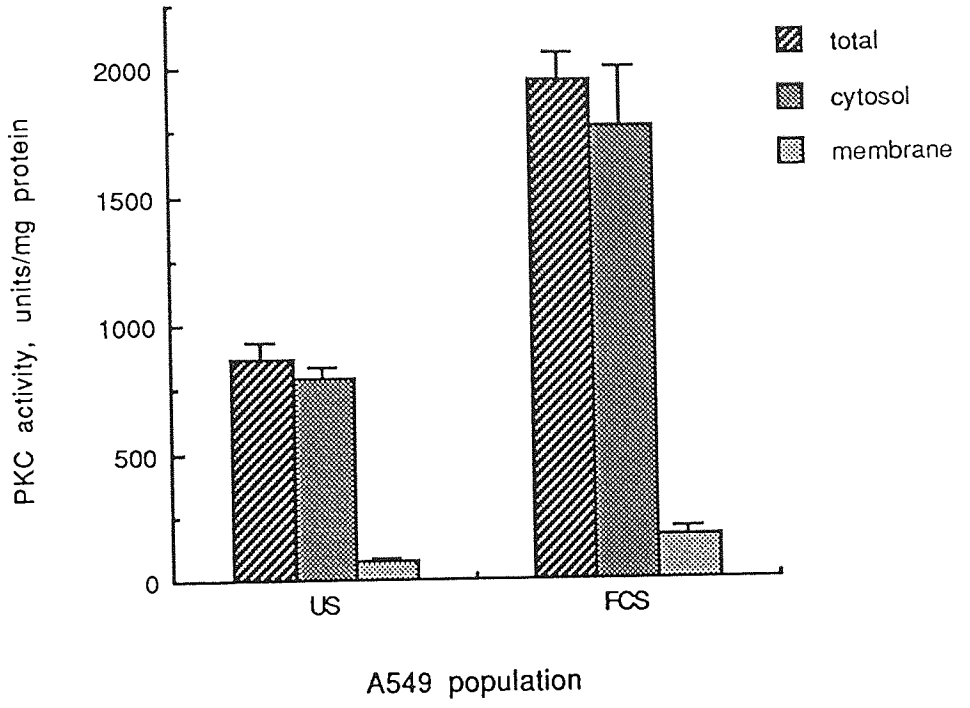


Figure 34. Translocation of PKC activity induced during exposure of A549-FCS and A549-US cells to increasing concentration of TPA for 30min. Activity was measured following partial purification by non-denaturing PAGE (n=2-3).

Figure 34

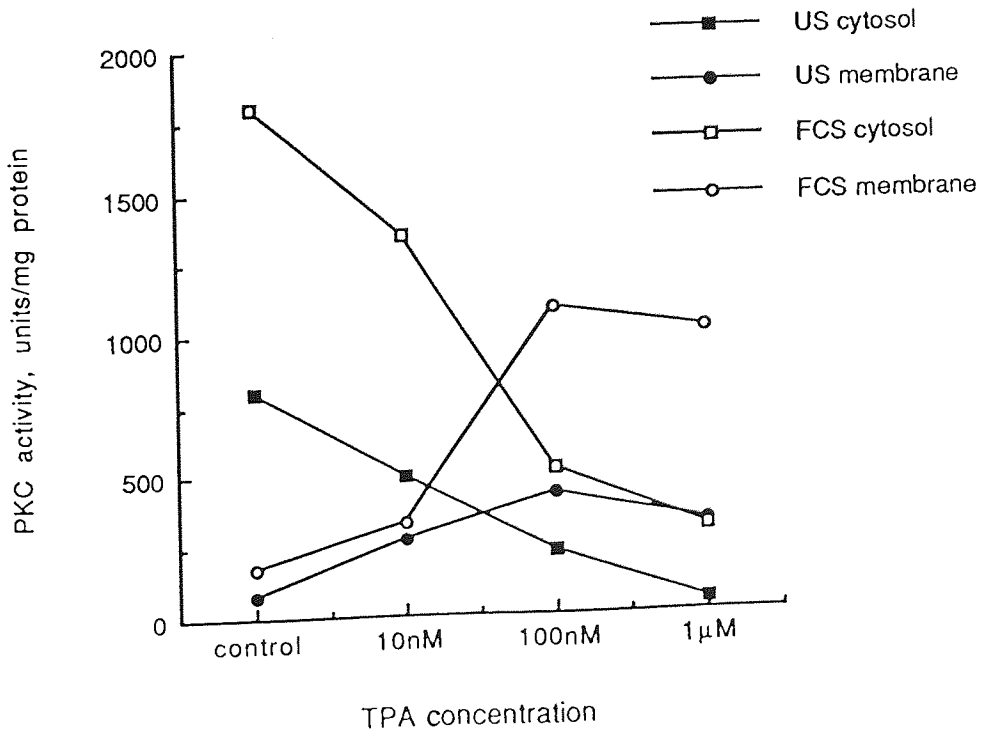


Figure 35. Translocation of PKC activity induced during exposure of A549-FCS cells to increasing concentrations of PDBu for 30min. Activity was measured following partial enzyme purification by non-denaturing PAGE (n=2).

Figure 36. Lack of translocation of PKC activity during exposure of A549-FCS cells to increasing concentrations of 4-α PDD for 30 min. Activity was measured following partial purification by non-denaturing PAGE (n=2).

Figure 35

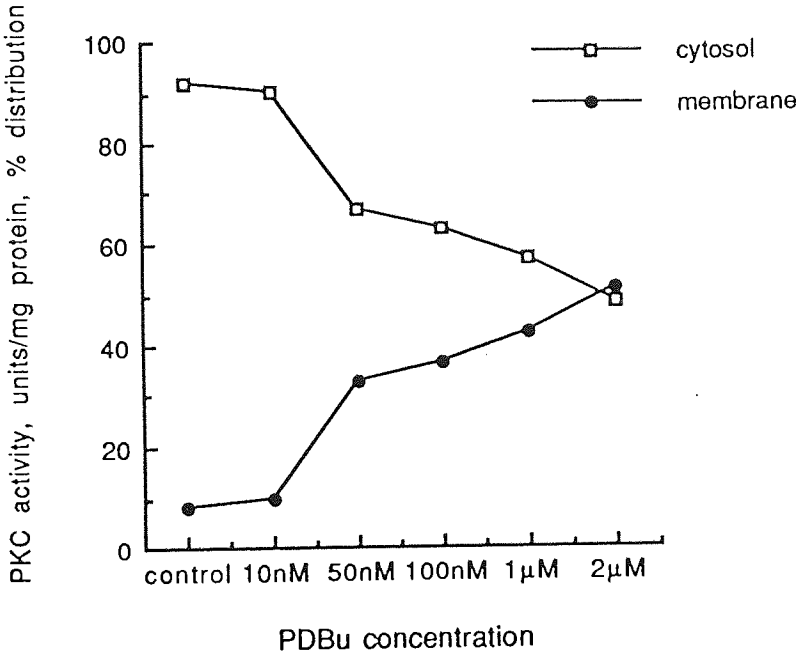


Figure 36

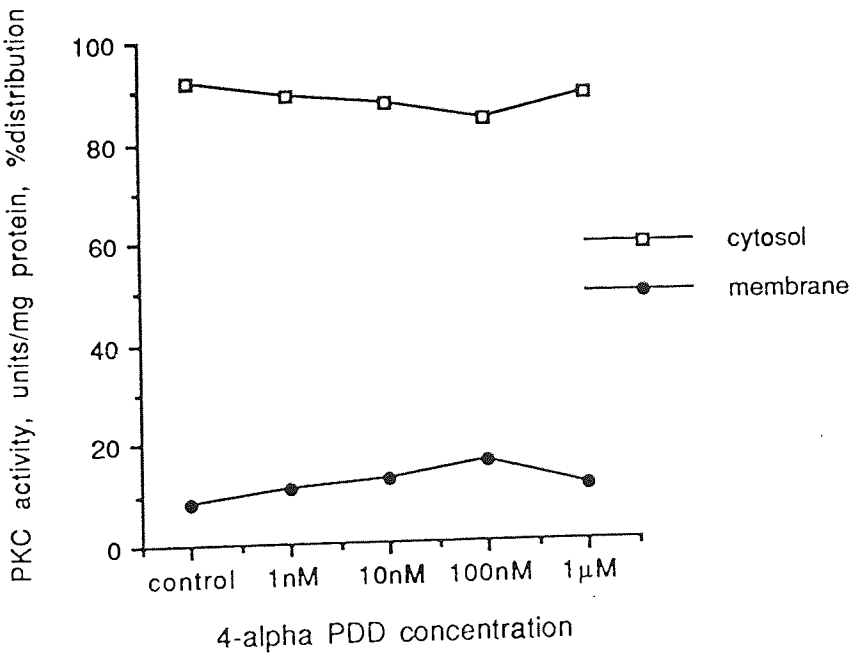


Figure 37. Translocation and down-regulation of PKC activity induced by TPA in A549-FCS cells. Cells were treated with a)300nM, b)100nM, c)10nM TPA for varying periods of time before PKC was partially purified and activity assessed in cytosolic and particulate fractions (n=2-3).

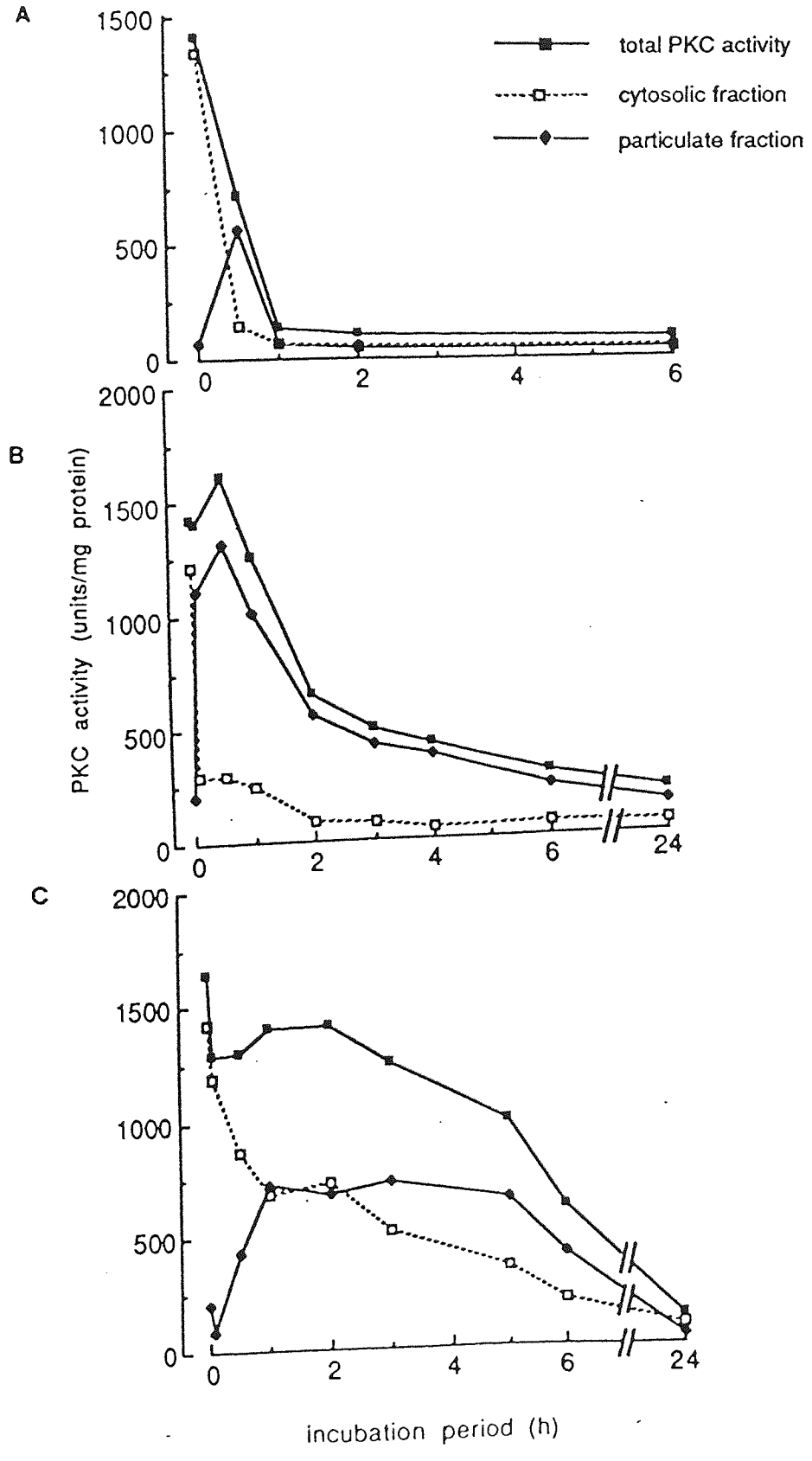


Figure 38

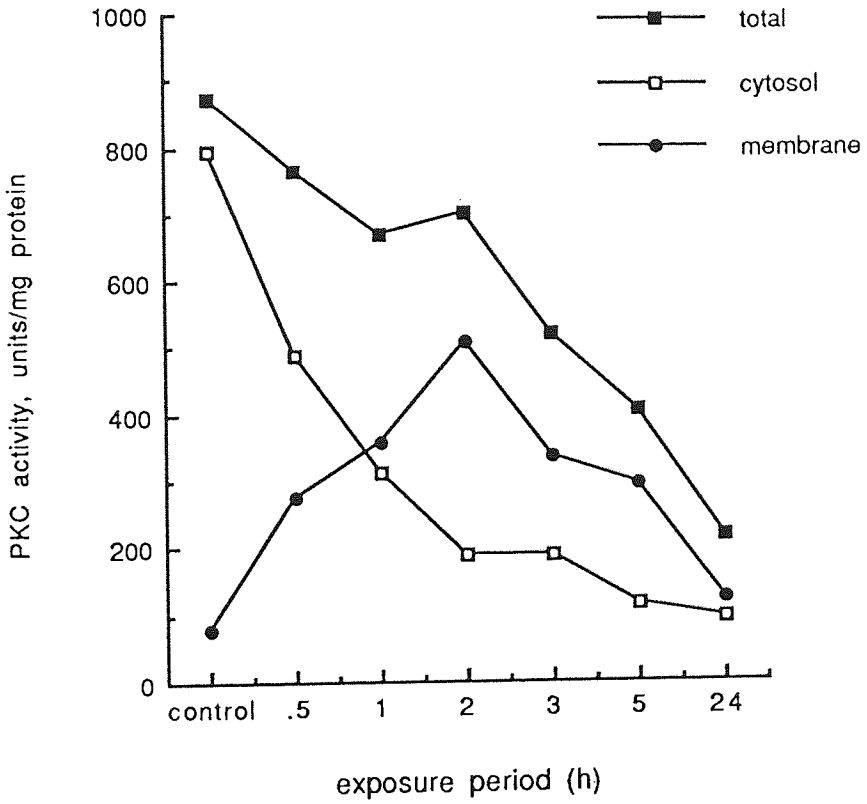


Figure 38. Translocation and down-regulation of PKC activity induced by 10nM TPA in A549-US cells. Cells were exposed to TPA for varying lengths of time before enzyme purification and measurement of activity (n=2).

modulation in the induction of cellular responses initiated by phorbol esters. The differentiation of a rhabdomyosarcoma cell line by TPA was not observed if the TPA-containing medium was replaced daily, indicating interaction of TPA with substances secreted by treated cells (Aguano *et al.*, 1990). Indeed, the results obtained from examination of A549 growth arrest by phorbol esters suggest a critical initial role for PKC activation but in addition, environmental factors are implicated which may be responsible for events distal to PKC activation which prolong inhibition of cell growth. Moreover, cells apparently devoid of active PKC, yet released from growth arrest, upon reseeding once again resume sensitivity to TPA (section 4.1.1).

4.2.2 Measurement of cytosolic phorbol ester binding.

Introduction.

Cytosolic phorbol ester receptor ligand binding was evaluated concurrently with assays measuring PKC activity. Using the physically defined, mixed micellar assay system described by Hannun *et al.* (1986) and Hannun and Bell (1987) and utilising [^{3}H]PDBu as ligand, specific and high affinity cellular receptors for biologically active phorbol esters have been measured in A549 cell populations.

Results and discussion.

Consistent with data reported by Hannun and Bell (1987), figure 39 illustrates that with increasing concentrations of labelled PDBu, an initial proportional rise in specific phorbol ester binding occurred which began to plateau at a concentration of 50nM, when saturation was achieved. Thence, 50nM [^{3}H]PDBu was adopted for all future binding assays. Moreover, binding assays performed using increased concentrations of [^{3}H]PDBu in the absence and presence 10nM TPA demonstrated consistently that between 49 and 57% labelled PDBu was displaced.

This assay was adopted to examine whether functional PKC detected in cytosol fractions correlated with cytosolic phorbol ester binding. Figure 40 illustrates that A549-US cells possessed a mean 36.2% cytosolic phorbol ester binding sites compared to the A549-FCS parent cell population.

Experiments revealed that cytosolic phorbol ester binding potential was lost in a time- and concentration-dependent manner following exposure of cells to TPA. Incubation of cells for 30 min with increasing concentrations of TPA, prior to the preparation of crude cytosolic extracts, resulted in a dose dependent decrease in specifically bound [^{3}H]PDBu in both A549 populations (figure 41). Consistent with the data obtained by measuring enzyme translocation from cytosolic to particulate fraction; concentrations of TPA able to reduce PDBu binding by 50% were calculated as 62.1nM and 43.5nM in A549-FCS and A549-US populations respectively. Cytosol prepared from A549-FCS cells pretreated for 30 min with 10nM, 100nM or 300nM TPA retained 69%, 47% and 21% binding potential respectively, compared with untreated controls (figures 41 and 42). Hence, the decline in specific PDBu binding was accelerated in cells exposed to higher TPA concentrations. Following 24h exposure to TPA, no phorbol ester binding was detected in cytosol extracts

prepared from A549-FCS or A549-US cells. These results correspond to the loss in PKC activity described (figures 37 and 38). Thus, A549-US cells, resistant to protracted growth inhibition by TPA, as well as A549-FCS cells were subject to time and concentration-dependent loss in cytosolic phorbol ester binding sites. Moreover, no specific phorbol ester binding could be detected in either cell population following 3, 6 or 10 days exposure to 10nM TPA, or in cytosol preparations of cells of A549-TPA populations. Figure 43 demonstrates that pre-treatment of cells with the inactive phorbol ester, 4-αPDD, up to concentrations of 1μM was unable to induce any decrease in PDBu binding. We have observed that data obtained from the mixed micelle assay for the measurement of cytosolic phorbol ester binding reflects the results obtained from assessment of enzyme activity in cytosolic extracts by PAGE. Thus, it can be concluded that cytosolic phorbol ester binding may represent a measure of active cytosolic PKC.

Loss of cytosolic specific phorbol ester binding corresponds in the short term to PKC translocation and in the long term to enzyme degradation, the latter a result of preferential proteolysis of active membrane bound PKC by calpain (Kishimoto *et al.*, 1983). The continued generation of PKC mRNA and the absence of protein in the presence of TPA has been documented (Isakov *et al.*, 1990). We wished to investigate whether, upon removal of phorbol ester from PKC-depleted cells, up-regulation of cytosolic phorbol ester binding occurred. It has previously been demonstrated by Dale (1989) that A549-FCS cells possessing a more permanent resistance to growth inhibition by phorbol esters revert back to the sensitive phenotype within 14 days, upon withdrawal of TPA from the surrounding medium. We wished to establish whether any correlation exists between this sensitivity and the re-emergence of cytosolic phorbol ester binding. Figure 4 demonstrated the reversible nature of phorbol ester-induced growth arrest upon removal of PDBu. The re-emergence of cytosolic phorbol ester receptors was examined in cells exposed to 100nM PDBu for 24h and in cell populations cultured permanently in the presence of 10nM TPA. Figure 44 illustrates that following 24h treatment of A549-FCS cells with 100nM PDBu, cytosolic phorbol ester binding represented 29.1% of control binding. At this time, the phorbol ester was thoroughly washed from cultures and cytosolic extracts prepared after a further 24, 48 and 72h. Specific [^{3}H]PDBu binding capacity of these preparations was 75.3%, 80.5% and 104.3% of control cytosolic [^{3}H]PDBu binding respectively, at which times 24%, 50% and 76% of the respective cell populations represented the progeny of the original cells exposed to 100nM PDBu. Hence, it appears that during the time when cells were sensitive to arrest by PDBu, its removal was associated with resumption of growth and up-regulation of cytosolic phorbol ester binding, to a degree exceeding that observed in controls. In contrast, in cells of a more TPA-resistant phenotype, removal of TPA from culture media led to a slower accumulation of cytosolic phorbol ester binding sites. Following 72h in a TPA-free environment, specific phorbol ester binding in cytosol prepared from A549-FCS cells was 28% compared to the untreated parent population. In the case of A549-US TPA-resistant cells, 52% of the binding potential had been recovered 72h after the removal of TPA. The observed reduced recovery of cytosolic binding capacity

in A549-FCS TPA-resistant cells, compared to phorbol ester-sensitive populations may be a consequence of the possible presence of trace amounts of TPA in the cell culture dishes, resulting from the higher lipophilicity of TPA compared to PDBu. Alternatively, the discrepancy may be a genuine phenomenon. The difference in ability to up-regulate cytosolic phorbol ester binding sites between briefly treated cells and those cultured in the continuous presence of TPA may correspond to an altered phenotype, a consequence of modified growth control in the absence of functional PKC. A third possibility could be that re-emergence of the phorbol ester receptor occurred in a ligand-specific manner, dependent upon the phorbol ester which induced enzyme down-regulation initially. Indeed, the exposure of cells to 100nM PDBU for 24h, unlike 10nM TPA, was unable to eliminate specific cytosolic phorbol ester binding completely. Reduced recovery of phorbol ester binding in A549-FCS, TPA-resistant cells compared to A549-US, TPA-resistant cells may be the consequence of differential enzyme up-regulation in a subspecies-specific manner, reflecting population-specific PKC isozyme content.

Cytosolic phorbol ester binding was measured in A549-US cells into whose environment 2mg/ml fetuin had been introduced 14 days prior to preparation of crude cytosol fractions. The hypothesis under investigation was that the presence of fetuin may increase cytosolic phorbol ester binding. The introduction of fetuin to serum-free medium resulted in a more rapid rate of growth of low density A549-US cells, and significantly enhanced the sensitivity of this cell population to growth inhibition by 10nM TPA (section 4.1.5.5). Thus it may be speculated that fetuin is an important factor contained in foetal calf serum which may be responsible for maintaining levels of PKC. Indeed, the induction of PKC-α and -ε expression by serum has been reported in a melanoma cell strain (Yamanishi *et al.*, 1990). There was a small but significant rise by 23% ($p < .001$) in specific [^{3}H]PDBu binding to cytosol prepared from cells cultured in an environment containing fetuin compared to cytosolic extracts of cells maintained in serum-free conditions only (figure 40). However, it is unlikely that this rise is directly linked to the increased sensitivity caused by fetuin to growth arrest by TPA, as cytosol prepared from cells cultured in medium fortified with 10% NBCS demonstrated no difference in specific [^{3}H]PDBu binding potential from cytosolic extracts of cells maintained in 10% FCS (figure 40). It has already been established that NBCS was less able to support the maintained growth inhibition by TPA than FCS (figure 21).

As discussed previously, RA is able to modulate responses to phorbol esters in several cell types. Indeed, it has been proposed that RA may interact with signal transduction mechanisms stimulated by phorbol esters. For example, RA enhanced the TPA-stimulated phosphorylation of the 48kDa and 80kDa PKC-substrates (Ways *et al.*, 1988). These authors concluded that RA induced amplification of phorbol ester signal transduction at the level of PKC activation, thus mediating the effects of this vitamin on phorbol ester-induced differentiation. It has been demonstrated by Niles and Loewry (1989) that RA treatment of B16 melanoma cells evoked large increases in both activity and amount of PKC. They proposed that such an increase may be part of the differentiation program induced by RA.

During the course of experiments in which the effect of RA on A549 cell growth was investigated, cytosolic phorbol ester binding potential in A549-FCS and A549-US cells, previously exposed to 1μM RA for 96h, was examined. The rationale behind this experiment was that amplification at the level of PKC by RA may confer greater sensitivity to growth inhibition by TPA on cells cultured under conditions of serum deprivation. Figure 27a demonstrated that no amplification of growth arrest by TPA was observed in A549-US cells by the presence of 1μM RA. In accordance with this observation, no increase in phorbol ester binding was recorded in crude cytosolic extracts prepared from A549-US cells. Indeed, slightly decreased phorbol ester binding was observed. However, cytosolic phorbol ester binding significantly increased following treatment of A549-FCS cells with 1μM RA for this period of time ($p = .001$) (figure 45). It could be speculated that this rise may result from the amplification of transcription for a PKC isozyme for which there is no message in A549-US cultures.

Using antipeptide antibodies that specifically recognize α, ß and γ isoforms, Makowske *et al.* (1988), demonstrated that all three isozymes increased in a co-ordinately regulated fashion, 3-fold in HL-60 cells following 96h treatment with 1μM RA. Interestingly, Rosenbaum *et al.* (1990) observed differential regulation of PKC genes during RA-induced F9 teratocarcinoma cell differentiation. Experiments indicated a rise in PKC-α mRNA and protein levels, whereas decreased levels of PKC-ß and -γ mRNA and protein levels were obtained; thus implicating regulation at the transcriptional level. The RA-receptor possesses characteristics of DNA binding proteins and is coded for by a gene which belongs to the family of steroid receptor genes, thus providing a potential pathway by which RA may influence the transcription of PKC (Petkovich *et al.*, 1987). In the study performed by Niles and Loewy (1989), RA was also able to enhance PKC activity in PKC-depleted cells, previously exposed to phorbol ester for 72h. It may be that the enhanced growth rate of PKC-depleted A549-FCS, TPA-resistant cells in the presence of 1μM RA occurs *via* a similar mechanism.

4.2.3 Examination of phorbol ester binding in intact cell cultures.

Introduction.

Previous investigations undertaken to examine the potential role of PKC in the observed inhibition of A549-FCS cell growth employed [^{3}H]PDBu to detect specific phorbol ester receptor binding to monolayer cultures (Gescher and Reed, 1985). It was reported that exposure of cells to 100nM PDBU prior to the receptor binding assay, resulted in a loss of receptor binding such that after 24h pretreatment, binding was reduced to 38% of controls. However, in cells desensitized towards phorbol ester-induced growth inhibition by treatment with 100nM PDBU for 6 days, receptor binding capacity was 75% of untreated controls. Moreover, in A549-TPA cells, more permanently resistant to growth inhibition, the ability of [^{3}H]PDBu to bind to receptors was only decreased by 23% following 24h pre-exposure to 100nM PDBu. This study led to the proposal that down-regulation of

Figure 39. Specific [^{3}H[PDBu binding to crude cytosolic extracts of A549-FCS cells. Binding is represented as a % 100nM [^{3}H]PDBu bound. The assay was performed excluding (-□-) and including (-■-) 10nM TPA in the assay mixture (mean ± SD, n=6-8).

Figure 39

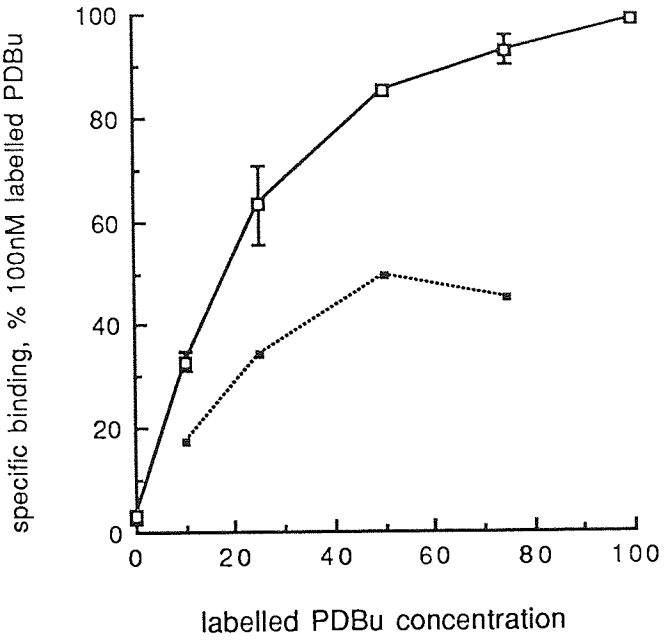


Figure 40. Specific [^{3}H[PDBu binding to crude cytosolic preparations from A549 cell populations. (mean ± SD, n=9).

Figure 40

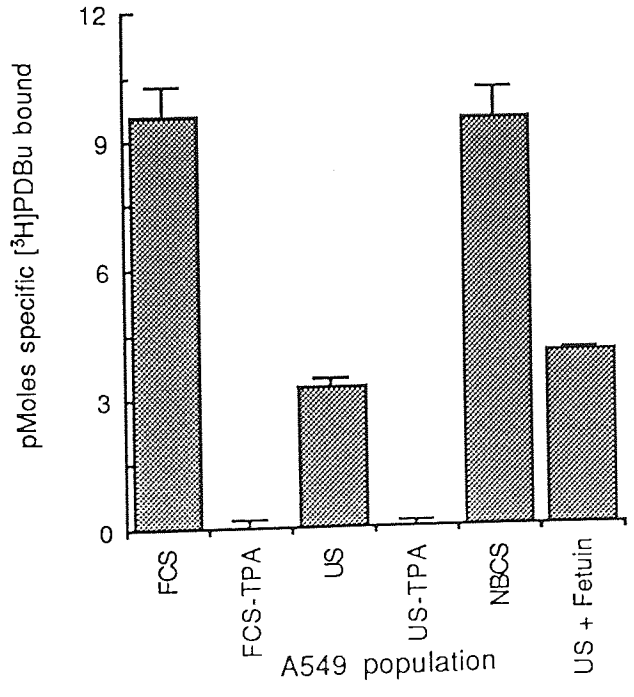
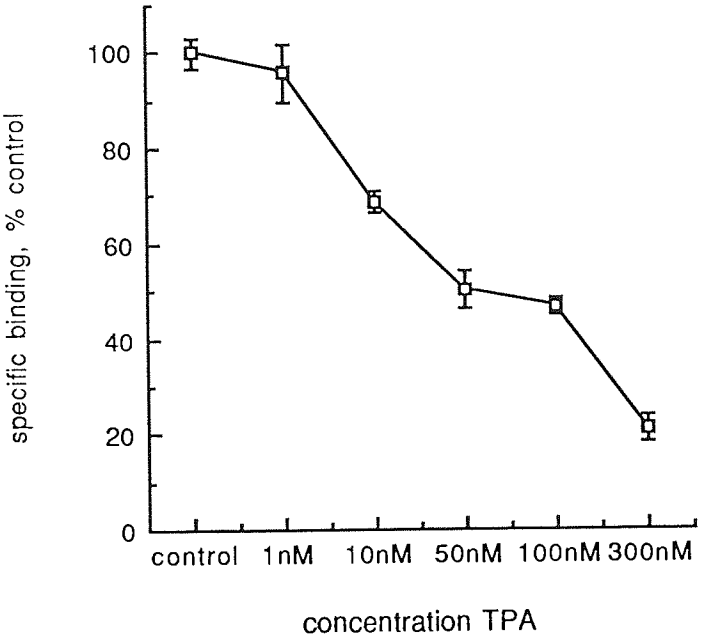


Figure 41. Concentration-dependent loss of cytosolic [^{3}H]PDBu binding potential following treatment of a) A549-FCS and b) A549-US cells with TPA for 30 min.(mean ∓ SD, n=9).

Figure 41a)



41b)

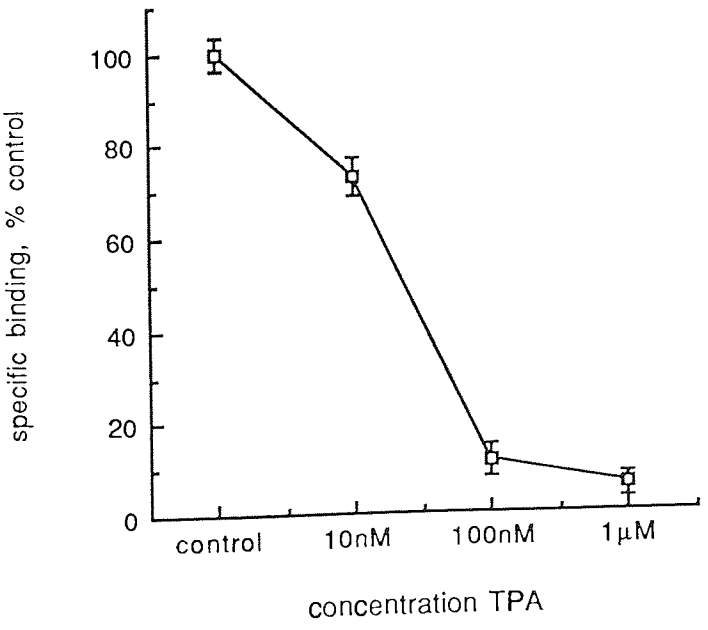


Figure 42

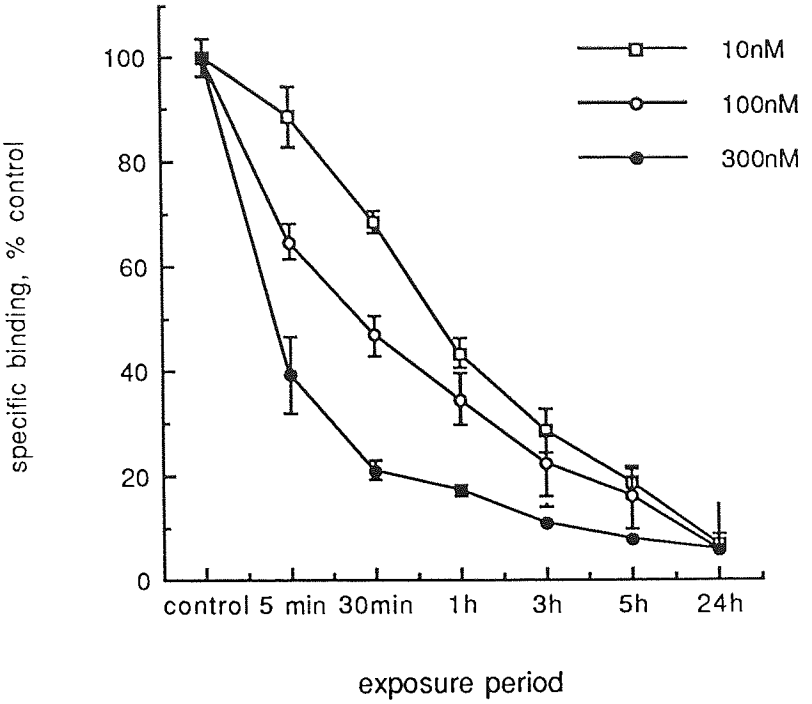


Figure 42. Loss of cytosolic [^{3}H]PDBu binding sites in A549-FCS cells pre-exposed to 10nM, 100nM and 300nM TPA for varying lengths of time. (mean ± SD, n=9).

Figure 43

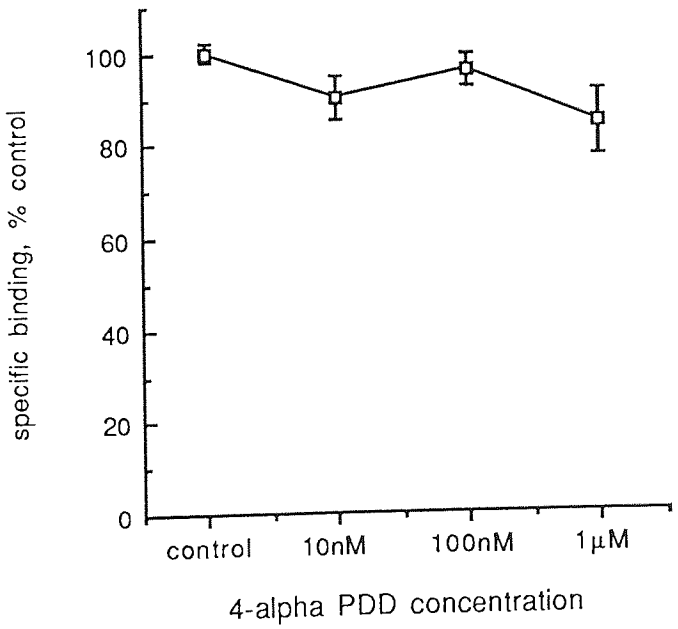


Figure 43. Cytosolic [^{3}H]PDBu binding potential in A549-FCS cells pre-exposed to increasing concentrations of 4-α PDD for 30 min. (mean ± SD, n=6).

Figure 44

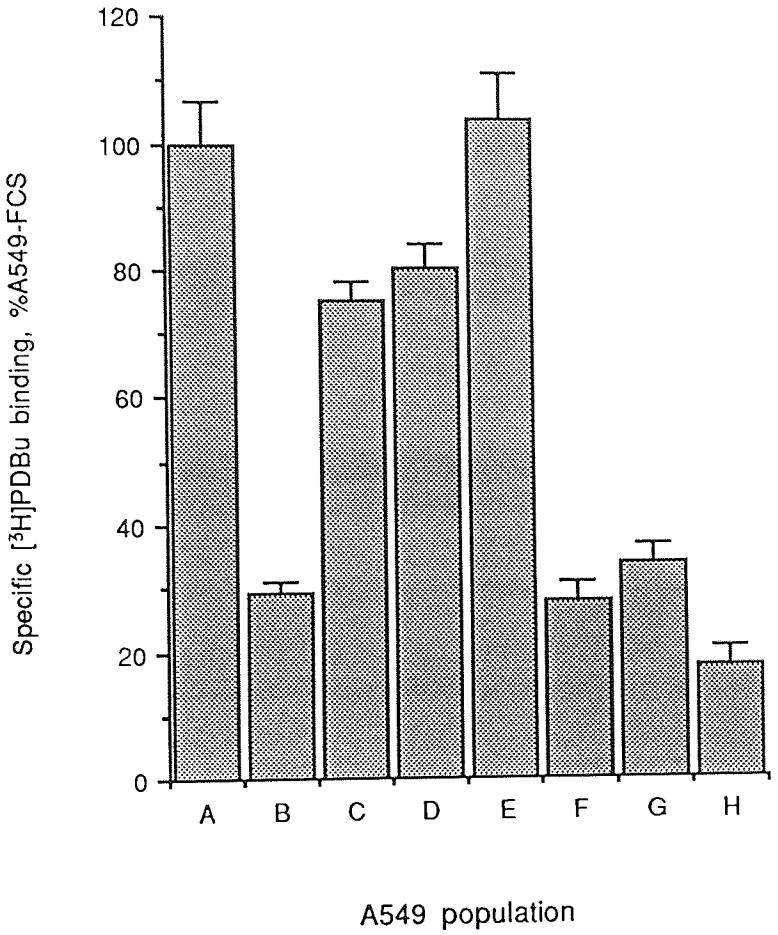


Figure 44. Phorbol ester binding potential in cytosolic preparations of:
A. A549-FCS cells
B. A549-FCS cells following 24h pre-treatment with 100nM PDBu
C. A549-FCS cells following 24h pre-treatment with 100nM PDBu and 24h recovery in PDBu-free medium
D. A549-FCS cells following 24h pre-treatment with 100nM PDBu and 48h recovery in PDBu-free medium
E. A549-FCS cells following 24h pre-treatment with 100nM PDBu and 72h recovery in PDBu-free medium
F. A549-FCS cells `resistant` to TPA following 72h in a TPA-free environment
G. A549-US cells
H. A549-US cells `resistant to TPA following 72h in a TPA-free environment (mean ∓ SD, n=6-9).

Figure 45

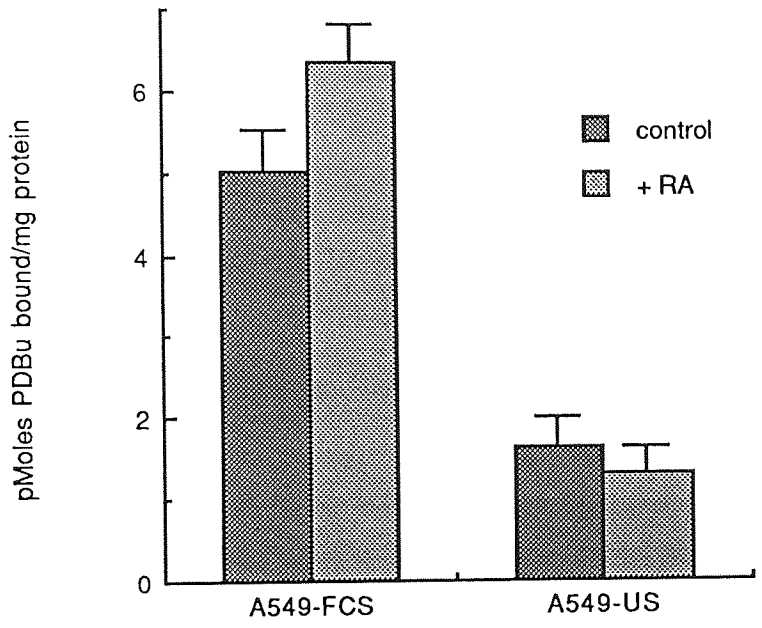


Figure 45. Cytosolic [^{3}H]PDBu binding potential in A549-FCS and A549-US populations following treatment of cells for 72h with 1μM RA immediately prior to cytosolic preparation. (mean ± SD, n=9).

phorbol ester binding capacity was part of events by which phorbol esters caused growth inhibition. Experiments described herein were performed to explore further the nature of specific phorbol ester binding to intact cell monolayer cultures, and compare the results with those observed using the methods described above, to elucidate more extensively the role of PKC in phorbol ester-induced A549 growth inhibition.

Results and discussion.

Consistent with the observations of Gescher and Reed (1985), but in direct contrast to the results obtained investigating cytosolic phorbol ester binding and enzyme activity, specific [^{3}H]PDBu binding was detected on cells cultured permanently in the presence of 10nM TPA. A549-FCS TPA-resistant populations were found to possess 81.8% of PDBu binding capacity compared to untreated A549-FCS monolayer cultures. Similarly, A549-US TPA-resistant cells displayed 86.1% binding potential compared to the A549-US parent population. Moreover, A549-US cultures displayed 38% increased specific [^{3}H]PDBu binding compared to A549-FCS cell populations from which they were derived (figure 46).

Down-regulation of [^{3}H]PDBu receptor binding was observed using this assay, when binding capacity was assessed during the initial 24h exposure to phorbol ester. The decrease in receptors seemed marginally more rapid in A549-US monolayer cultures, for example, after 3h exposure to 10nM TPA [^{3}H]PDBu binding was 56.2% in A549-FCS cells and 44.3% in A549-US cells (figure 47). However, complete loss of specific binding was not achieved. Following exposure of A549-FCS and A549-US cells for 24h to 10nM TPA, 29.6% and 24.4% [^{3}H]PDBu binding potential was retained respectively. Moreover, residual phorbol ester binding appeared to be dependent on the concentration of TPA; high concentrations were able to diminish [^{3}H]PDBu binding by a greater degree (result not shown). It has been reported by Mattingly *et al.* (1987), that down-regulation of phorbol ester binding to NG115-401L neuronal cells was dependent on concentration, exposure time and phorbol ester; with greater potency displayed by TPA compared to PDBu. Evidence obtained during this study suggests that the re-emergence of specific [^{3}H]PDBu binding, in the continued presence of 10nM TPA was a gradual phenomenon, established over varied periods of time, specific for the particular A549 cell batch. Contrary to the observations of Reed and Gescher (1985), cells, cultured for 10 days in presence of 10nM TPA, released from temporary growth inhibition induced by tumour promoting phorbol esters, demonstrated no specific PDBu binding.

Co-incubation of cells for 30 min with increasing concentrations of TPA led to the displacement of 50nM [^{3}H]PDBu from specific receptors in a dose-dependent manner, illustrated in figure 48. It was calculated that 50% of cell surface receptors would be occupied by 4.2nM in A549-FCS cells, and by less than 1nM TPA in A549-US cells. In contrast, figure 49 shows that the inactive phorbol ester, 4-α PDD was unable to inhibit specific [^{3}H]PDBu binding to any extent at concentrations up to and including 1μM.

The results suggest that down-regulation of phorbol ester binding sites may not occur or be maintained in parallel with the degradation of functional PKC activity, in response to treatment of cells with TPA. The biochemical processes underlying this observed

discrepancy remain to be elucidated. Experiments were performed by Adams and Gullick (1989), to examine the correlation between loss of PKC protein and loss of phorbol ester binding sites. These authors demonstrated that the decrease in [^{3}H]PDBu binding following treatment of cells with TPA may not be as large as the decrease in immunologically detectable PKC. It was suggested that limited proteolysis of activated PKC may release a phorbol ester binding fragment which could be retained in the presence of phorbol ester. However, antiserum recognizing the 32kDa fragment containing the phorbol ester binding domain failed to detect such a fragment (Parker *et al.*, 1986).

Down-regulation of immunoprecipitable PKC has been studied in various human breast cancer cell lines that displayed differential growth inhibitory responses toward TPA (Fabbro *et al.*, 1988). Enzyme translocation and down-regulation was revealed without diminishing PKC synthesis. It was demonstrated that during prolonged exposure to TPA, 20-80% of the total 80kDa PKC of control cells was synthesized as a membrane bound 74/80kDa protein doublet. Both proteins lacked kinase activity and phorbol ester binding capacity, but revealed structural similarity with active 80kDa PKC from untreated cells. Data from this study has suggested that the presence of TPA, resulted in immediate docking of non-functional PKC to membranes. Interestingly, the proportional amounts of 74 and 80kDa PKC-related proteins varied widely among cell lines; synthesis of the 80kDa protein, following PKC down-regulation, correlated inversely with the extent of growth inhibition induced by TPA. Thus, it has been postulated that the 74/80kDa PKC-related proteins may play a role in the differential growth responses to phorbol ester. It may be speculated that the 74/80kDa protein doublets were generated following post translational modification of PKC to render the enzyme inactive. An alternative explanation may be that the 74/80kDa protein doublets are putative PKC precursors. Removal of TPA from the cellular environment resulted in the resumption of cell growth and the reappearance of the functional 80kDa PKC protein.

An *in vitro* method has recently been developed by Chakravarthy *et al.* (1990) by which PKC activity can be measured directly in isolated membranes, using a specific synthetic peptide substrate. This method could be adopted to resolve the paradoxical nature of enzyme activity and phorbol ester binding in A549 cells by quantitatively measuring the proportion of both active and inactive PKC in membranes.

4.2.4 Detection of PKC protein by Western Blotting.

Introduction.

Western blot experiments were performed in order to determine whether monolayer phorbol ester binding capacity, or PKC possessing kinase potential represented immunologically detectable protein in A549 cell populations. We wished to establish any further constancy, or discrepancy between indices of PKC quantification; to aid resolution of the nature of intact A549 phorbol ester binding sites. Detection of protein was performed using an anti-PKC primary Ab recognizing the cleavage domains of intact PKC-α and ß isoforms.

Figure 46. [^{3}H]PDBu binding to TPA-sensitive and -resistant A549-FCS and A549-US cell monolayers. Confluent cell monolayers were incubated at 37^{0}C for 30min in the presence of 50nM [^{3}H]PDBu. Non-specific binding was determined in the presence of excess unlabelled PDBu. (mean + SD, n=8-12).

Figure 46

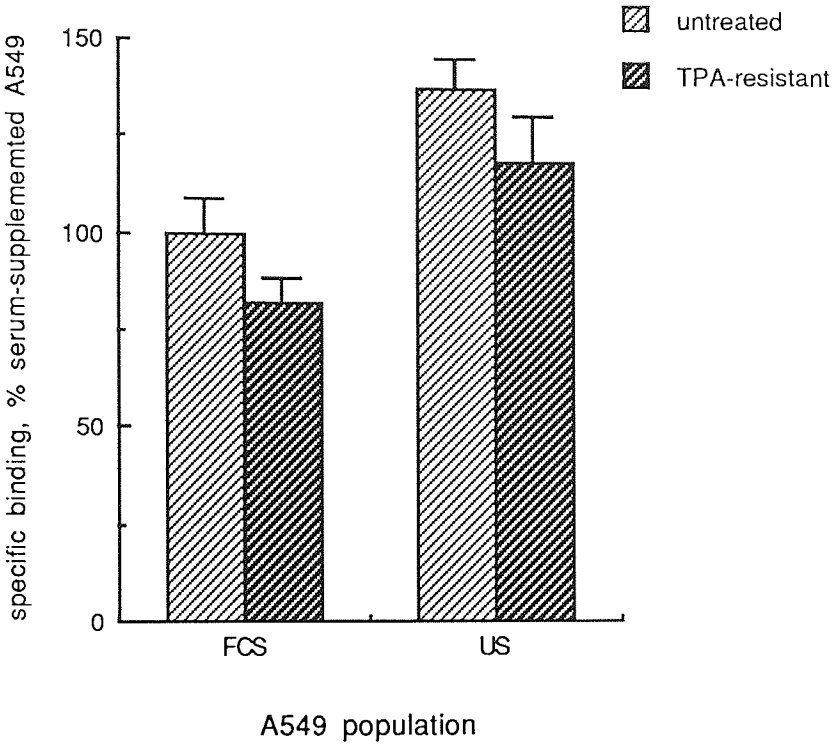


Figure 47. Down-regulation of [^{3}H]PDBu binding potential to intact cell monolayers following prior incubation of cells with 10nM TPA for increasing periods of time. (mean ∓ SD, n=8-12).

Figure 47

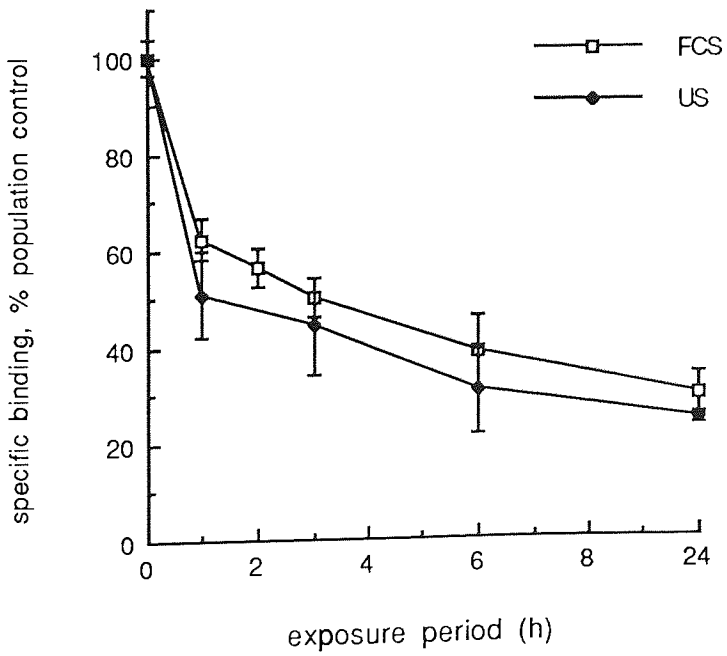


Figure 48. Displacement of 50nM [^{3}H]PDBu binding from intact A549-FCS and A549-US cell monolayers by co-incubation with TPA. (mean ∓ SD, n=8-12).

Figure 49. Lack of displacement of 50nM [^{3}H]PDBu binding from intact A549-FCS cell monolayers following co-incubation with 4-α PDD. (mean ∓ SD, n=8).

Figure 48

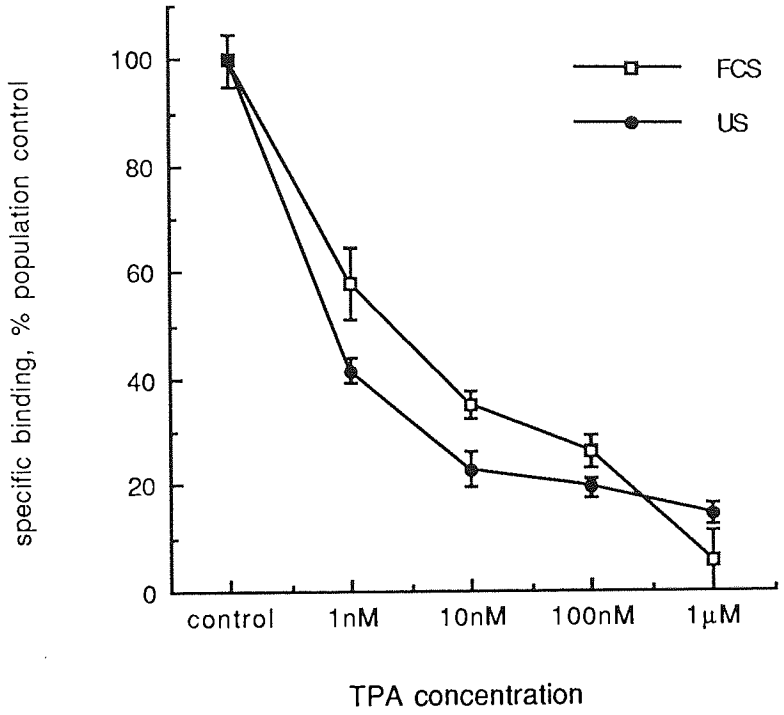
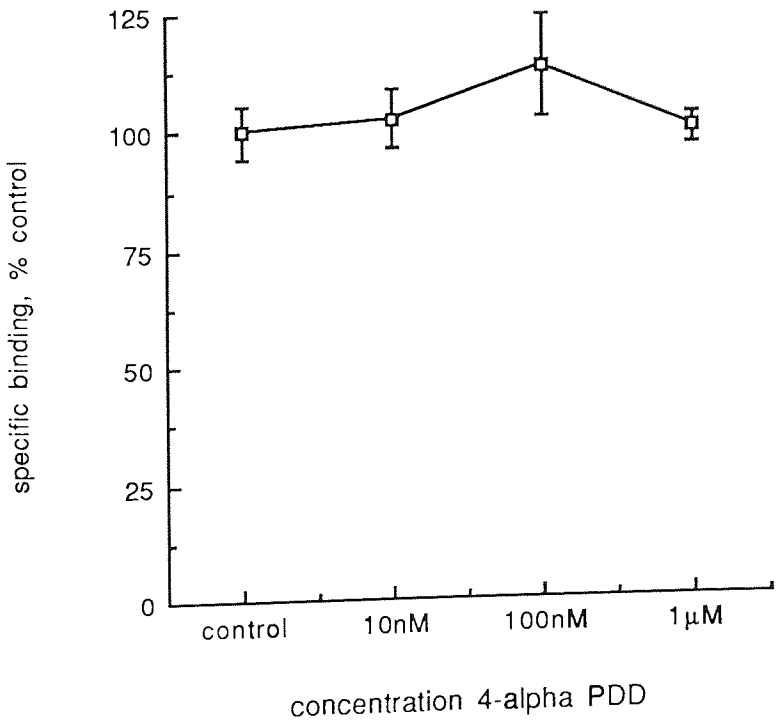


Figure 49



Results and discussion.

Experiments confirmed that the major portion of PKC resides in the cytosol of untreated cells. Translocation of holoenzyme from cytosol to membrane extracts was evident following 30 min exposure to 100nM TPA (figure 50, 1 and 2). Whole cell (not shown), cytosolic and particulate lysates, prepared following exposure of cells to 10nM TPA for 24h, revealed residual recognition of the α/ß cleavage domain (figure 50, 1); hence, it appeared that enzyme down-regulation was not complete. However, non-specific interaction of primary Ab with protein bands was evident during the detection procedure. Additionally, it was demonstrated by this technique that the cytosol of A549-US cells possessed a mean 40% immunodetectable PKC protein compared to A549-FCS populations. Lysates from equal numbers of cells were loaded into each lane and band intensity was evaluated by means of densitometry. PKC was not detected in A549-FCS or A549-US TPA-resistant cells (figure 50, 3).

Experiments performed by Adams and Gullick (1989) have revealed cell type-specificity in both rate and extent of PKC down-regulation induced by 100nM TPA. Absolute down-regulation of immunodetectable PKC was observed in V79 Chinese hamster lung fibroblasts and Swiss 3T3 mouse fibroblasts following 5h and 30h exposure respectively. After 10h treatment, maximal PKC down-regulation of 30% occurred in bovine kidney epithelial cells. In a rat glioma cell line however, no enzyme down-regulation was detected following an exposure period of 30h. These authors also demonstrated no correlation between concentrations of TPA able to evoke enzyme degradation and abundance of PKC in a particular cell line. It may be speculated that differential patterns of down-regulation are a consequence of cell type-specific expression of PKC isozymes in different ratios. Evidence lending support to this hypothesis has been reported (Ase *et al.*, 1988; Cooper *et al.*, 1989; Huang *et al.*, 1989). Differential rates of PKC degradation were obtained, resulting from isozyme-specific sensitivity to proteolysis. PKC-α yielded the greatest resistance to proteolytic degradation (Ase *et al.*, 1988). Consistent with this observation, the observed rate of down-regulation in V79 cells was substantially faster than enzyme degradation in Swiss 3T3 cells (Adams and Gullick, 1989). The latter possess a single isoform; PKC-α (Nishizuka, 1988).

It has been established that the 7 isoforms of PKC discovered to date display differential regional and cellular expression (Kikkawa *et al.*, 1988). Moreover, various permutations and combinations of the α and ß subspecies exist in many cell types (Nishizuka, 1988; Sawamura *et al.*, 1989), indicative of the fact that each subspecies may possess a unique role in the cellular response to TPA. Interestingly, of the two peaks of PKC activity eluted in HL-60 cells by hydroxyapatite column chromatography by Beh *et al.* (1989), peak-1 was maximally activated by lower TPA concentrations than peak-2.

Primary antibodies specific to PKC-α and PKC-ß were employed in order to determine the ratio of the presence of these two isoforms in A549-FCS and A549-US cell populations. We wished to examine the hypothesis that the loss of sensitivity to protracted growth arrest induced by tumour promoting phorbol esters may be a consequence of specific depletion of

a functional isoform of PKC in cells cultured in serum-free conditions. Additionally, we wished to explore the possibility of differential isozyme down-regulation following treatment of A549-FCS cells with different activators of PKC.

It was established that there was no presence of PKC-ß in A549 cell populations (figure 51b). To test the viability of the Ab, HL-60 cytosolic and membrane lysates were examined, whose isozyme content is known to include PKC-ß. Indeed, PKC-ß was recognized and predominantly found in the cytosolic extract of untreated HL-60 cells (figure 52b). The presence of PKC-α was also detected in cytosolic HL-60 lysates (figure 51a, 2), although the band was much fainter, consistent with the proportional α, ß and γ isozyme content established in this promyelocytic cell line of 11, 80 and 9% respectively (Nishikawa *et al.*, 1990). The α isozyme of PKC was clearly detected in untreated A549-FCS and A549-US cell cultures (figure 51a). Cytosolic extracts from the latter population yielded a protein band with an intensity 40% of that displayed by cytosol lysates from A549-FCS cells. Hsieh *et al.* (1990) have discovered increased levels of endogenous PKC-α and decreased levels of PKC-ε mRNAs in tumourigenic clones of rat liver epithelial cell lines when compared to parental cells. Increased PKC-α mRNA was also detected in rat liver tumours and regenerating rat liver. It is speculated that such enhanced expression of PKC-α may play a role in cell proliferation and hepatocarcinogenesis. This is extremely interesting in the light of the re-expression of AFP in hepatocarcinomas and regenerating liver. Evidence has been provided by Eldar *et al.* (1990) suggesting a role for PKC-α in the regulation of EGF receptor expression. Swiss 3T3 cells stably over expressing PKC-α and displaying an enhanced growth rate, possessed reduced levels of high and low affinity EGF receptors. It was observed (section 4.1.5.4) that EGF may augment inhibition of A549 cell growth by TPA under conditions of serum deprivation. We may speculate that the inhibition of A549-US cell growth in response to TPA and EGF may be a consequence of a rise in cell surface EGF receptor number. This hypothesis remains to be tested. Translocation of PKC-α to the membrane fraction of A549-FCS cells was seen following 30 min exposure to 100nM TPA (figure 51a, 2). Complete down-regulation of immunodetectable PKC-α occurred resulting from prolonged treatment (24h) with TPA (10nM). In neither TPA-resistant A549 population were traces of PKC-α detected (results not shown).

Thus, in cells resistant to growth inhibition by TPA, neither immunologically detectable PKC-α, nor enzyme activity, nor specific cytosolic phorbol ester binding was detected. In A549-US cultures these three indices of PKC activity were greatly reduced. However, TPA-resistant cells displayed specific phorbol ester binding in the assay performed upon intact cell monolayers. If one considers the hypothesis put forward by Borner *et al.* (1988), such binding may reveal the presence of inactive PKC precursors, or may possibly be a consequence of post translational modification of PKC. In the light of this suggestion, we

may postulate that environmental factors, such as the presence foetal growth factors, may promote conversion or retention of functional PKC. We may speculate that mRNA synthesis remains unaltered in A549 cells, in view of the observations of Mitzuguchi *et al.* (1988) and Isakov *et al.* (1990). Isakov *et al.* (1990), have adapted a human leukaemic T-cell line to continuous growth in the presence of phorbol ester. These cells displayed a 6-fold reduction in cellular PKC enzymatic activity and a change in expression of cell cycle genes. Immunodetection indicated the expression of α, ß and γ isozymes of PKC in the parent population. However, phorbol ester-mediated selective loss of PKC-α protein only was reported in the phorbol ester-resistant subline, supporting the hypothesis that PKC isozymes are independently regulated and possess unique biological roles. The correlation between phenotypic and functional changes and the selective reduction of PKC-α led to the speculation that expression of cell cycle-regulated genes and functional activation may be regulated preferentially by PKC-α.

Decreased expression of PKC-ß has been reported in HL-60 cells resistant to the induction of differentiation by phorbol ester (Nishikawa *et al.*, 1990). Melloni *et al.* (1989), have reported a functional link between the expression of PKC-ß and erythroleukaemia cell differentiation. Cells displaying accelerated rates of differentiation were enriched in this particular isoform.

Retention of PKC-ß and PKC-dependent vinculin phosphorylation in BC3H-1 myocytes, following chronic phorbol ester treatment has been reported (Cooper *et al.*, 1989). Degradation of PKC-γ was sustained however, with the loss of acute TPA effects on glucose transport and PKC-directed histone phosphorylation. However, identical isozyme expression has been demonstrated in epidermal cells and tissues from 2 strains of mice differing by several hundred-fold in their sensitivity to the tumour promoting effects of TPA (Ashendel *et al.*, 1990).

It is possible that A549-FCS and A549-US cells may express isozymes of PKC differentially. This hypothesis can neither be accepted nor dismissed in the light of findings presented in this study. Examination for the presence of PKC-subtypes γ, δ, ε, and ζ remains a future aim of this project.

If A549 cells possess solely the PKC-α isozyme, they may provide an ideal system in which to investigate further the physiological function of this PKC subspecies. Experiments are beginning to reveal cell type-specific functions for PKC-α; for example as an inhibitor of colonic Cl^- secretion (van den Berghe *et al.*, 1990).

However, for clearer elucidation of mechanisms underlying the cellular responses elicited by tumour promoting phorbol esters, events triggered by esters such as TPA, independent of, or distal to the activation of PKC and the possibility of signal transduction crosstalk which may contribute to the differential growth responses of A549 populations in the presence of TPA must be considered. The PKC-catalysed phosphorylation of serine or threonine residues in many cellular proteins results in profound changes in gene expression (Smith and Denhardt, 1989; Woodgett and Hunter, 1987). Genes whose transcription is

induced by TPA possess a TPA-responsive element (TRE) in the 5` flanking region, a binding site of the AP-1 protein complex. Studies have shown that the cellular response to TPA is mediated by transcriptional factors such as the activator protein AP-1. The *fos* and *jun* proteins are constituents of the AP-1 complex (Franza *et al.*, 1988; Hata *et al.*, 1989). It has been established that tumour promotion sensitive JB6 cells display TPA-inducibility of AP-1 dependent gene expression (Bernstein and Colburn, 1989). Moreover, a serum response element, in the c-*fos* gene enhancer, activated by the c-Ha-*ras* protein and independent of PKC has been demonstrated (Fukumoto *et al.*, 1990).

Techniques employing fluorescently labelled PKC activators may be adopted to investigate directional enzyme translocation in cell populations, following stimulation by different activators of PKC. Such investigations may lead to the localization of activator-specific PKC substrates. Additionally, one could compare protein phosphorylation following TPA treatment in A549 populations. This may possibly offer a more sensitive technique to establish the presence of enzymatically functional PKC.

4.2.5 The effect of staurosporin on A549 cell growth and on A549 cell growth inhibition elicited by TPA.

Intrduction.

PKC activity has been implicated in the action of tumour promoters (section 4.2.1), in the regulation of differentiation and proliferation and in certain aspects of oncogenesis (section 1). Elucidation of the molecular mechanisms of regulation of PKC activity has assumed urgency in the light of these implications. Inhibitors of PKC are employed in experimental procedures to facilitate the determination of the physiological importance of this enzyme. However, many inhibitors have been described which are neither specific nor potent for PKC (section 1.3.9). These compounds are active only in the μM range for example trifluoperazine, chlorpromazine and polymixin B demonstrated inhibitory action against PKC with IC_{50} values of 60μM, 80μM and 10μM respectively (Tamaoki *et al.*, 1986). Sangivamycin, a nucleoside analogue, demonstrated selectivity for PKC compared to cyclic AMP-dependent kinase, but again, inhibition of PKC activity occurred at μM concentrations (Loomis and Bell, 1988).

Staurosporin (STSPN), a microbial alkaloid with antifungal activity, produced by a *Streptomyces* sp. is a potent but non-specific inhibitor of PKC. STSPN possesses a flat polyaromatic aglycone structure which is resposible for PKC inhibition (Davis *et al.*, 1989). It has been found to inhibit rat brain PKC with an IC_{50} of 2.7nM (Tamaoki *et al.*, 1986). STSPN does not interfere with phorbol ester binding to PKC (Tamaoki *et al.*, 1986), instead, it interacts with the catalytic moiety of PKC and induces enzyme association with the membrane (Wolf and Baggiolini, 1988). These authors demonstrated that protein phosphorylation by STSPN-translocated PKC was inhibited in the absence or presence of phorbol esters.

In an attempt to elucidate in more detail the role of PKC in A549 cell growth arrest elicited by TPA, we have conducted experiments using STSPN.

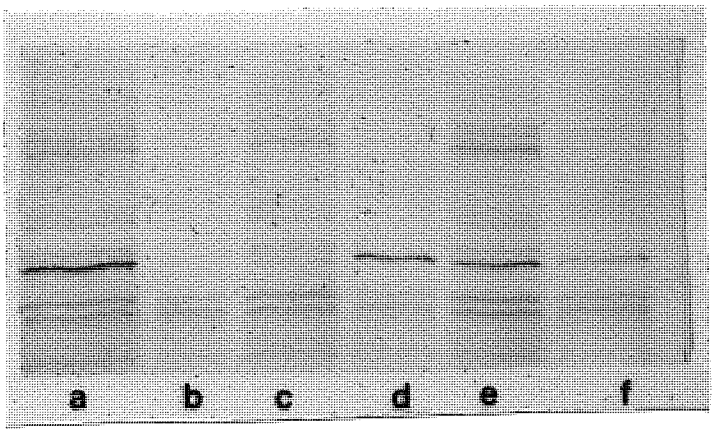
Figure 50. Detection of Protein kinase C using a monoclonal Ab recognizing the cleavage domain of PKC isoforms -α and -ß. Cytosol and particulate fractions were prepared from A549 cell cultures following appropriate treatments with modulators of PKC. Proteins were separated by PAGE and blotted onto nitrocellulose before the detection procedure commenced.

1. a, c, e: A549-FCS cytosol; b, d, f: A549-FCS membrane fractions;
a,b: control; c,d: 30 min 100nM TPA; e,f: 24h 10nM TPA.

2. a, c, e, g: A549-FCS cytosol; b, d, f, h: A549-FCS membrane fractions;
a, b, g, h: A549-FCS; c, d: 30 min 100nM TPA; e, f: 30 min 100nM bryo 1.

3. a, c, e, g: cytosol; b,d,f,h: membrane fractions;
a, b: A549-FCS; c, d: A549-US; e, f: FCS-TPA; g, h: US-TPA.

1



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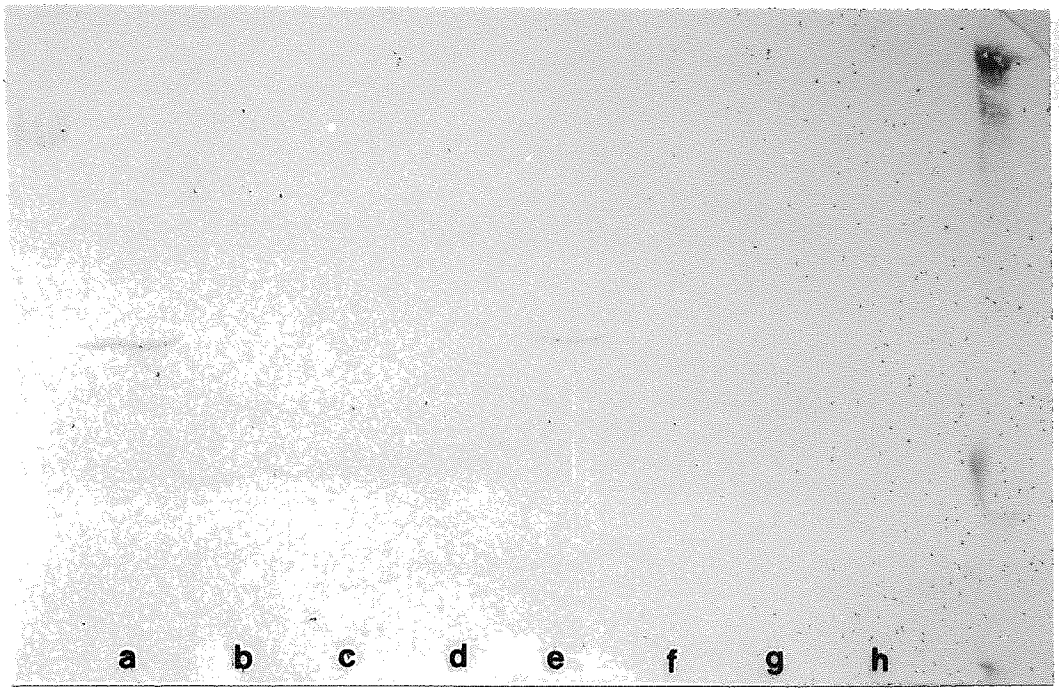
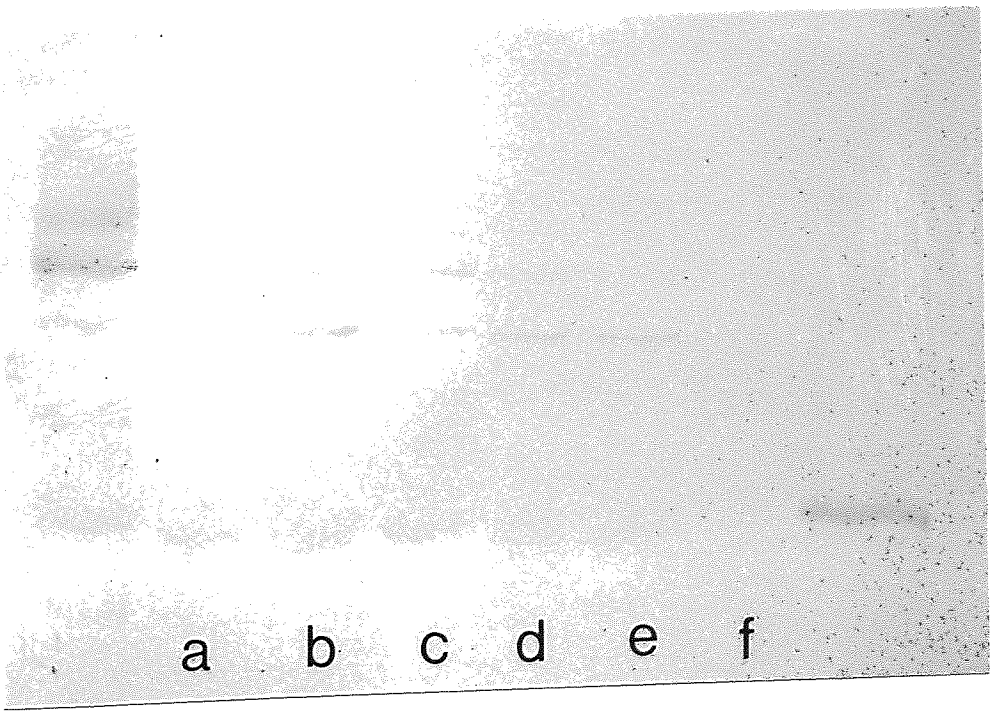


Figure 51a). Detection of Protein kinase C-α using a monospecific monoclonal Ab. Cytosol and particulate fractions were prepared from A549 cell cultures following appropriate treatments with modulators of PKC. Proteins were separated by PAGE and blotted onto nitrocellulose before the detection procedure commenced.
1. a, b, d, e: cytosol; c, f: membrane fractions;
a, b, c: A549-FCS; d, e, f: A549-US.

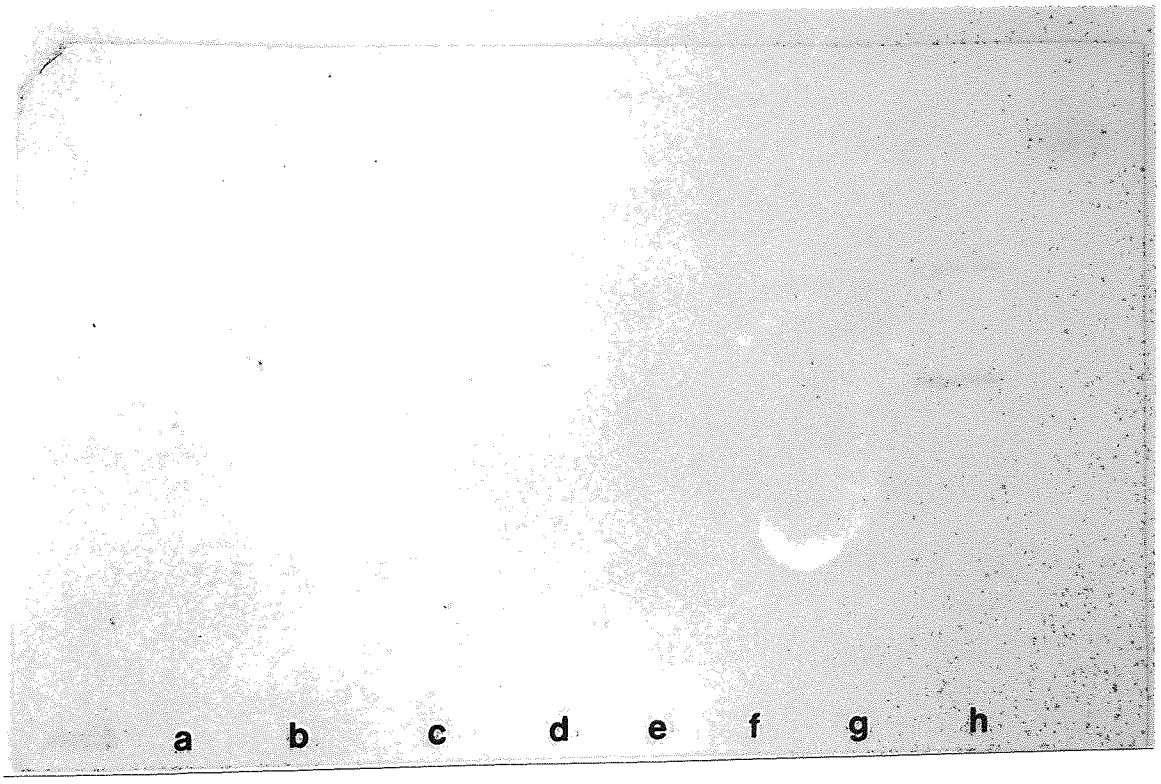
2.a, c, e, g: cytosol; b, d, f, h: membrane fractions;
a, b: HL-60; c, d: A549-FCS; e, f: A549-FCS 30 min 100nM TPA; g, h: A549-FCS 30 min 100nM bryo 1.

1



2

Figure 51b). Detection of Protein kinase C-ß using a monospecific monoclonal Ab. Cytosol and particulate fractions were prepared from A549 and HL-60 cell cultures. Proteins were separated by PAGE and blotted onto nitrocellulose before the detection procedure commenced.
b, d, f, h: cytosol; a, c, e, g: membrane fractions;
a, b: A549-FCS 30 min 100nM bryo 1; c, d: A549-FCS 30 min 100nM TPA; e, f: A549-FCS; g, h: HL-60.



Results and discussion.

Initially, it was necessary to determine the effect of STSPN alone on A549 cell growth. Cells were incubated for 24h with various concentrations of STSPN, in the presence or absence of 10nM TPA before incorporation of labelled thymidine was measured. The results are illustrated in figure 52. Cell growth was monitored daily, for 9 days in the presence of 10nM STSPN. Following an incubation period of 96h in the presence of either STSPN (10nM) or TPA (10nM) or both agents combined, cell numbers were determined. Figure 52 reveals that STSPN itself evoked a concentration-dependent inhibition of [^{3}H]TdR incorporation, with an IC_{50} value calculated as 0.9nM. No inhibition of TPA-induced growth arrest was observed after 24h exposure, on the contrary, an additive arrest of DNA synthesis was observed. A similar observation was made when cells were counted following 96h exposure to 10nM STSPN and 10nM TPA (figure 53). Figure 54 demonstrates that 10nM STSPN exerts a potent cytostatic effect upon A549 cells. Following 9 days exposure there was no indication that cell growth would resume. It remains to be determined therefore, whether growth arrest by 10nM STSPN is irreversible, at which phase in the cell cycle growth arrest is imposed, and moreover, whether the effect is a cytotoxic or a cytostatic one. The morphology adopted by A549 cultures in the presence of STSPN didnot resemble the characteristic protruding, rounded form following TPA or bryo treatment (figure 55).

The effect of STSPN on the cellular proliferation of other neoplastic cell lines has been documented. The growth of HeLa S3 cells was inhibited with an IC_{50} value of 4.08pM after 72h and 280nM after 1h exposure. STSPN arrested the growth of B16 melanoma cells and P388 leukaemia cells to the same degree (Tamaoki *et al.*, 1986). These authors conclude that the potent cytotoxic activity may be due to inhibition of PKC, however, STSPN also inhibited cyclic AMP dependent protein kinase which may contribute to cytotoxicity. Interestingly, it has been demonstrated that the growth of DEMEL metastatic melanoma cells, which is temporarily inhibited by TPA, is arrested by nM concentrations of STSPN (Coppock *et al.*, 1990). Moreover, following 24h incubation, the inhibitory response in the presence of both these agents was more potent than the effect of either agent alone.

Thus far it has been established that PKC activators (tumour promoting phorbol esters) and the PKC inhibitor STSPN may evoke potent A549 cell growth arrest. We wished to investigate the time course for the onset of inhibition of DNA synthesis by these agents. A549-FCS cells were exposed for increasing periods of time to 10nM TPA, 10nM STSPN and 10nM TPA plus 10nM STSPN. Incorporation of [^{3}H]TdR was assessed and the results illustrated in figure 56.

The initiation of inhibition of [^{3}H]TdR incorporation by 10nM TPA was immediate. By 2h exposure, DNA synthesis was between 50% and 60% of control values and maintained at this level during the first 6h incubation. After this time, a rapid decrease in [^{3}H]TdR incorporation was observed and maintained throughout the 72h time period investigated. In contrast, in the presence of 10nM STSPN, the proliferative potential during the first 6-9h remained at levels not significantly different from those observed in control cells. After this

time a rapid decline in the cells` ability to incorporate [^{3}H]TdR was observed until, following 24h incubation, equal potencies were achieved by 10nM TPA and 10nM STSPN. Inhibition of DNA synthesis by STSPN was sustained at levels below 20% of control values for each exposure period examined. Co-incubation of cell cultures with 10nM TPA and 10nM STSPN led to the following observations: during the initial 6h, incorporation of [^{3}H]TdR was impeded by between 11 and 20%, thus proliferative capacity was lower than control values or 10nM STSPN alone but sigificantly higher than 10nM TPA alone. Between 6 and 12h exposure, rapid loss of proliferative potential was detected and after 12h, an inhibitory response more potent than that induced by either agent alone was observed: consequently, negligible incorporation of [^{3}H]TdR was observed up to 72h. The same protocol was adopted in A549-US cells, to test the hypothesis that inhibition of PKC may prevent the initial inhibition of [^{3}H]TdR incorporation induced by TPA. As described previously (section 4.1.3) the proliferative potential of this cell population in the presence of 10nM TPA begins to return to control values following 24h exposure. STSPN (10nM) evoked inhibition of [^{3}H]TdR incorporation in the A549-US population similar to that observed in A549-FCS cells. Co-incubation of A549-US cells with TPA and STSPN led to partial protection from the inhibition of DNA synthesis evoked by TPA during the initial 6h incubation period. Further exposure to STSPN alone or to STSPN and TPA resulted in a rapid decline in [^{3}H]TdR incorporation until negligible incorporation of [^{3}H]TdR was obtained after 18h and maintained, such that no increase in DNA synthesis was detected. In contrast, cells exposed to TPA alone displayed the ability to regain proliferative potential following 24h exposure (figure 57). It is demonstrated in figure 55 that the characteristic alteration in A549 morphology, present during the time of TPA-induced growth arrest was not observed when STSPN was also present in the incubate. Cells had the appearance of control cultures, or that of cells treated with STSPN alone. The TPA-induced rounding up of cells was prevented. From the results obtained thus far we can speculate that the activation of PKC is important for the initiation of growth arrest induced by TPA.

One may tentatively predict that the inhibition of PKC and the down-regulation of PKC may generate the same cellular responses. Indeed, this conclusion has been drawn by Kitajima *et al.* (1988). The effect of TPA on the morphology of human epidermal carcinoma cells, cultured in conditions of low calcium, was biphasic. Initially, cell-cell contact was induced, followed by a fibroblastic morphological change with a decrease in the number of cell-cell contacts. These phases can be attributed to, initially the activation, then down-regulation of PKC. Addition to the medium of the PKC inhibitor H-7 blocked the TPA-induced formation of cell-cell contacts and moreover, in cultures grown in an environment of normal calcium concentrations, H-7 caused a decrease in the number of cell-cell contacts accompanied by fibroblastic morphological changes.

Certain parallels may be drawn between these observations and the results obtained using A549 cells. In A549-FCS cultures, one may describe the phenomenon of growth arrest by TPA as occurring in phases. The initial 6h may be referred to as phase 1, during which time approximately 45% inhibition of [^{3}H]TdR incorporation is maintained after rapid

onset. The 2nd phase may be described between 6h and 18h exposure to TPA. A decline in arrest of DNA synthesis, which is mimicked by STSPN, is observed. These stages in growth arrest were initially attributed to the activation, then down-regulation of PKC. However, we cannot draw the same conclusions as A549 growth arrest evoked by TPA is only temporary. Growth resumes following 5-6 days culture in the continued presence of TPA, in the absence of measurable PKC activity and immunodetectable PKC-α or -ß. Moreover, TPA in A549-US cells, and bryo in A549-FCS cultures (sections 4.1.3 and 4.6), induce only very transitory growth arrest, effective for less than 48h. One could speculate therefore that the initial stages of growth inhibition are solely dependent on the activation of PKC.

Wolf and Baggiolini (1988) have described enhanced translocation of PKC in the presence of TPA and STSPN, but enzyme-dependent phosphorylation was inhibited. One may postulate that PKC activation was therefore prevented and thus enzyme down-regulation would not ensue. The magnitude of response generated following co-incubation of cells with STSPN and TPA was in between those responses elicited by either agent alone, during the initial 6h. STSPN and TPA occupy separate sites on PKC. A result of competition at the level of activation may determine the ensuing cellular response. Such a possibility has been indicated by Davis *et al.* (1989). These authors obtained a concentration-dependent antagonism of phosphorylation of a 47kDa protein by TPA. One may hypothesize that inhibition of PKC down-regulation may enable more persistent activation of PKC. Hence, TPA in A549-US cultures and bryo in A549-FCS cultures may maintain their ability to arrest cell growth in the presence of STSPN. This is only speculation as growth arrest may be as a consequence of cytotoxicity induced by STSPN, which may be a result of interference with protein kinases other than PKC. Experiments will be designed to address these hypotheses in A549 cells in the future, in addition, the effect of STSPN on the growth of PKC-depleted cells will be examined.

Cases have been reported in which STSPN not only failed to inhibit the cellular response induced by TPA, but elicited a similar response. For example, 24h after exposure to 10nM STSPN, all cultured primary keratinocytes had differentiated and detached from the substrate, in addition, the formation of cornified envelope was induced. Interestingly, bryo partially inhibited this response (Dlugosz *et al.*, 1990). It was inferred from this study that STSPN may provide a useful antitumour agent *in vivo*. The induction of ornithine decarboxylase in isolated mouse epidermal cells occurred following treatment of cells with either TPA or STSPN (Kiyoto *et al.*, 1987). Differential effects elicited by STSPN with respect to TPA-induced responses have been described by Yamamoto *et al.*, 1989. The induction of ornithine decarboxylase caused by TPA was not inhibited by STSPN, but rather augmented by this agent. Topical application of STSPN prevented TPA-dependent tumour formation, in a dose dependent fashion, but failed to inhibit oedema formation induced by TPA.

Efforts have been undertaken to design more selective derivatives of STSPN. Davis *et al.* (1989) have succeeded in generating potent, selective inhibitors of PKC which are being evaluated in cellular systems and animal models as potential agents for therapeutic

intervention in diseases such as malignancy, immune disorders and inflammation. The STSPN analogue termed CGP 41 251 (Meyer *et al.*, 1989) possessed reduced ability to inhibit PKC activity compared to the parent compound but demonstrated a high degree of selectivity when assayed for inhibition of other kinases. These authors have illustrated growth inhibition by STSPN and CGP 41 251 in the T-24 bladder carcinoma cell line, in HL-60 cells and in bovine corneal endothelial cells at concentrations which correlated well with *in vitro* PKC inhibition. It has been concluded from this study that an association exists between PKC inhibition, anti-proliferative and anti-tumour activity. This conclusion is supported by work characterising growth inhibition and cytotoxicity induced by sphinganine (Stevens *et al.*, 1990). The anticarcinoma agent dequalinium has been shown to inhibit rat brain PKC-α, ß and γ with equal potencies (IC_{50} concentration of 11μM) (Rotenberg *et al.*, 1990). Moreover, studies using intact cells have demonstrated that dequalinium is able to protect cells against phorbol ester-induced morphology changes. Thus, it has been proposed that PKC could be a critical *in vivo* target of dequalinium. The microbial compound calphostin C, a highly potent and specific inhibitor of PKC is reported to possess potent cytotoxic and antitumour activity (Kobayashi *et al.*, 1989). To conclude, although universally, there is little doubt that the activation of PKC is an important cellular mediator of phorbol ester-induced responses, including inhibition of growth of many malignant cell lines (section 1.6.4); it may transpire that the inhibition of PKC activity will result in a more permanent arrest of malignant cell growth and thus may prove potentially more beneficial therapeutically.

4.2.6 Investigating the effect of Okadaic acid on A549 cell proliferation.

Introduction.

Okadaic acid, isolated from the marine sponge *Halichondria okadai* , is a potent tumour promoter but not an activator of PKC (Sugunuma *et al.*, 1988). It is a powerful inhibitor of protein phosphatases-1 and -2A (PP-1 and PP-2A) (Bialojan and Takai, 1988). These proteins are dominant protein phosphatases which have been found to act on a broad spectrum of phosphoproteins *in vivo* (Haystead *et al.*, 1989). It is reported that PP-1 and PP-2A are likely to be the major enzymes which reverse the actions of PKC. Furthermore, PP-2A are reported to selectively dephosphorylate PKC (Parker *et al.*, 1986) and may therefore be involved in regulating PKC activity (Parker *et al.*, 1987). Thus it is attractive to speculate that tumour promotion may arise from increased phosphorylation of one or more proteins that are substrates for PKC and dephosphorylated by PP-1 or PP-2A. Enhanced phosphorylation of membrane-bound proteins involved in human T-cell activation has been reported following activation of PKC by TPA and following treatment of cells with okadaic acid (Alexander *et al.*, 1988). We wished to test the hypothesis that okadaic acid may mimic the effects of tumour promoting phorbol esters in cultured A549 cells in order to establish whether growth arrest is a consequence of phosphorylation events subsequent to PKC activation or phosphatase inhibition.

Results and discussion.

Figure 52

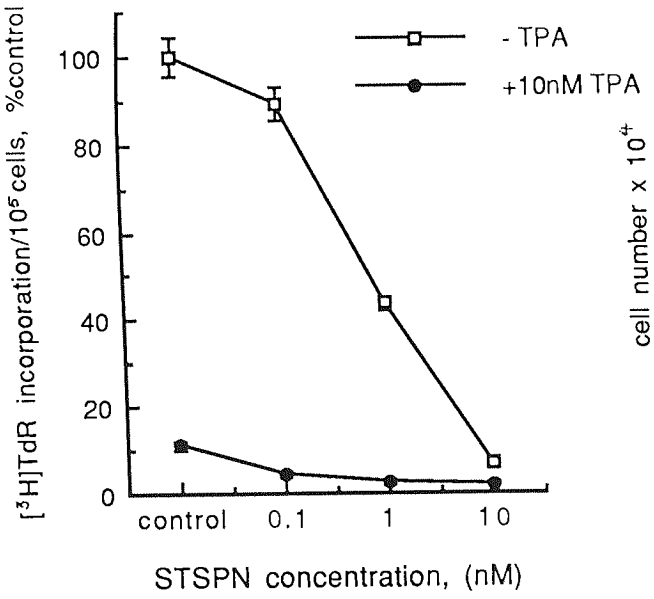


Figure 53

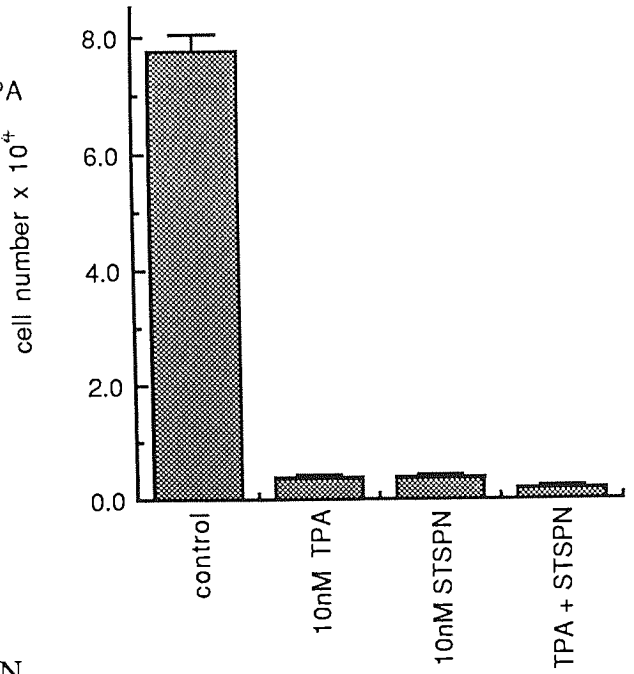


Figure 54

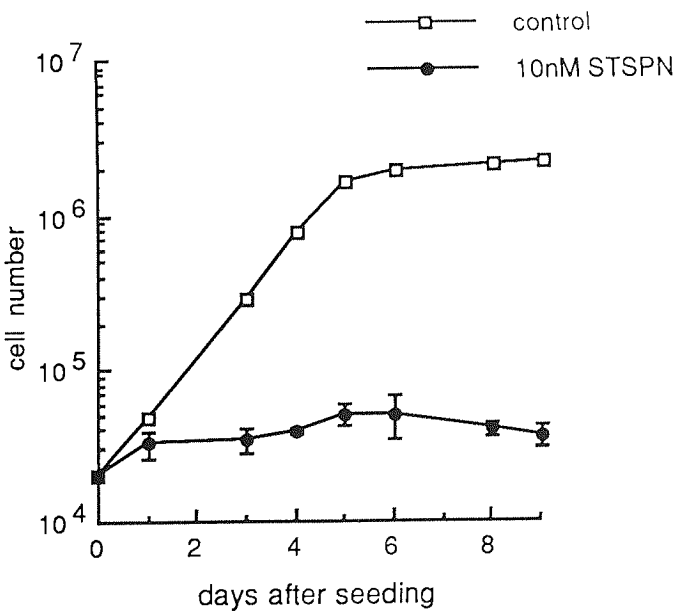


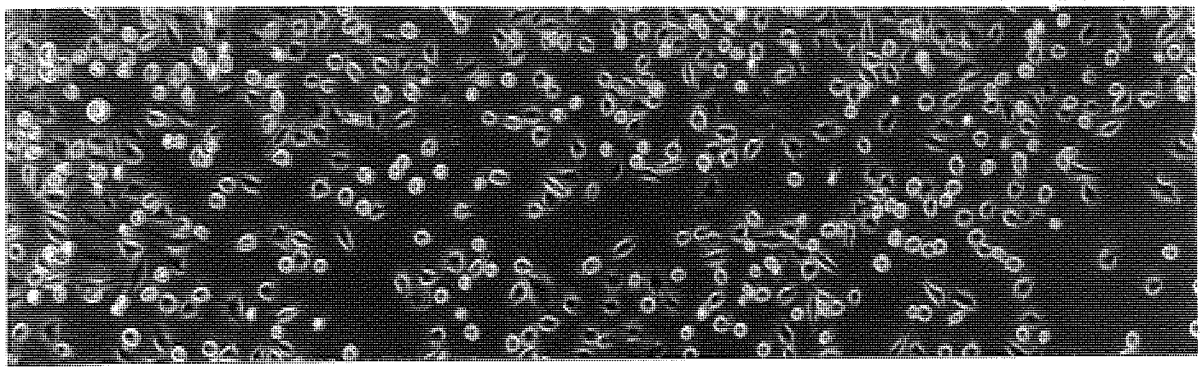
Figure 52. Dose response of A549 cells to STSPN.
Cells, 2 x 10^5 were seeded and treated with increasing concentrations of STSPN in the presence -●- or absence -□- of 10nM TPA for 24h before incorporation of [^{3}H]TdR was measured (mean ∓ SD, n=9).

Figure 53. Effect of STSPN and TPA on cell growth.
Cells, (2 x 10^4) were seeded and exposed to 10nM STSPN and/or 10nM TPA for 96h before cell counts were performed (mean ∓ SD, n=4).

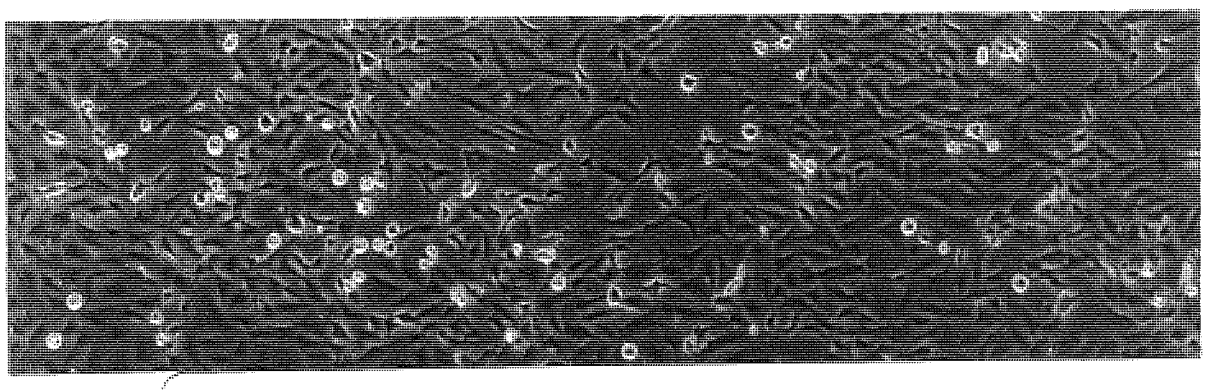
Figure 54. The effect of 10nM staurosporin on the A549 cell growth.
Cells (2 x 10^4) were seeded, cultured in the presence -●- or absence -□- of 10nM STSPN and counted at the time intervals indicated. Medium and STSPN were replenished every 48h. (mean ∓ SD, n=3).

Figure 55. Phase contrast micrographs of A549 populations following treatment with 10nM STSPN and / or 10nM TPA.
A, B, C, G A549-FCS; D, E, F A549-US
A, D 12h TPA; B, E 12h STSPN; C, F 12h TPA plus STSPN; G 3d STSPN
A, B, C, D, E, F cells seeded 2 x 10^5; G cells seeded 2 x 10^4.

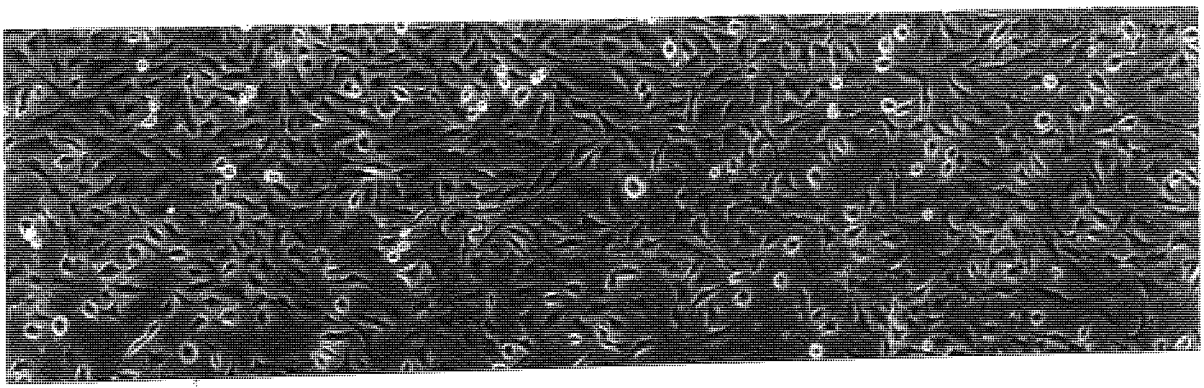
A



B



C



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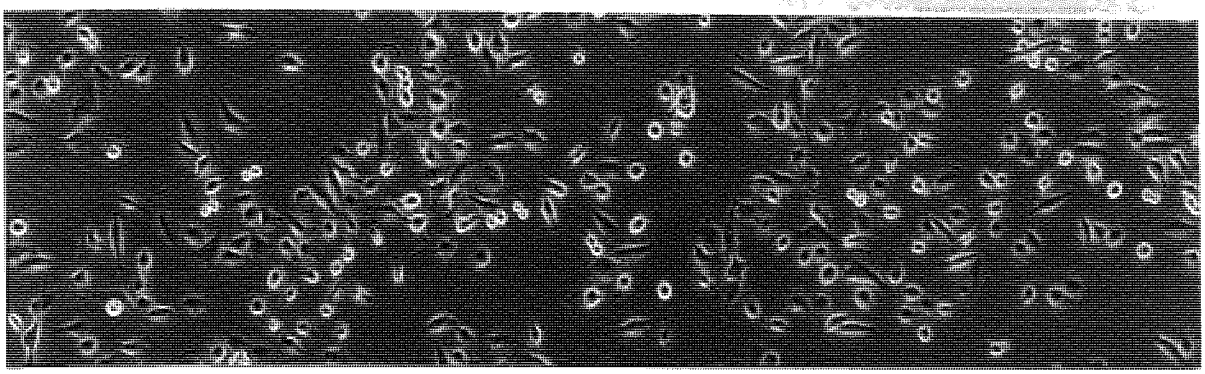
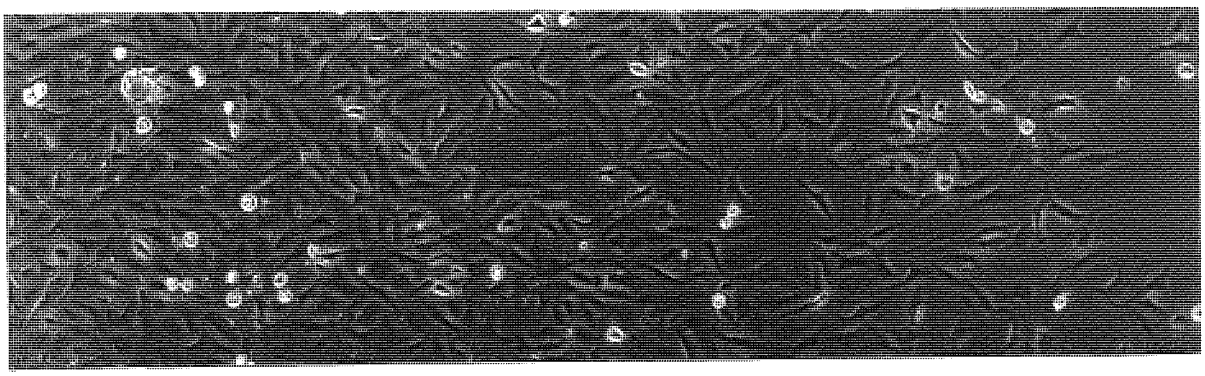
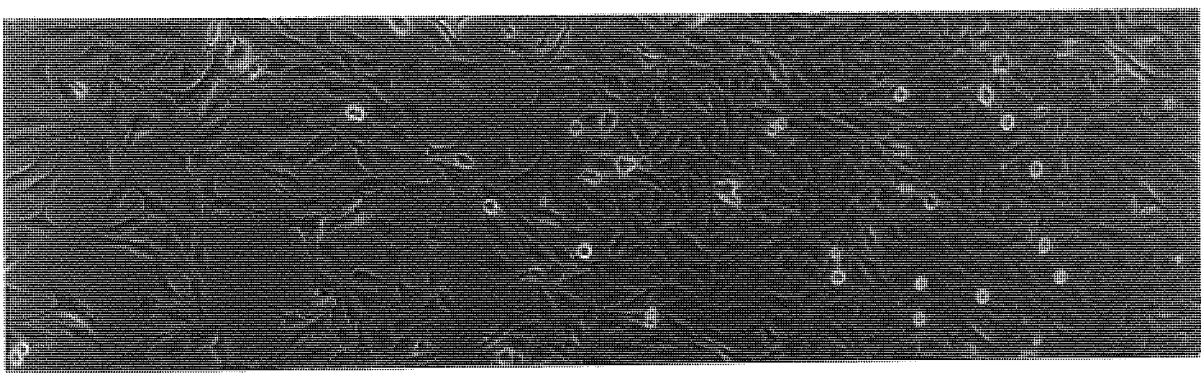


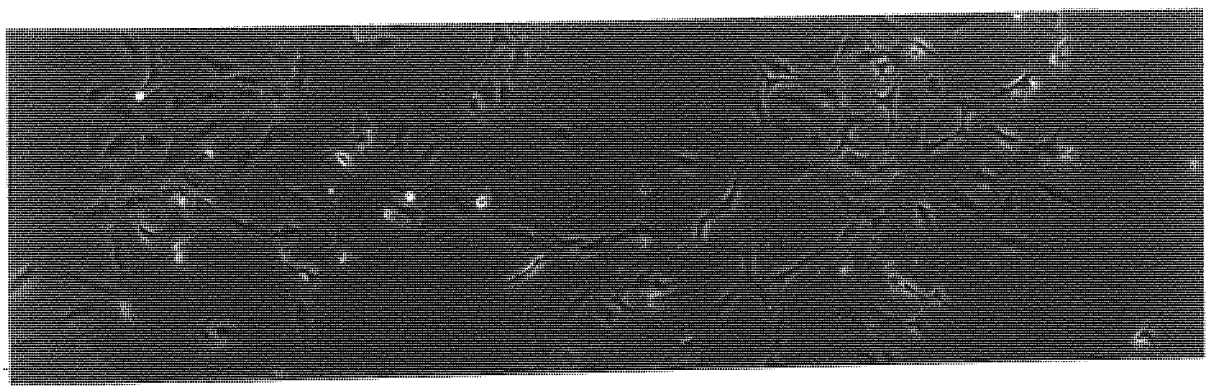
E



F



G



Figures 56 and 57. Time course of [^{3}H]TdR incorporation following treatment of 56; A549-FCS 57; A549-US cells with 10nM STSPN - -, 10nM TPA - - or these agents combined - -.
Treatment began 4h after cells (10^5 or 2x 10^5) had been seeded. Following the indicated exposure period, incorporation of [^{3}H]TdR was measured (mean ± SD, n=6).

Figure 56

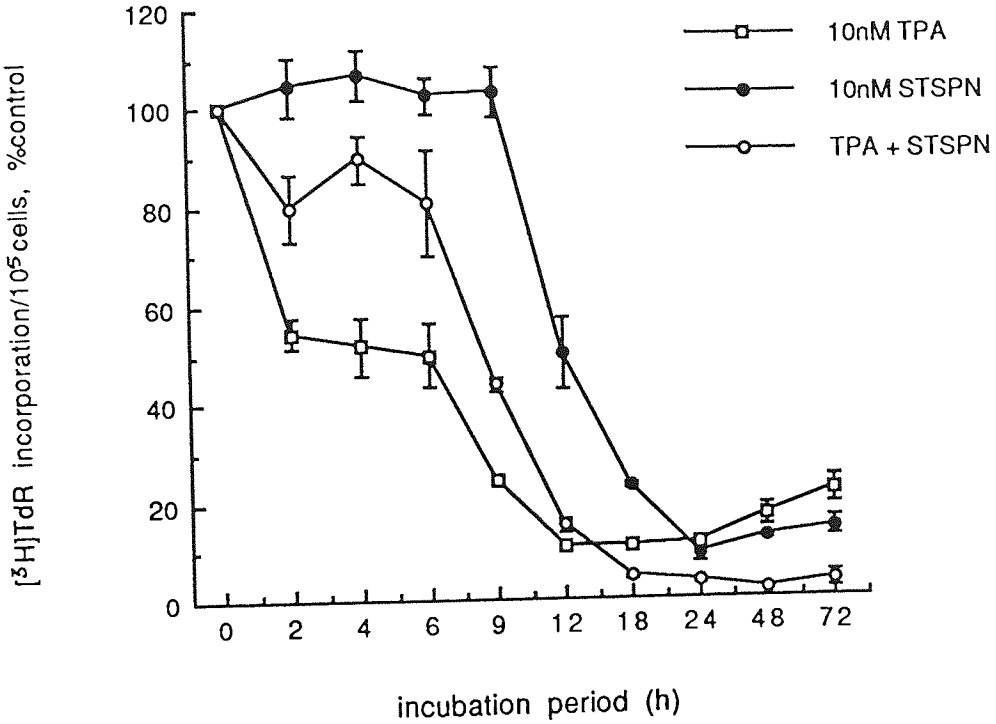


Figure 57

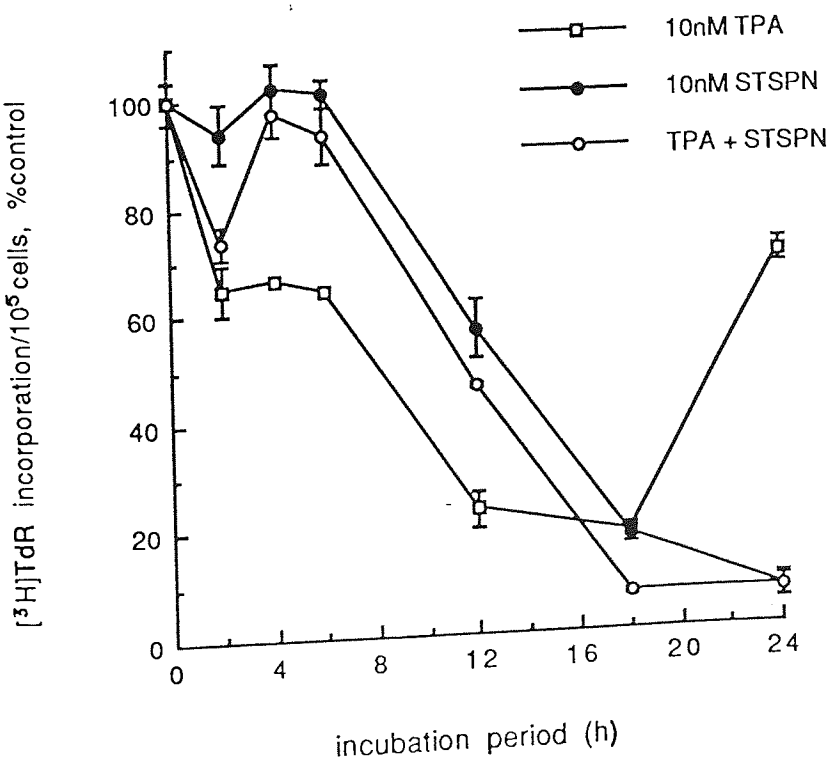


Figure 58 illustrates the effect of increasing concentrations of okadaic acid on A549 cell numbers following 96h exposure period. A dose-dependent decrease in growth was observed. The concentration of okadaic acid, derived from the values represented in figure 58, able to inhibit cell growth by 50% was calculated as 19.8nM. Negligible cellular debris was observed, thus indicating a cytostatic rather than cytotoxic effect.

We wished to examine the inhibition of DNA synthesis by 10nM okadaic acid in A549 cells and to test the hypothesis that bryo 1 is able to block the inhibition of [^{3}H]TdR incorporation which may occur. It has been reported in A549 cells that following 24h exposure, 1μM bryo 1 was unable to inhibit [^{3}H]TdR incorporation; moreover, it was able to abolish the growth arrest elicited by 10nM TPA, at this time (Dale and Gescher, 1989). The effect of bryos on A549 cell growth is discussed in detail in section 4.6. Guided by the above observation, cells were incubated for 24h in medium containing 1μM bryo 1 and 10nM okadaic acid, alone and combined. Figure 59 demonstrates that bryo 1 decreases [^{3}H]TdR incorporation to a negligible extent. After this incubation period, okadaic acid was seen to increase slightly the proliferative capacity. However, when both compounds were present, DNA synthesis was inhibited by 48%.

Experiments described in section 4.6 report that 1μM bryo 1 is able to induce a very transient arrest in DNA synthesis. This response may be a consequence of the transient phosphorylation of certain proteins. The level of phosphorylation of any isolated protein reflects the dual regulation of both kinases and phosphatases. It may be possible that the inhibition of [^{3}H]TdR incorporation observed in the presence of bryo 1 and okadaic acid is a result of okadaic acid prolonging the growth arrest induced by bryo 1, by maintaining proteins in the phosphorylated state.

The time course, assessing [^{3}H]TdR incorporation in the presence of 10nM okadaic acid, reveals that no inhibition of DNA synthesis occurred during the initial 24h exposure period (figure 60). In fact, raised proliferative capacity was recorded in the presence of 0.1nM, 1nM (result not shown) and 10nM okadaic acid. This is in contrast to the decrease in ability of cells to synthesize DNA during the initial hours of exposure to 10nM TPA. Following an exposure period of 48h, [^{3}H]TdR incorporation was inhibited by 46.7% in the presence of 10nM okadaic acid.

The hypothesis was tested that following treatment of cells for 24h with PDBu (50nM), okadaic acid is able to elicit inhibition of A549 cell growth. The results are shown in figure 61. Exposure of cells for 48h to PDBu resulted in 71.2% inhibition of DNA synthesis. Treatment for 24h with PDBu followed by 24h in phorbol ester-free medium resulted in a proliferative capacity of 58.2% compared to control values. PDBu treatment (24h) followed by exposure to okadaic acid for 24h led to [^{3}H]TdR incorporation values which didnot significantly differ from the capacity to synthesize DNA following either 48h exposure to okadaic acid or 24h exposure to PDBu followed by 24h in medium alone. However, [^{3}H]TdR incorporation was reduced by 57.9% compared to 24h treatment with okadaic acid only. Thus, it cannot be established from experiments conducted here whether pre-treatment of cells with PDBu influences the cellular response to okadaic acid. In a study carried out by Cope *et al.* (1990), pre-activation of PKC by TPA antagonized the okadaic

acid inhibition of protein phosphatases and resulted in the observed loss of protein phosphorylation.

It may be argued that the proteins phosphorylated following TPA treatment, responsible for the initial inhibition of DNA synthesis, are not those proteins whose dephosphorylation is inhibited by okadaic acid. Tumour promotion may be a long term response unrelated to the initial, rapid arrest in DNA synthesis observed in A549 cells following treatment with bryo 1 and TPA. Indeed, bryo 1 has been reported to be non tumour promoting (Hennings *et al.*, 1987). Longer term inhibition of [^{3}H]TdR incorporation was observed in A549 cells following exposure to the tumour promoting agents, okadaic acid and TPA only.

Experiments to pursue further the effects of okadaic acid on A549 cell growth would examine the effect of okadaic acid on the proliferative potential of A549 cells beyond 48h and observe the combined effect of bryo 1 and okadaic acid on DNA synthetic activity at these time points. It would be interesting to establish whether growth inhibition is a reversible phenomenon. Elucidation of mechanisms which underlie tumour promoter-induced growth inhibition could be sought by comparing protein phosphorylation profiles in treated cells. However, experiments performed using okadaic acid were very limited as only a minimal amount of this tumour promoter was available. Thus, no firm conclusions can be drawn from this work.

Figure 58. Effect of increasing concentrations of okadaic acid on A549 cell growth. Cells, $2x10^4$ were seeded and allowed 4h to attach before being exposed to various okadaic acid concentrations for 96h. Counts were performed on a coulter counter (mean∓SD, n=6).

Figure 58

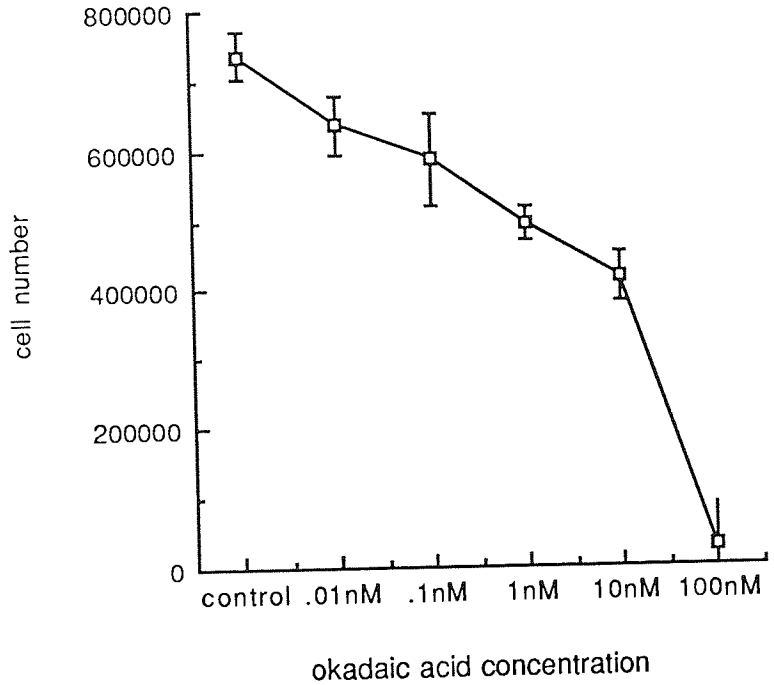


Figure 59. Effect of bryo 1 and okadaic acid on [^{3}H]TdR incorporation. Cells, $2x10^5$ were seeded and allowed 4h to attach. Incubations were carried out with 1µM bryo 1, 10nM okadaic acid and these compounds combined, for 24h before [3H]TdR was assessed (mean ∓ SD, n=6).

Figure 59

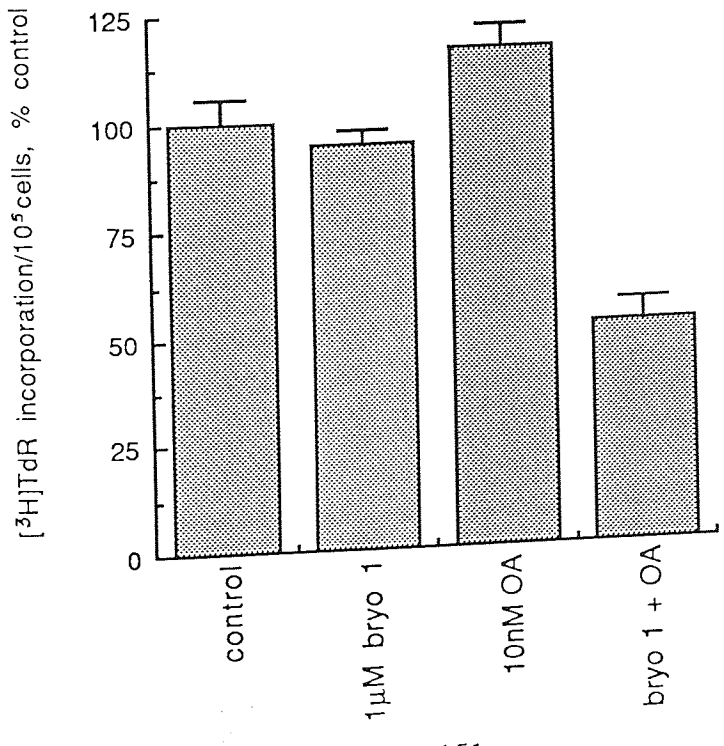


Figure 60. Time course [3H]TdR incorporation in cells exposed to 10nM okadaic acid. Cells, 10^5 were seeded and allowed 4h to attach. Incubations were carried out with 10nM okadaic acid for increasing periods of time before [3H]TdR incorporation was measured (mean ∓ SD, n=6).

Figure 60

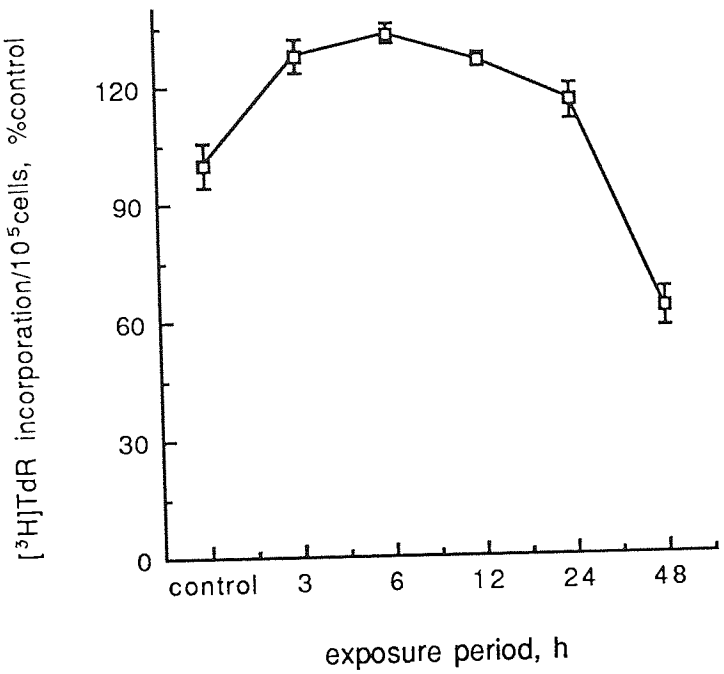
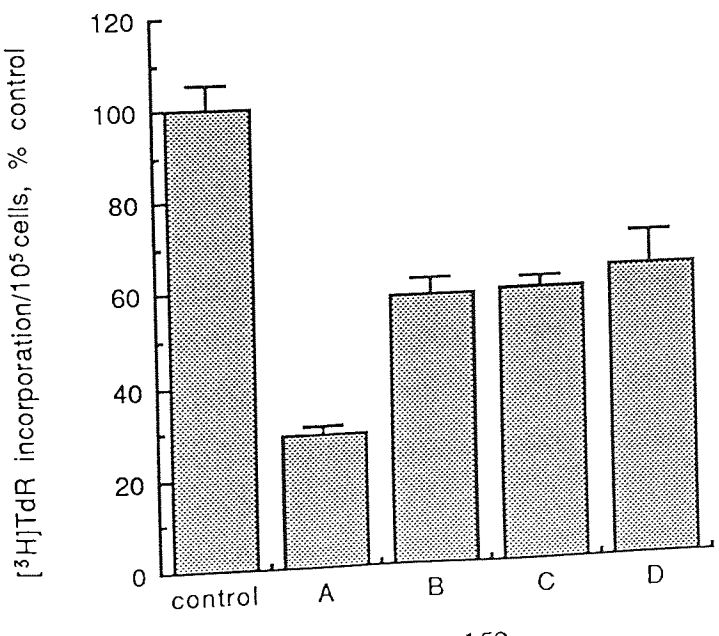


Figure 61. Effect of PDBu and okadaic acid on [3H]TdR incorporation.
Cells, 10^5 were seeded and allowed 4h to attach. After the following incubation conditions, [3H]TdR was assessed.
A. Cells were treated for 48h with 50nM PDBu.
B. Cells were treated for 24h with 50nM PDBu, washed thoroughly, and in cubated in a drug-free environment for a further 24h.
C. Cells were treated for 24h with 50nM PDBu, washed thoroughly, and treated for a further 24h with 10nM okadaic acid.
D. Cells were treated for 48h with 10nM okadaic acid (mean ∓ SD, n=6).

Figure 61



4.3 Cell cycle analysis during TPA-induced growth arrest.

Introduction.

Significant progress has been made towards elucidating signal transduction pathways leading to PKC activation. However, steps between enzyme activation and the ensuing nuclear events remain obscure. To determine the phase of the cell cycle at which point TPA interacts, may assist our understanding of the mechanisms which are interfered with by tumour promoting phorbol esters. The effect of TPA on vascular smooth muscle cells requires the activation of PKC, as TPA-induced growth inhibition was absent in PKC-depleted cells (Huang and Ives, 1987). In this study, in was concluded that TPA probed an event late in the G_1 phase of the cell cycle, or at the G_1-S transition. During G_1, a high rate of DNA transcription, RNA and protein synthesis occur and cell volume is restored to its normal size. Synthesis and replication of DNA and centrioles takes place during S phase (Junqueira and Carneiro, 1983; Hopkins, 1978).

We wished to examine whether the profound growth inhibition elicited by TPA in A549-FCS cells occurred during a specific phase of the cell cycle. Analysis was performed upon cells exposed to TPA for varying periods of time. Additionally, cell cycle distribution was examined in cells cultured continually in the presence of TPA and in the A549-US cell population.

Results and discussion.

In control A549-FCS cell cultures, approximately 58% of the population resided in the G_1 phase of cell cycle, 14% were undergoing DNA synthesis (S phase) and 28% occupied the combined G_2/M phases. Figure 62 demonstrates that upon exposure to the growth arresting agent TPA, alterations in population profiles with respect to cell cycle distribution occurred. Following 3-6 h exposure a decrease in S phase was apparent accompanied by an accumulation of events in the G_2/M cycle stages. The initial observation was a decline in G_1 events which would be expected if cells were prevented, by the G_2/M block, from entering this phase. Presumably therefore there was a block during G_1 also, as the contents of S phase remained negligible for at least 4 days. Accordingly, minimal incorporation of labelled thymidine occurred during this period in cells exposed to TPA. After 4 days treatment, in the continued presence of 10nM TPA, a fall in the number of events blocked at G_2/M began and the number of cells entering S phase steadily increased as near normal cell growth rate resumed. Following 6 days treatment, a similar cell cycle distribution as untreated controls was observed.

These data suggest that following TPA treatment, an accumulation of events occurs in the G_2/M cell cycle phase, thus preventing progression through the cycle and subsequent cell division. G_2 phase follows S phase and may account for the observed lag in attaining potent inhibition of [^{3}H]TdR incorporation when measuring thymidine uptake in the presence of growth arresting phorbol esters (Dale and Gescher, 1989). A549-FCS cells, permanently exposed to 10nM TPA displayed a cell cycle distribution profile as follows: a

slightly higher proportion of cells resided in the G_1 phase when compared to naïve A549-FCS cells (61.6%); with fewer events occupying the S and G_2/M phases, (11.5% and 26.9% respectively) reflecting the greater length in population doubling time. Profiles representing the cell cycle distribution of control A549-US cell cultures, demonstrated fewer cells undergoing DNA synthesis (10.2%), and mitosis (G_2+ M, 21.74%) reflecting the increased doubling time of these cells compared to cells cultured in medium fortified with serum. A comparatively higher proportion of cells occupied G_1 (68.0%) (figure 63). TPA elicited a potent but very transient inhibition of cell growth in serum-free cultures (section 4.1.3); accordingly, a brief disappearance of events from S phase was observed and only a small and transient rise was seen in the proportion of cells in the G_2/M phases (figure 64). It has been suggested that there exists a correlation between cells actively cycling and the susceptibility to growth arrest by TPA (Beckwith *et al.*, 1990). Following the period of A549-US growth arrest, a modest shift in cell cycle distribution was observed with a small increase in cells entering S phase and G_2/M accompanied by a brief and small decline in the G_1 compartment. After 4 days exposure to TPA cell cycle distribution once more resembled the control population profile. Assays measuring thymidine incorporation in A549-US cells during exposure to TPA have verified this immediate and temporary increase in mitotic activity, above control levels, following their growth arrest.

The apparent accumulation of growth arrested cells in G_2/M is not unique to A549 cells. However, that this block is temporary is unusual, as an accumulation of a population of cells during this phase of the cell cycle normally preceeds cell death; for example cells which have been subjected to toxic insult (Ruddon, 1987). Evidence has shown that inhibitors of topoisomerase II cause an arrest at the G_2/M interface in Chinese hamster ovary (CHO) cells (Constantinou *et al.*, 1989). Reduced topoisomerase II activity has also been associated with the induction of differentiation in HL60 cells by TPA; thus may be mediated by PKC. It has been reported that TPA-induced HL-60 cell differentiation was accompanied sequentially by the inhibition of cell cycling from G_1 to S phase, followed by a temporary block in G_2 and the final growth arrest of most cells in G_1 within 2-3 days exposure to TPA (Yun and Sugihura, 1986). Interestingly, Coppock *et al.* have demonstrated a temporary inhibition of DNA synthesis in a metastatic melanoma cell line and using flow cytometry have established two blocks in the cell cycle: one preventing entry to S phase and the second block upon exit from G_2/M. Beckwith *et al*, (1990) have demonstrated, upon analysis of phorbol ester treated lymphoma cells, a G_2/M accumulation of cells from 2 lines and a G_1/S block in 1 line. Thus it appears that TPA is able to block cell cycle progression at 2 points. It is possible that PKC activation by phorbol ester results in phosphorylation of a key regulatory protein whose function is one of control over cell cycle progression and that the phosphorylation state of this protein is altered in malignant transformation. It has recently been proposed that phosphorylation of a mammalian protein $p34^{CDC2}$ homologous to the yeast cell cycle control protein cdc2+ may be involved in regulation of exit from (G_2/M) and re-entry into (G_1) the mitotic cycle (Draetta and Beach,

1988; Lee *et al.*, 1988).

It has been established in this study, that A549 cells contain PKC-α (section 4.2.4). Isakov *et al.* (1990) propose that PKC-α may play a regulatory role in the expression of several cell cycle regulated genes. In human leukaemic cells, depleted of this PKC isoform, a reduction in c-*myc* , ornithine decarboxylase and *lck* mRNA was observed. The latter encodes a lymphocyte-specific tyrosine kinase that has been implicated in T cell maturation, differentiation and growth regulation. In contrast, c-*myb* mRNA levels increased. Interestingly, Levin *et al.* (1990), have isolated a single gene from yeast *Saccharomyces cerevisiae*, encoding a putative PKC closely related to the α, ß and γ subspecies of mammalian PKC. Cells depleted of this gene product displayed a uniform phenotype, characteristic of cell division cycle (cdc) mutants and arrested cell division subsequent to DNA replication but prior to mitosis. These authors have concluded that in this species, the PKC locus is essential for growth and moreover, for the G_2/M transition of the cell division cycle.

We may hypothesize that upon treatment with agents capable of causing PKC down-regulation, a temporary defect in cell division cycle may be imposed onto A549 cell cultures; a supposition supported by the observed uniform phenotype, characteristic of TPA-arrested cells. However, cells manifest rapid adaptability to their environment and are capable of release from growth inhibition. Such an observation suggests that activation of PKC may regulate only one of the mechanisms by which mammalian cell division is governed. Further, cells maintained in the serum-free environment whose content of PKC is significantly lower than the A549-FCS parent line exhibit a very transitory arrest in cell cycle, which may be interpreted as additional support for this hypothesis.

Figure 62

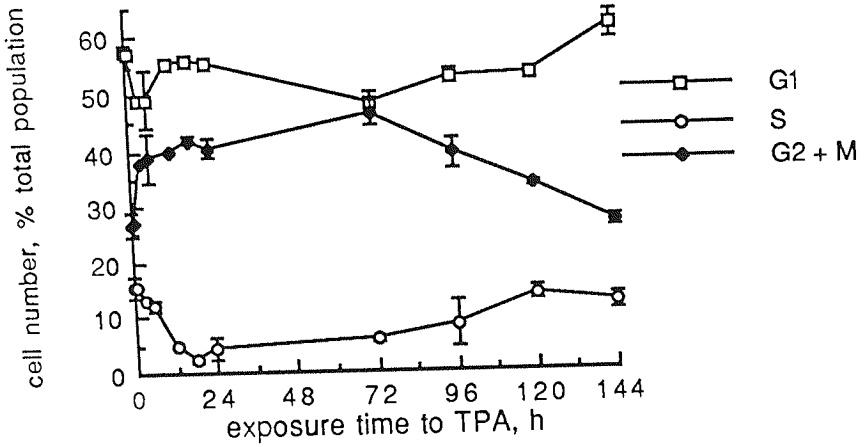


Figure 63

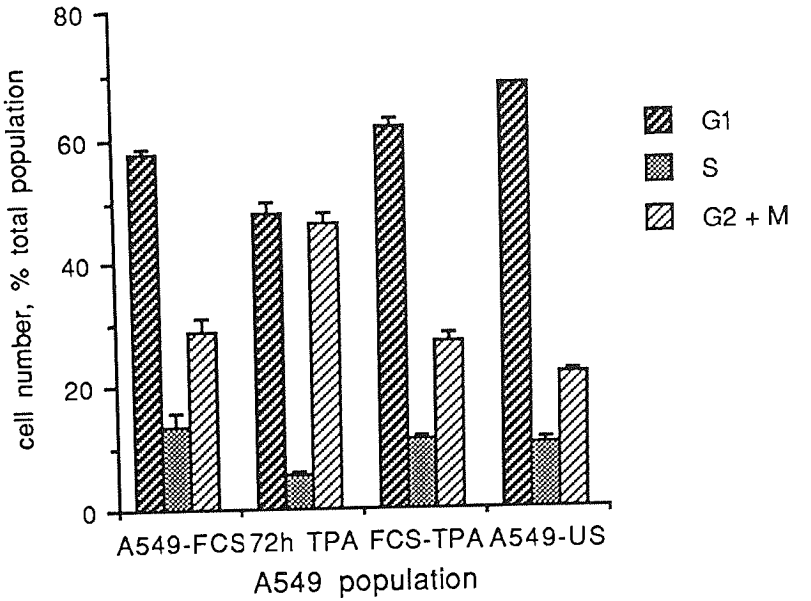


Figure 64

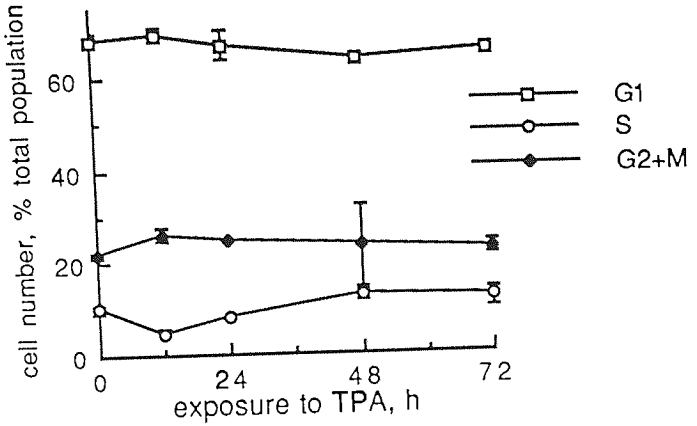


Figure 62. Cell cycle distribution of A549-FCS cells during treatment with TPA.

Figure 63. Analysis of cell cycle distribution in A549 populations.

Figure 64. Cell cycle distribution of A549-US cells during treatment with TPA.

A549 cell cultures were treated with 10nM TPA for varying periods of time as required, harvested and prepared for analysis by FCM. Results are represented as a% of total cell population (mean ± SD, n≥3).

4.4 Effect of TPA on the ultrastructure of A549 human lung carcinoma cells.

Introduction.

In a physiological environment, type II alveolar cells are found interspersed among squamous epithelial cells, contact with which is maintained by junctional complexes comprising desmosomes and occluding junctions (Junqueira and Carneiro, 1983). Cytologically, type II alveolar cells resemble typical secretory cells, possessing well developed Golgi apparatus, rough endoplasmic reticulum (RER), mitochondria, microvilli on their free apical surface and multilamellar bodies which comprise and secrete pulmonary surfactant.

It was reported in 1976 that A549 tumour cells had maintained certain characteristics of the parent cell from which they were derived. They contained multilamellar bodies and synthesized surfactant after approximately 1000 cell generations (Lieber, *et al.*, 1976). Transmission electron micrographs were taken of A549 cell populations cultured during the course of this study.

Results and discussion.

A549-FCS cells were polygonal in shape and appeared to possess irregular surfaces with microvilli visible. A549-US cells appeared more elongated, a less irregular cell surface was observed. Lightly stained nucleoli could be detected within a large nucleus. Lamellar bodies were not found in cytosol from either population. In both populations, where 2 cells were juxtaposed, it was difficult to decipher the plasma membrane. Junctional complexes could not be distinguished (figure 65, A and C).

Following 48h exposure to 10nM TPA, A549-FCS cells only were subject to growth arrest. Morphologically, light microscopy revealed that cells were rounded, protruding, condensed and apparently singular. Severely reduced intercellular contact was detected whilst cells were in the growth arrested state. Such external characteristics were maintained over a much longer period in A549-FCS cells. Interestingly, type II alveolar cells, without surfactant would round up, a general phenomenon exhibited by cells in response to the need to reduce the energy expenditures which are required to maintain the extensive surface area of flattened cells (Junqueira and Carneiro, 1983).

Transmission electron microscopy of A549-FCS and A549-US cells was performed following 48h exposure to 10nM TPA. Micrographs demonstrate that the presence of TPA caused cells to become rounded, where two cells lie together, negligible contact was maintained and cell boundaries were clearly defined (figure 65, B and D). Further examination is required to decipher clearly the affect of TPA on intercellular communicative mechanisms in A549 cultures. A transitory reduction of gap junctions and reduced junction coupling have been reported in primary chick embryo hepatocytes during the first 3h exposure to 100ng/ml TPA, with partial recovery during the following 3h (van der Zandt *et al.*, 1990). In cultured chick hepatocytes, TPA caused the disappearance of gap junctions during the first 6h of exposure, with a slow recovery observed following 36h treatment. Additionally, it has been demonstrated that activators of PKC were able to disrupt cell-cell

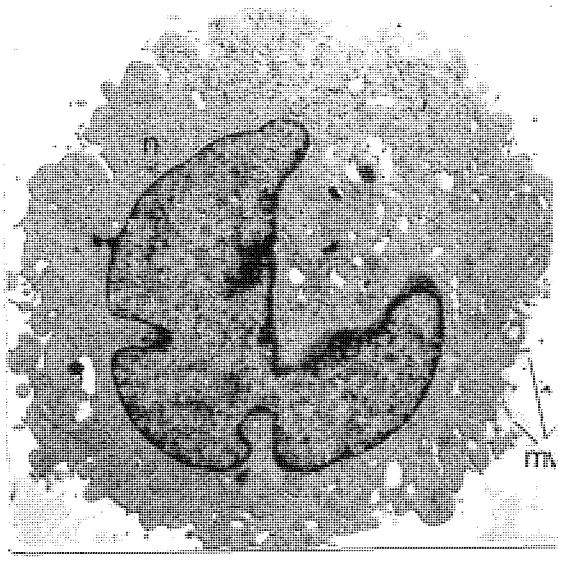
communication by the observed suppression of Lucifer yellow dye transfer following its microinjection into primary epidermal cells (Pasti *et al.*, 1988).

A high proportion of electron dense heterochromatin was revealed in the nuclei of TPA-treated A549-FCS cells, a typical appearance of cells in G_2 phase of the cell cycle, characteristic of quiescent nuclei, not actively transcribing RNA (Junqueira and Carneiro, 1983). It may be tentatively concluded therefore that cells are arrested by TPA in the G_2 cell cycle phase. G_2 phase follows replication of DNA. During this brief premitotic interval, cell growth and the production and accumulation of energy occurs, to be utilized for the synthesis of tubulin and during mitosis (Junqueira and Carneiro, 1983; Hopkins, 1978). For gene transcription to occur, the association between the gene to be transcribed and RNA polymerase must take place (Ruddon, 1987). RNA polymerase represents one example of several enzymes demonstrated to be *in vitro* substrates for PKC and capable of altering gene transcription (Chuang *et al.*, 1989; see section 1.3.8). In contrast, untreated A549-FCS cells possess lighter staining nuclei (figure 65, A), the higher proportion of euchromatin presents a greater DNA surface area available for transcription of genetic material. Additionally observed in A549-FCS cells undergoing treatment with TPA were well defined, dark staining acidophilic nucleoli. This organelle lies free within the nucleoplasm of a non-mitotic nucleus and disperses during cell division. (Hopkins, 1978; Junqueira and Carneiro, 1983). Larger nucleoli are encountered in the majority of rapidly growing malignant tumours. In A549-US cells exposed to TPA for 48h, more densely stained chromatin is observed within the nuclei compared to the untreated A549-US cells. Additionally, defined nucleoli are seen. However, the differences between untreated and treated cells appear less dramatic than observed in A549-FCS cells. A549-US, TPA treated cells do not display such uniformity as A549-FCS cell populations exposed to TPA for 48h; certain cells do not appear so rounded or regular in shape.

It should be noted that very few concrete conclusions can be drawn from the EM work, as the cells photographed represent a tiny sample of the population, thus the discussions induced by these observations are only speculative.

Figure 65. Electron micrographs of A549 cells.
A and B: A549-FCS
C and D: A549-US
A and C: untreated
B and D: following 48h exposure to 10nM TPA (magnification x 10 000)
N: nucleus, Nu: nucleolus, Mi: microvillus,
ec: euchromatin, hc: heterochromatin.

A



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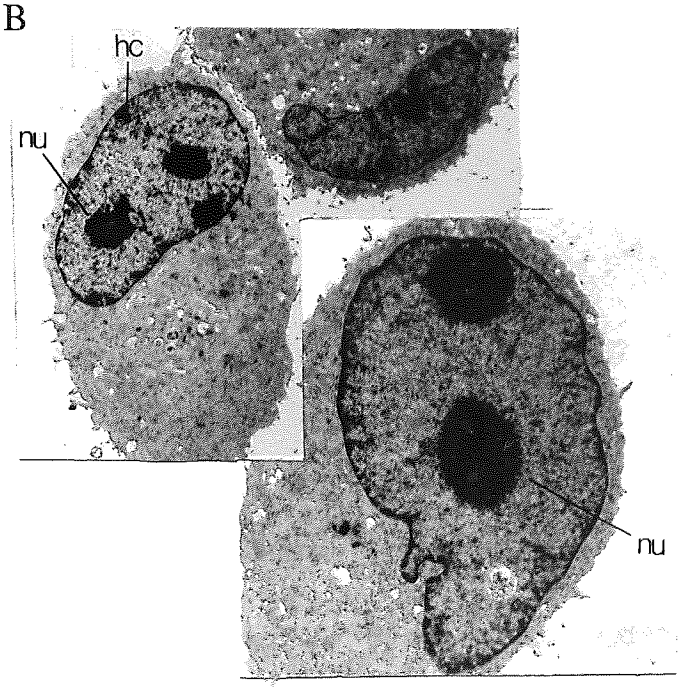

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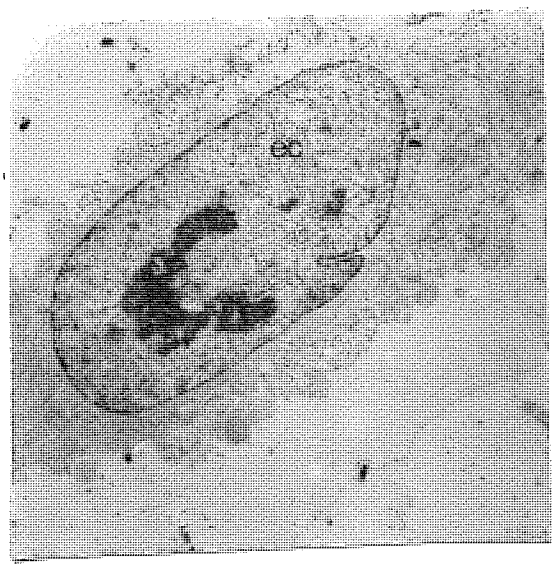

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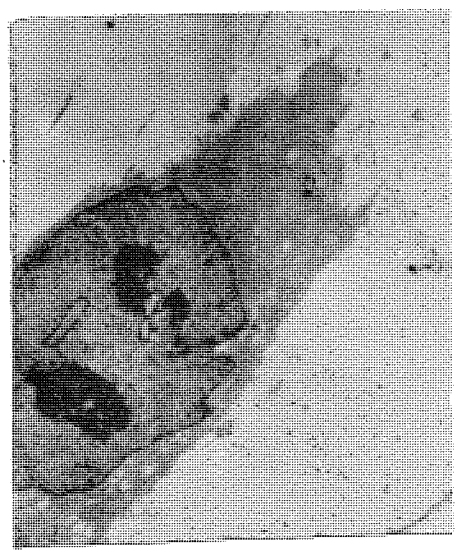


4.5 Examination of the affinity of synthetic DAGs and analogues for PKC and their effects on A549 cell growth.

Introduction.

Tumour promoting phorbol esters are able to elicit growth inhibition at non toxic concentrations in A549 cells. They are able to induce the differentiation of certain malignant cell lines (discussed in section 1.6.3). Moreover, an abundance of literature exists implicating the phorbol ester receptor, PKC in the process of tumourigenesis. Together, these facts have led to the proposed hypothesis that pharmacological modulation of PKC may be exploited therapeutically in malignant disease.

DAGs are the endogenous activators of PKC (Nishizuka, 1984). The interaction between DAG and PKC is stoichiometric (Hannun *et al.*, 1985), stereospecific, with the S-enantiomer being active, and transient. The tumour promoting phorbol esters, ingenols, aplysiatoxins, teleocidins and bryos are structurally diverse molecules which appear to act by mimicking DAGs. Indeed, the receptor site for DAG, located on the regulatory portion of PKC is also the receptor for the tumour promoting phorbol esters (Bonser *et al.*, 1988, Cazaubon *et al.*, 1989). However, in contrast to DAGs, phorbol esters are able to activate PKC in a permanent and unregulated manner such that the biological responses they evoke differ qualitatively and quantitatively from those elicited by DAGs. The rapid metabolism of endogenous PKC activators precludes prolonged activation and enzyme down-regulation afforded by these exogenous activators, which may be necessary for the ensuing physiological effects (May *et al.*, 1986). Furthermore, TPA may inhibit DAG kinase. 1,2-DAGs have been reported to activate a sphingomyelinase which is able to reverse PKC activation. The failure of phorbol esters to activate this negative effector system may account for certain differences between these agents (Kolesnick and Clegg, 1988). Computer modelling of the putative phorbol ester pharmacophore has shown similarities between PKC activators in the relative positions of certain heteroatoms and hydrophobic groups. For the phorbol ester TPA, this mapping consists of C-4, C-9 and C-20 hydroxyl groups and a hydrophobic region filled by a long chain acyl functionally attached to either the C-12 or C-13 positions (Wender *et al.*, 1986). Such spatial arrangement of heteroatoms appears crucial for binding and is consistent with the ability of DAGs to fit this model in a stereospecific manner.

Previously, the effects of TPA, DAGs, and certain synthetic cyclohexanetriol diesters, conformationally locked analogues of DAGs, on the growth of A549 cells have been compared (Laughton *et al.*, 1989). The study described here represents an extension of these structure-activity investigations using i) cyclohexanetriol diesters, ii) 4 newly synthesized DAG analogues in which the free hydroxyl group is sterically hindered by attachment of 1 or 2 alkyl moieties onto the glyceride backbone, iii) an analogue of DAG in which the free hydroxyl group is replaced by NH_2. We have investigated the ability of synthetic DAGs to interact with PKC and tested the hypothesis that these compounds are able to inhibit the binding of PDBu to its receptors in A549 human lung carcinoma cells. The possibility that these analogues, like TPA but unlike DAGs, are able to interfere with

the growth of A549 cells was examined.

Results and discussion.

Figure 66 illustrates the structure of DiC8, OAG, and the analogues synthesized by Dr. C.A.Laughton. Figure 67 and Table 1 illustrate the ability of diC8 and DAG analogues to compete with [^{3}H]PDBu for phorbol ester binding on A549 cell monolayers. The binding of diC_8 to phorbol ester receptors occurs in a dose dependent manner with 50% [^{3}H]PDBu displaced by 44μM diC_8. The sterically hindered analogues show decreased affinity for the phorbol ester receptors on intact A549 cells. Non-specific binding in this assay was relatively high, reaching values of up to 40% total binding. Therefore, the ability of DAG and its analogues to displace [^{3}H]PDBu from A549 cytosolic phorbol ester receptors was examined using the mixed micelle assay of Hannun and Bell (1987). The affinity of diC_8 and OAG (figure 68) for this receptor preparation increased with concentration of competitor. DiC_8 displayed the higher affinity with 50% [^{3}H]PDBu displacement by 28.8μM.

The monomethyl substituted analogues retained some ability to displace [^{3}H]PDBu (figure 69), but all other analogues displayed little binding potential for this preparation of phorbol ester receptor (table 1). Non-specific binding in the micelle assay was consistently low (<13% total binding).

Mixed micelle assays were performed using pure rat brain PKC, comprised of α, $ß_I$, $ß_{II}$ and γ isozymes as a source of phorbol ester receptors. Specific binding increased with increasing concentrations of PKC (figure 70a). Routinely, a final concentration of 3 units/ml was used. As was the case when A549 cells provided the source of receptors, 50nM [^{3}H]PDBu was used in this assay, as specific PDBu binding to this pure receptor source was dose-dependent and saturated at this concentration (figure 70b). DiC_8 displaced [^{3}H]PDBu with greater affinity when compared to phorbol displacement in an A549 cytosolic receptor preparation (figure 71). Displacement of 50% [^{3}H]PDBu was incurred by 7.8μM diC_8. The increased affinity may possibly arise because of preferential affinity of diC_8 for certain isozymes of PKC which might not be present in A549 cytosol. In addition, the crude preparation may have possessed proteins able to inhibit the interaction between DAG and phorbol ester receptor. The cyclohexanetriol diesters 053 and 082 and the hindered DAG analogues 146 and 153 exhibited negligible binding to PKC at concentrations comparable with those of diC8. However, analogues 139 and 145 showed some binding ability but with decreased affinity when compared to diC8 (table 2).

Work by Bonser *et al.* (1988) focused on the stereochemical requirements for the interaction of novel DAG analogues at the recognition site on PKC. The dihexanoyl analogues of 145 and 146 were used and purified PKC was the source of phorbol receptors. In this study it was concluded that in addition to the need for a natural 1,2-diacyl-*sn* -glycerol configuration, there is a preferred stereochemical arrangement at the 3 position in the methylated analogues. We cannot draw such a conclusion, possibly

because of the racemic nature of analogues used in our study, whereas the pure S-enantiomers were used by Bonser *et al.* (1988).

According to the hypothesis of Nishizuka (1984), the hydroxyl group at position C-9 of phorbol ester correlates with the free hydroxyl group of DAGs. The competing hypothesis of Wender *et al.* (1986) suggests that it is the hydroxyl group at C-20 that is mapped onto the free hydroxyl at position 3 of DAG. Computer-aided molecular modelling revealed that in phorbol esters the hydroxyl group at C-9, which is tertiary, is in a sterically crowded environment while the hydroxyl group at C-20, which is primary, is in an exposed position (Laughton *et al.*, 1989). Our results suggest that the introduction of some steric hindrance affecting the hydroxy moiety in the DAG molecule causes a decrease in ligand binding affinity, which seems to suggest that the interaction between DAG and the phorbol receptor requires an hydroxyl group in the exposed position. Substitution of the exposed hydroxyl moiety with $-NH_2$ rendered the compound unable to compete with phorbol ester for receptor binding in A549 cells. Ganong *et al.* (1986) proved an absolute requirement for the 3-hydroxyl moiety, as replacement of this group with an aldehyde function confers neither affinity nor efficacy upon this analogue.

The design of the cyclohexanetriol diester analogues 153, 052 and 053 to resemble ring C of the phorbol ester molecule, was guided by the hypoyhesis of Nishizuka (1984) that this is the important structural moiety of the phorbol ester pharmacophore. Synthesis of these DAG analogues was performed to test the hypothesis that the greater potency of phorbol esters is associated with the cyclical structure bearing the diester moiety. However, the lack of significant receptor binding activity of cyclohexanetriol diesters suggests that the receptor binding affinity of glyceride type molecules is not increased by introduction of a cyclical structure. A recent report describes the synthesis of a series of 1,2-cyclohexanediol diesters as potential high affinity activators of PKC (Kerr *et al.*, 1990). These compounds, designed to mimic the 6,7-double bond and C-20 hydroxy of phorbol ester, failed to bind to or activate PKC. Thus, the pursuit of simple cyclohexane analogues appears unjustified and new compounds would need to include more of the critical molecular framework of the phorbol ester pharmacophore.

The *in vitro* evidence that DiC_8 binds to the phorbol ester receptor leads to the prediction that elevation of intracellular DAG would induce phorbol ester like effects. It has been reported that elevated levels of DAG are important for maintenance of the transformed phenotype (Wolfman and Macara, 1989). Indeed, the synthetic DAG *sn* -1,2-didecanoylglycerol has been evaluated as a complete tumour promoter in dimethylbenz[a]-anthracene-initiated CD-1mouse skin (Smart *et al.*, 1989). The ability of diC_8, OAG, and analogues 145, 146, 139, and 153 to interact with cytosolic PKC following treatment for 30 mins of intact A549 cell monolayers with these compounds was investigated. Phorbol ester binding to cytosolic fractions was measured; in addition, PKC assays were performed to determine enzyme activity in both cytosolic and particulate fractions following partial purification by non-denaturing PAGE.

In stark contrast to TPA, which, following incubation with cells for 30 mins caused a concentration-dependent shift in enzyme distribition from cytosol to membrane (Dale *et al.*,

1989) and a concentration-dependent disappearance of phorbol ester binding sites (section 4.2); negligible effect on cytosolic PKC was observed after this period of time, with concentrations of DAG (figure 72) and analogues which are non-toxic to the cell. Neither translocation to the membrane of cytosolic PKC, nor enzyme down-regulation was induced by DAGs or its analogues. Introduction of DAG in this manner to the extracellular environment creates an artificial situation as the compound whose half life is finite has first to be intercalated into the membrane; therefore it remains unknown whether physiologically relevant concentrations of DAG have entered the cell. In accordance with our results, the oleoylester analogues of 145 and 146 were unable to activate purified PKC (Molleyres and Rando, 1988). Additionally, diC_8 was unable to promote PKC translocation in platelets, although it inhibited both phorbol ester binding and the thrombin-induced Ca^{2+} transient (Erne *et al.*, 1987). However, it has been established that DAG-induced enzyme translocation is a transient phenomenon with fractional PKC redistribution to the membrane, followed by its return to the cytosol within minutes, and no down-regulation (Issandou and Darbon, 1988; Murray *et al.*, 1987). O`Flaharty *et al.* (1990) have described two sequential mechanisms for the translocation of PKC in human polymorphonuclear neutrophils. The first involved Ca^{2+} transients producing a rapid reversible response. The second more slowly evolving Ca^{2+}-independent signal contributed to the regulation of PKC. Phorbol esters stimulate stable membrane-bound PKC that can only be solubilized with detergents (Kraft and Anderson, 1983). Therefore, it is possible that following exposure to DAGs and analogues for 30 mins certain negative effector mechanisms have been enforced to inhibit further PKC activation. Bazzi and Nelsestuen (1989) have reported the formation of an active PKC, reversibly bound to the membrane with both activity and membrane binding being terminated by the addition of calcium chelators. These workers have established that phorbol esters were also able to promote constitutive PKC activation by formation of an irreversible PKC-membrane complex. These results led to the proposal that the different behaviours of DAG and phorbol esters may be consistent with their different effects in whole cells and tissues.

Evaluation of the ability of the DAG analogues to inhibit the growth of A549 cells yielded the IC_{50} levels demonstrated in table 3. The concentrations of DAG analogues required to cause 50% release of LDH from the cells are also represented. For compounds 145, 146, and 153, LC_{50} values could not be attained; 100μM caused 31 + 2%, 36+ 8% and 30+ 6% LDH leakage from cells respectively.The concentration-dependent arrest of cell growth and LDH release from cells following their treatment with compound 135 are illustrated in figure 73a) and b). Although the LC_{50} values obtained were somewhat greater than the IC_{50} values, there was no apparent difference in order of magnitude. The difference in initial cell densities; $2x10^4$ and 10^5 in inhibition and cytotoxicity studies respectively, may account for the apparent variance in sensitivity. The NH_2 substituted analogue was synthesized as the ammonium bromide salt. The presence of bromine anions may be responsible for the greater toxicity of compound 135. The results suggest that the analogues tested, like diC_8 and OAG, but unlike the tumour promoting 4-ß phorbol esters,

inhibit A549 cell growth only at μM concentrations by non-specific cytotoxic mechanisms. These results were not unexpected in the light of the compounds inability, like diC_8 and OAG, to cause translocation of PKC to the membrane of A549 cells.

Metabolic studies were carried out by Dr.C.A. Laughton on diC_8 and derivatives 145, 146, 139, 153 and 053 in A549 cultures, using a gas liquid chromatography method for analytical detection (Laughton *et al.*, 1989). The half life of diC_8 was 2h. It was concluded that cells rather than serum affected the rate of metabolism. Lipases and kinases within the cell therefore play a greater role in the metabolism than do serum lipases or non-enzymic hydrolysis. In the absence of cells the stability of the DAG analogues was similar to that of diC_8, but in the presence of cells, the most conformationally hindered analogue 153 and the cyclohexanetriol diester 053 demonstrated increased stability. These results suggest that it is the activity of the kinases rather than the lipases which are responsible for the greater part of the metabolism of these DAG analogues, and that metabolism can be suppressed by introducing sufficient steric hindrance around the free hydroxyl group. The observation that analogues 153 and 053 possess neither the growth-inhibitory characteristics of TPA nor the ability to bind to the phorbol ester receptor suggests that structural incompatibility with the phorbol ester receptor on PKC rather than metabolic instability accounts for their inability to mimic TPA.

In conclusion, the results presented here support the view that binding of glyceride-type molecules to PKC exhibits stringent specificity. Compounds tested in this study possess structural features inadequate to fit the phorbol ester pharmacophore. Thus they fail to induce similar cellular responses, and are unable to activate PKC or effectively compete with either phorbol ester or DAG for their receptors. The results also suggest that the design of novel activators of PKC or potent ligands competing for the phorbol ester receptor requires the placement of the DAG pharmacophore into a molecular framework which is more complicated than glycerol or ring C of phorbol.

It should be noted that there are disadvantages involved when using the A549 cell line as a model system with which to screen novel compounds. DAGs cause no detectable enzyme translocation. The lack of correlation between translocation and biological effects mediated by PKC has been argued by Bosca *et al.*, (1989). According to this report, the full expression of some PKC mediated biological responses in lymphoblastic cells may be achieved by less than 5% PKC activation. It is possible that methods employed in this study for detection of PKC translocation from cytosol to the membrane fraction are not sensitive to detect such small shifts of enzyme activity.

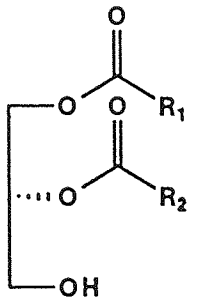
Furthermore, DAGS elicit negligible effects on A549 cell growth (Dale, 1989). A system in which the administration of DAGs evokes a specific cellular response would be a better alternative to conduct growth investigations using DAG analogues. For example, inhibition of MCF-7 cell growth has been observed following treatment with diC_8 and TPA. Activation of PKC has been demonstrated in both cases, however, distinct phosphorylation patterns were induced and down-regulation of PKC was achieved following exposure to TPA only (Issandou *et al.*, 1990). Repeated application of DAGs to M5076 tumourigenic

murine macrophage cells did result in the down-regulation of PKC and accompanied potent cell growth inhibition, mimicking the action of phorbol esters such as TPA (Goode and Hart, 1990).

Recent experiments have provided preliminary evidence which suggests that A549 cultures contain PKC-α (section 4.2.4) as source of phorbol ester receptor, there was no detection of PKC-ß. It is possible that DAG and analogues may exhibit isozyme specificity, possessing greater affinity for isozymes not present in A549 cells. In support of this hypothesis, it was observed that compound 139 displayed negligible binding affinity for A549 derived phorbol ester receptor but was able to modestly displace [^{3}H]PDBu from pure PKC derived from bovine brain comprising α, ß and γ isozymes. Moreover, in the mixed micelle assay, diC_8 exhibited significantly higher affinity for phorbol ester receptors when pure PKC containing α, ß and γ isozymes was included in the assay mixture than when A549 cytosol provided a crude source of phorbol ester receptors ($P<.001$). It has been reported by Sekiguchi *et al* . (1988) that PKC-α and -γ demonstrated much less activation by dioleoylglycerol than PKC-ßI and -ßII. In this study, PKC-α displayed highest sensitivity to activation by 1-steroyl-2-arachidonoylglycerol and PKC-γ was activated most efficiently by the synthetic permeable DAGs diC_{10} and diC_8. In order to reach a final conclusion concerning the ability of DAG analogues to interact with PKC, additional screening systems are necessary including binding studies using pure samples of each isozyme. Future screening techniques should include experiments to investigate protein phosphorylation by PKC activators and compounds designed to compete for phorbol ester binding. If it becomes established that PKC-α exclusively is expressed within A549 cells, this cell line would provide an excellent model with which to investigate competitive ligand binding to PKC-α and characterize certain cellular functions triggered by PKC-α activation. The combination of analysis of isozyme tissue specificity and synthesis or isolation of isozyme targetted molecules would make the pursuit for selective chemotherapy a more tangible goal.

Figure 66. Structure of DiC_8, OAG and synthetic DAG analogues used in this study.

diC_8	cal 139
OAG	cal 153
cal 135	cal 053
cal 145	cal 052
cal 146	cal 082

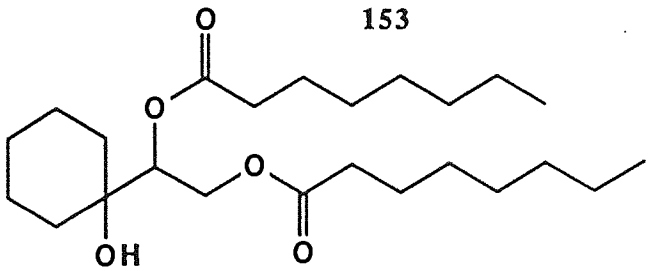


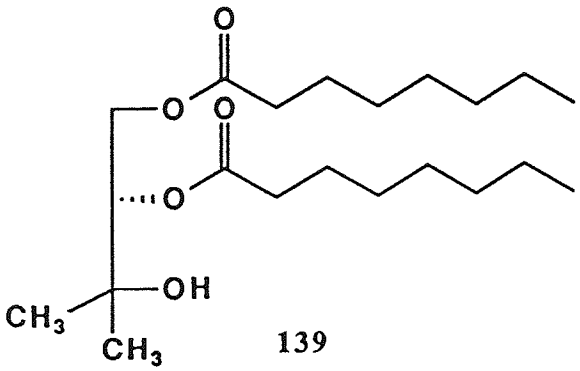
OAG

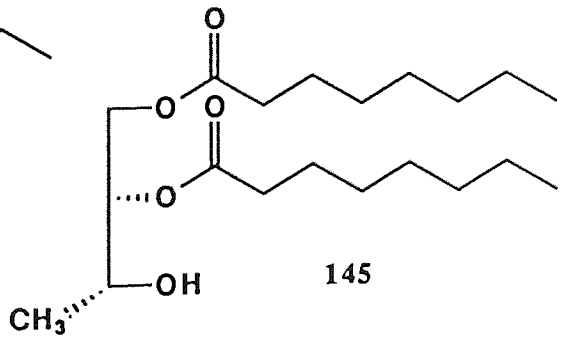
$R_1 = -(CH_2)_7CH=(CH_2)_7CH_3$
$R_2 = -(CH)_3$

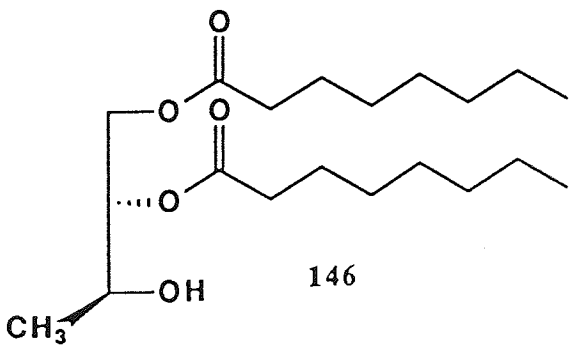
DIC_8

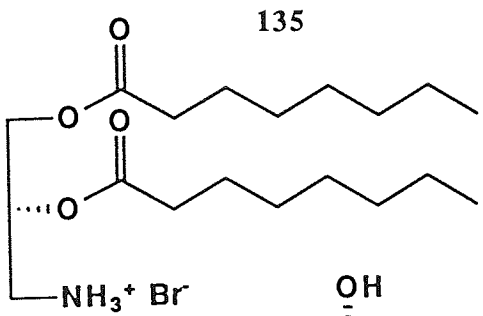
$R_1 = R_2 = -(CH_2)_6CH_3$

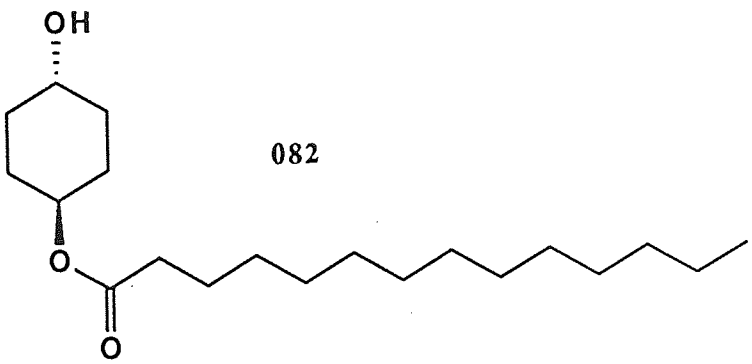









135
O
O
O
O
NH3+ Br-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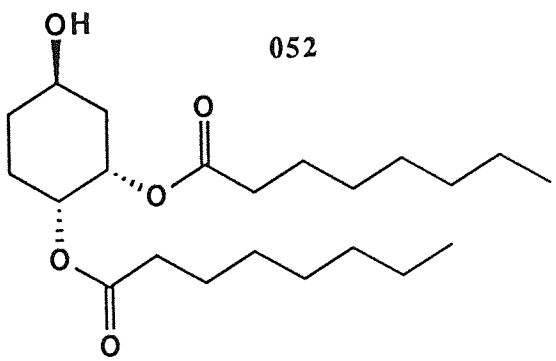
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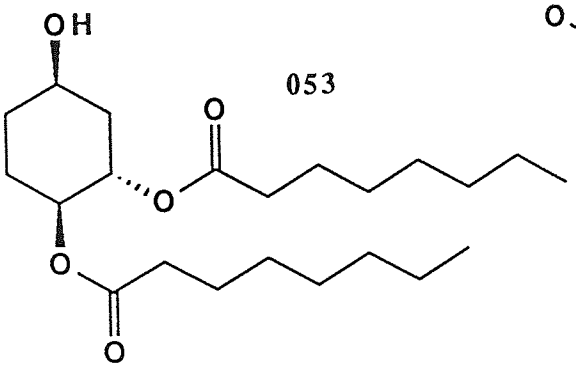
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Figure 67

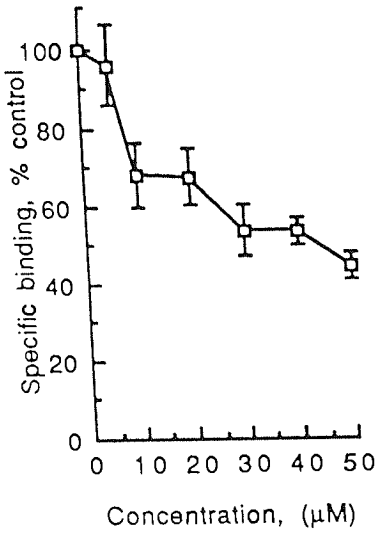


Figure 68

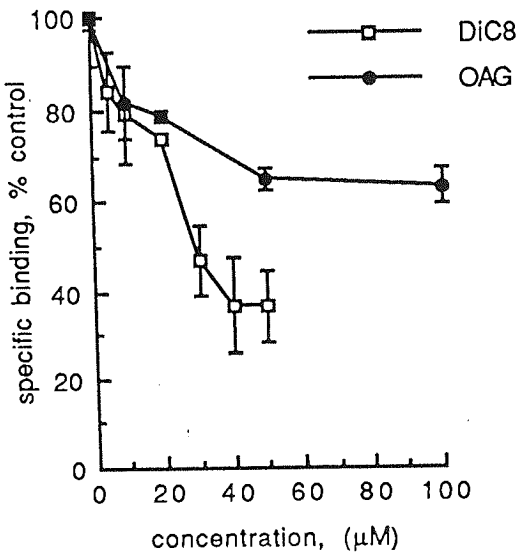


Figure 69

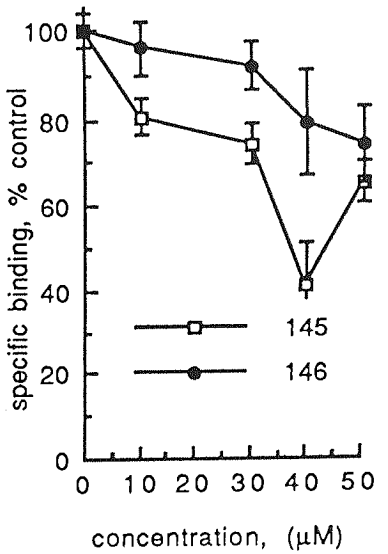


Figure 67. Displacement of [^{3}H]PDBu by diC$_8$ from confluent A549 monolayers.
Cells were co-incubated for 30 mins at 37^{O}C with 50nM [^{3}H]PDBu and increasing concentrations of diC$_8$. Non-specific binding was determined in the presence of excess unlabelled PDBu (mean ∓ SD, n=9).

Figure 68. Displacement of [^{3}H]PDBu by diC$_8$ and OAG from cytosolic A549 receptors.
Figure 69. Displacement of [^{3}H]PDBu by compounds 145 and 146 from cytosolic A549 receptors.
Crude cytosolic extracts were prepared. The binding reaction was performed in the presence of mixed micelles, 50nM [^{3}H]PDBu and increasing concentrations of synthetic DAG or analogues, before bound [^{3}H]PDBu was separated from free. Non-specific binding was determined in the presence of excess unlabelled PDBu (mean ∓ SD, n=9).

Table 1 demonstrates the % inhibition of [^{3}H]PDBu binding by 50μM diC_8 and its analogues.

Compound	In whole cells	In cytosolic extract
DiC_8	52.2 + 3.3 (n=6)	63.5 + 8.2 (n=7)
145	17.6 + 8.4 (n=8)	34.6 + 4.6 (n=6)
146	23.3 + 6.7 (n=8)	26.0 + 8.9 (n=4)
139	36.4 + 2.7 (n=6)	14.9 + 0.8 (n=3)
153	21.6 + 4.8 (n=4)	11.4 + 4.2 (n=2)
053	11.1 + 4.6 (n=4)	10.4 + 2.4 (n=4)
082	25.2 + 5.6 (n=4)	10.1 + 3.5 (n=4)
135	negligible (n=4)	negligible (n=2)

Table 2 demonstrates % [^{3}H]PDBu displacement from pure PKC by compounds 145 and 139 (n=3).

concentration	145	146
30μM	15.73 + 3.6	23.8+ 6.9
50μM	24.1 + 3.8	35.3 + 6.1

Table 3 demonstrates the calculated concentrations of compounds which would inhibit cell growth by 50% and, where possible, evoke 50% LDH release from cells.*Laughton *et al.*, 1989.

Compound	LC_{50} (μM)	IC_{50} (μM)
*DiC_8	56	44
*OAG	75	70
145		31
146		28
139	78.5	32
153		52
053	36	23
052	47	49
135	7.5	3.3

Figure 70. Measurement of specific [^{3}H]PDBu binding to pure rat brain PKC.
a) The concentration of PKC was increased and [^{3}H]PDBu concentration was maintained constant at 50nM. Data were represented as % specific binding to 4u/ml PKC.
b) The concentration of [^{3}H]PDBu bound to 3u/ml PKC was increased and data represented as % PKC bound to 100nM [^{3}H]PDBu (mean + SD, n=6).

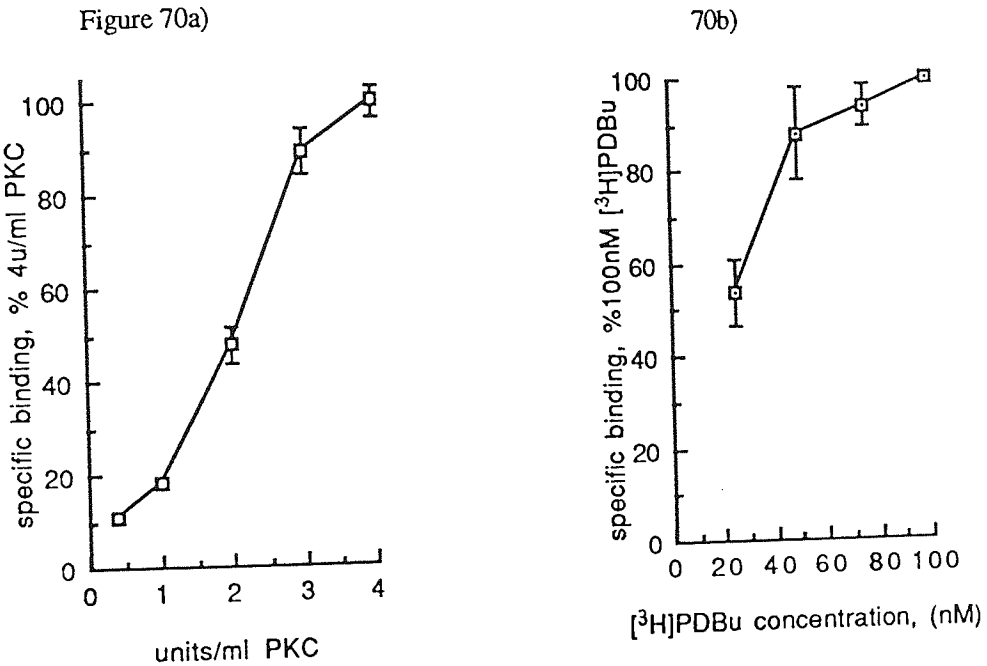


Figure 71. Displacement of 50nM [^{3}H]PDBu from 3u/ml PKC by increasing concentrations of diC_8. (mean $\pm$ SD, n=9).

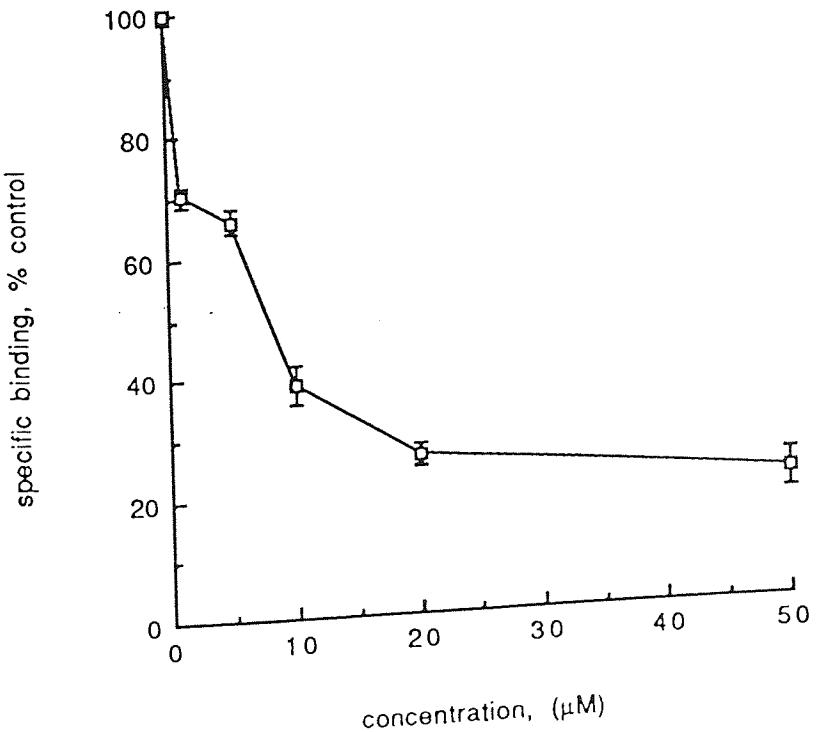


Figure 72. Effect of synthetic DAGs on cytosolic [^{3}H]PDBu binding.
Cells were pretreated for 30mins with increasing concentrations of diC_8 or OAG. Cytosolic extracts were crudely prepared and assayed for phorbol ester binding potential.
(mean ∓ SD, n = 9).

Figure 72

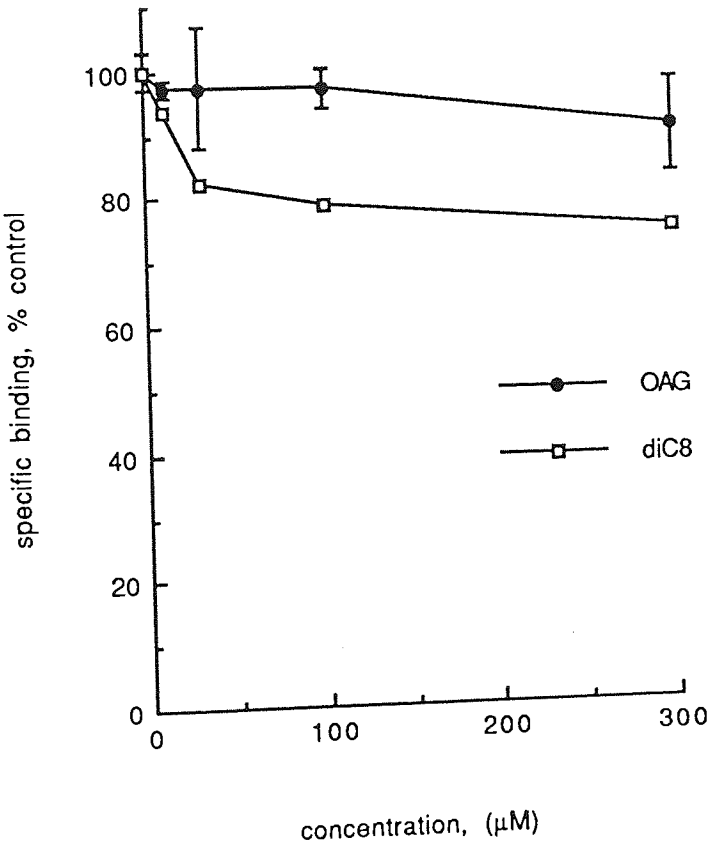


Figure 73a). Determination of IC_{50} dose of compound 135.
Cells ($2x10^4$) were seeded and treated for 3 consecutive days with increasing concentrations of compound. Cells were allowed a 48h period of growth before counts were performed (mean + SD, n=9).

Figure 73a)

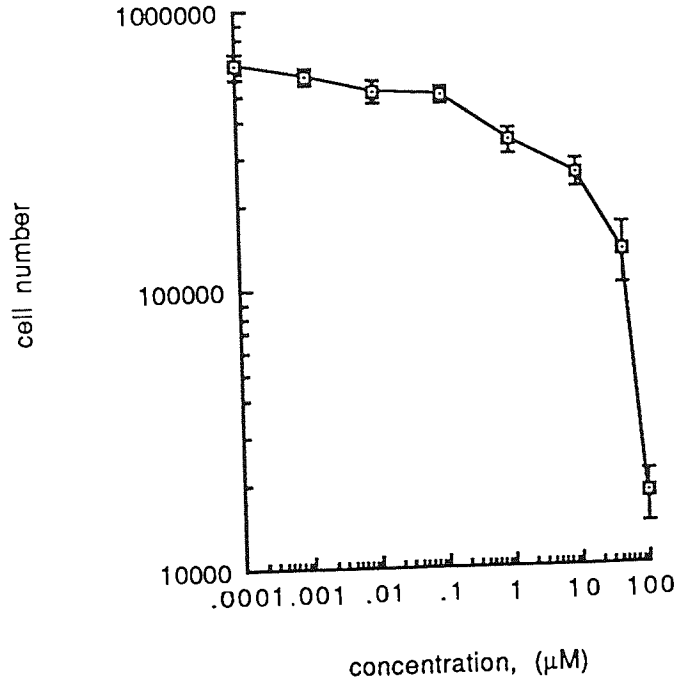
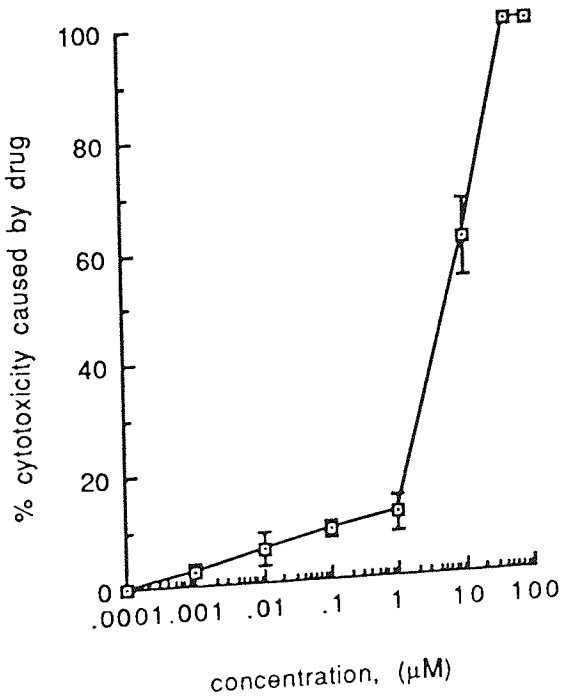


Figure 73b). Determination of LC_{50} dose of compound 135.
Cells (10^5) were seeded and administered 2 treatments of a specific concentration of compound over a 24h period. LDH release into medium was determined and concurrent cell counts performed (mean ∓ SD, n=9).

73b)



4.6 Investigation of the effects of bryostatins on A549 cells in culture.

4.6.1 Effect of bryos on the growth and cell cycle distribution of A549-FCS cells.

Introduction.

Bryos are macrocyclic lactones containing a 26 membered ring, isolated from the marine bryozoans *Bugula neritina* and *Amanthia convoluta* (Pettit *et al.*, 1982, 1983, 1984, 1985, 1987). To date 17 such compounds have been purified and found to exhibit antineoplastic activity (Pettit *et al.*, 1970) (section 1.7.1). Bryos bind to the phorbol ester receptor with exquisite affinity and activate PKC in a fashion similar to TPA. The C-4, C-9 and C-20 oxygens of phorbol constitute the putative phorbol ester pharmacophore (Wender *et al.*, 1986). Extension to the bryos reveal that the C-26, C-1 and C-19 oxygen atoms yield an excellent spatial correlation with this model (Wender *et al.*, 1988). However, bryos induce only a subset of the responses triggered by phorbol esters, moreover, they are able to block those phorbol ester responses that they themselves fail to induce. Like TPA, bryo 1 activates DNA synthesis in quiescent cultures of murine fibroblastic cell lines (Smith and Smith, 1985) and induces phosphorylation and down-regulation of transferrin receptors (May *et al.*, 1986). Antagonistic to the effect of TPA, bryo 1 has been reported to block phorbol ester-induced HL60 differentiation (Kraft *et al.*, 1986). Experiments have confirmed the hypothesis that the exogenous PKC activators, bryo 1 and bryo 2 are able to interfere with A549 cell growth at non-toxic concentrations; whereas OAG and 1,2-diC_8, synthetic analogues of the physiological ligands of PKC, inhibit growth only when present at toxic concentrations (Dale and Gescher, 1989). Following 24h exposure, bryo 1 inhibited incorporation of [^{3}H]TdR maximally at a concentration of 10nM. At concentrations above 10nM, arrest of DNA synthesis was diminished or abolished. No change in cell morphology was observed at high, non-inhibitory concentrations. The time course of inhibition of DNA synthesis caused by 10nM bryo 1 demonstrated minimum incorporation of [^{3}H]TdR after 12h exposure, with increasing levels of DNA synthesis by 24h. Similarly, bryo 2 affected incorporation of [^{3}H]TdR into cells maximally at 100nM following 24h exposure. At concentrations exceeding those which cause maximal inhibition of DNA synthesis, bryos abolished not only their own antireplicative effect but also that of TPA (Dale and Gescher, 1989). In A549 cells therefore, bryos 1 and 2 are able both to mimic and antagonise the effects of TPA.

The particular aims of the experiments described below were to investigate the nature of cell growth inhibition induced by bryos 1, 4 and 5 and to investigate the effect of bryos on cell cycle distribution. The hypothesis has been examined that differences exist between bryos in their phorbol ester antagonistic effects on cell growth.

Results and discussion.

Results presented previously have demonstrated inhibition of DNA synthesis by bryo 1 within a defined concentration window. We wished to investigate whether the arrest of DNA synthesis upon exposure of A549 cultures to different concentrations of bryo 1 was time dependent. Incorporation of [^{3}H]TdR by A549 cells was monitored at specific time intervals during treatment with 10nM and 1μM bryo 1.

Figure 76 demonstrates that [^{3}H]TdR incorporation was markedly inhibited in cells following treatment with 1μM bryo 1. However, the potency elicited by 10nM bryo 1 was not achieved. Maximum inhibition of DNA synthesis was observed after 6h exposure (59.7%). Following 9h treatment, [^{3}H]TdR incorporation was inhibited by 45.5% compared to control cultures. By 18h exposure, complete recovery of growth potential had occurred and [^{3}H]TdR incorporation slightly exceeded control values. Altered morphology of cultures was observed within 1h of treatment with 1μM bryo 1 and this was maintained during the early hours of exposure. However normal morphology was observed after 24h. Cells exposed to 10nM bryo 1 incorporated minimum [^{3}H]TdR after 18h exposure. Following 12, 18 and 24h treatment with 10nM bryo 1, DNA synthesis was inhibited by 84.7, 88.5 and 34.2% respectively. After 48h continued exposure to 10nM bryo 1, normal morphology had resumed and incorporation of [^{3}H]TdR was slightly increased when compared to control cultures. Thus, A549 cell growth arrest by bryo 1 displays time and concentration dependency. It appears that each concentration induces inhibition of [^{3}H]TdR within a defined period of time.

Inhibition of A549 cell growth by bryos 4 and 5 was assessed by cell counting following 96h incubation (figure 74). Biphasic dose responses were obtained, characteristic of those observed after treatment with bryos 1 and 2. Maximum inhibition of growth (69.3%) was achieved by incubating cells with 10nM bryo 4. Bryo 5 caused maximum inhibition at concentrations between 1 and 5nM, cell counts were 30.7 and 36.9% of controls respectively. At a concentration of 1μM, bryos 4 and 5 decreased cell growth by 23.4 and 18.3% respectively during this period of time.

Measurement of [^{3}H]TdR in cells following 24h exposure to bryos 4 and 5 yielded interexperimental variability. Inconsistently, bryo 5 appeared able to inhibit DNA synthesis extremely potently at low (0.1nM) concentrations. Assessment of DNA synthetic capacity following a 12h incubation period was performed (figure 75). The concentration-dependent responses appeared only slightly biphasic in nature. Examination of the time course of inhibition of [^{3}H]TdR incorporation into cells exposed to 10nM and 1μM bryo 4 and 5 revealed the transient nature of growth inhibition and thus explained the above observations. Bryo 4 and 5 (10nM) evoked very similar patterns of inhibition in DNA synthesis. Arrest of DNA synthesis was potent and transient. Maximal inhibition was achieved following 18h exposure to bryos 4 and 5, after which time [^{3}H]TdR incorporation was 7.4 and 6.1% of untreated cells respectively. After 24h incubation with these agents, release from growth arrest was observed. The potency of agents at 10nM was not achieved with 1μM bryos 4 and 5. Repression of DNA synthesis by 1μM bryo 4 and 5 was very similar to the inhibition profile observed following treatment with 1μM bryo 1. DNA synthesis was maximally inhibited following 9h exposure. [^{3}H]TdR incorporation was 27.7 and 25.5% of control values in the presence of bryo 4 and 5 respectively. By 12h incubation, inhibition of [^{3}H]TdR incorporation had decreased to 46.5 and 46.9%. Thus, reversal of growth inhibition had begun and at 18h no significant inhibition of [^{3}H]TdR incorporation was observed (figure 77). Bryos 4 and 5 induced the same transient morphology changes characteristic of TPA and bryo 1. It can be concluded that the precise

incubation time and concentration of bryo 4 and 5 determine the nature of the growth inhibitory response. Transient inhibition of [^{3}H]TdR incorporation accompanied by morphology changes, typical of morphology changes following exposure to TPA, has also been reported in SH-SY5Y neuroblastoma cells in response to treatment with bryo 1 and 2 (Jalava *et al.*, 1990).

Cell cycle analysis was performed using cells at various stages of bryo treatment. Figure 78 demonstrates selected representative profiles obtained. There was an initial rise of A549 cells in G_2 upon exposure to bryos. Following 3h treatment with 10nM bryo 1, the proportion of cells in G_2 had risen from the control value of 28.5 to 34.6% (result not shown) and after 6h had increased such that 41.0% of the total population resided in G_2. The fraction of the total population in S phase fell from control levels of 13.6% to 8.9% after 6h treatment. Following 18h exposure to 10nM bryo 4, only 2.1% of the total cell population occupied S phase, no significant difference in cell number residing in G_2 phase was observed at this time point when compared to the control cells. Cell cycle distribution following 18h incubation of cells with 1μM bryo 1 was virtually indistinguishable from the profile obtained in control cultures.The disappearance of cells from S phase of the cell cycle reflects data obtained measuring DNA synthesis by means of [^{3}H]TdR incorporation. However, when one compares the effect of bryo on cell cycle distribution with that seen following treatment of A549-FCS cells with TPA (section 4.3), it appears that bryos are unable to maintain the G_2 block induced by TPA. A brief rise only in the population occupying G_2/M phase is observed during the initial hours of bryo exposure. Interestingly, exposure of A549-US cultures to TPA did not lead to a prolonged accumulation of events in the G_2 /M phase and induced potent but transient growth arrest (section 4.1.3).

We wished to test the hypothesis that bryos 4 and 5, like bryos 1 and 2, are able to abolish the maintained growth arrest induced by TPA. Figure 79a illustrates that 10nM and 1μM bryo 4 and 5 are partially able to abolish the growth arrest induced by 10nM TPA. It appears that the restoration of growth incurred by 10nM and 1μM bryo 4 and bryo 5 occurred in a non-competitive manner, as in all cases cell growth in the presence of both TPA and bryo represented between 40.3 and 42.8% of cell growth in the presence of bryo alone. Assays were conducted to measure the incorporation of labelled thymidine into A549 cultures following 48h exposure to bryo 5 (10nM or 1μM) and TPA (10nM or 200nM) and the results are represented in figure 79b. The ability of bryo 5 to block TPA-induced growth arrest appeared to be proportional to its own pro-replicative effect at that concentration, thus DNA synthesis in cultures containing 1μM bryo 5 exceeded the proliferative capacity of cultures containing 10nM bryo 5. In the presence of 10nM TPA, incorporation of [^{3}H]TdR into cell cultures containing 10nM or 1μM bryo 5 was 70.1 and 79.3% compared with cells exposed to bryo alone. These figures were decreased to 62.2 and 64.2% in the presence of 200nM TPA. An alternative description, in accordance with results obtained from cell counts could be that TPA, at a given concentration appeared equally able to partially inhibit the proreplicative effect of 10nM and 1μM bryo 5. Paradoxically, 200nM TPA alone evoked significantly less inhibition of DNA synthesis

than 10nM TPA (p = .002). Hence, the ability of bryo 5 (10nM or 1μM) to block the growth inhibition by 200nM TPA was significantly less efficient than the bryo 5-induced repression of growth arrest elicited by 10nM TPA (10nM bryo 5, p = .001; 1μM bryo 5, p = .005). Thus it appears that the cellular response obtained as a consequence of co-exposure to bryo 5 and TPA is achieved via mechanisms which may be competitive in the presence of high concentrations of phorbol ester.

In Friend erythroleukaemia cells, bryo 1 restored differentiation, inhibited by varied concentrations of phorbol ester (Dell`Aquilla *et al.*, 1987). The results were interpreted in the following manner: the antagonism of phorbol ester action by bryo 1 was noncompetitive and may be mediated *via* a high affinity site that does not recognize phorbol esters with good efficiency.

It is noteworthy that unlike bryo 1, which was able to abolish TPA-induced growth arrest completely (Dale, 1989), bryos 4 an 5 behaved like bryo 2 which induced only a partial blockade of TPA-induced response. To date 17 naturally occurring bryostatin derivatives have been described which appear to differ in their relative phorbol ester-mimetic and phorbol ester antagonistic activities (Blumberg et *al.*, 1989). Dell`Aquilla *et al.* (1988) described the induction of arachidonic acid metabolite release in C3H $10T^1/2$ cells by phorbol ester. This response was blocked entirely by bryo 1 and partially by bryos 2, 3, 4 and 10. The ability of bryos 2, 3, 4 and 10 to block the phorbol ester response was inversely proportional to their own ability to induce the release of arachidonic acid metabolites. Moreover, bryo 1 was able to inhibit the response of bryo 3 indicative of differences in the structure-activity relationships of bryos with respect to phorbol ester-mimetic and phorbol ester-inhibitory actions. Consistent with this hypothesis and the observations described regarding figure 79b, one may speculate that the high affinity site may be recognized with varying degrees of potency and specificity, and saturated at lower concentrations of bryos, but recognized by phorbol esters at higher concentrations. In the light of the above hypothesis, it would be interesting to determine the nature of A549 growth behaviour in response to bryo 3.

4.6.2 Investigation of the interaction between bryos and the phorbol ester receptor and the effect of pM concentrations of bryo on cell growth and phorbol ester binding.

Introduction.

Several lines of research have been pursued to address the paradox of the observed differential growth responses evoked by bryos compared to TPA in A549 cells. The affinity of bryos 1, 2, 4 and 5 for the phorbol ester receptor in A549 cells is described in this section. The hypothesis was examined that concentrations of bryo in the pM range may displace phorbol ester binding and retard cell growth.

Results and discussion.

Figure 80 demonstrates the specific binding of 50nM [^{3}H]PDBu to confluent A549 cell monolayers following 30min co-incubation in the presence of increasing concentrations of bryos 1, 2, 4 and 5. Displacement of [^{3}H]PDBu, represented as % of specific binding in

Figure 74. The effect of increasing concentrations of bryo 4 -□- and bryo 5 -●- on A549 cell growth. Cells (2 x 10^4) were seeded and treated 4h later with bryos. Following 96h exposure, cell counts were performed (mean ∓ SD, n=6).

Figure 74

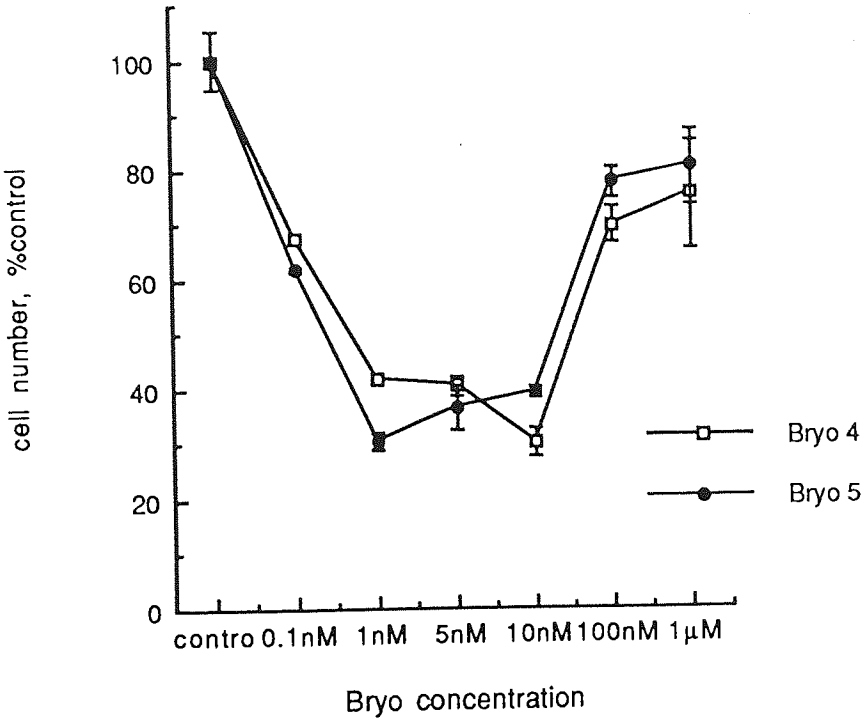


Figure 75

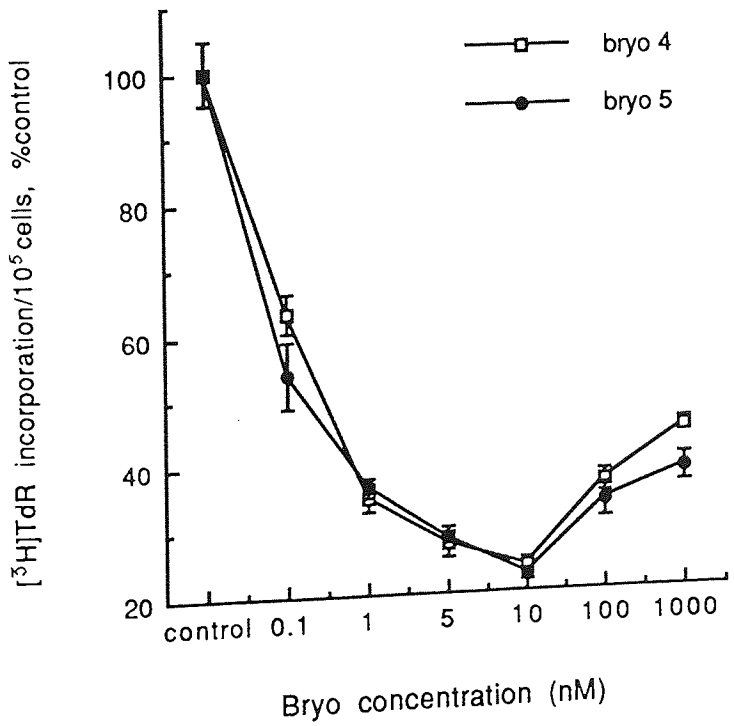


Figure 75. Effect of bryos 4 and 5 on DNA synthesis. Cells (2 x 10^4) were seeded and treated with increasing concentrations of bryos 4h later. Following an exposure period of 12h, [^{3}H]TdR was assessed (mean ∓ SD, n=9).

Figure 76

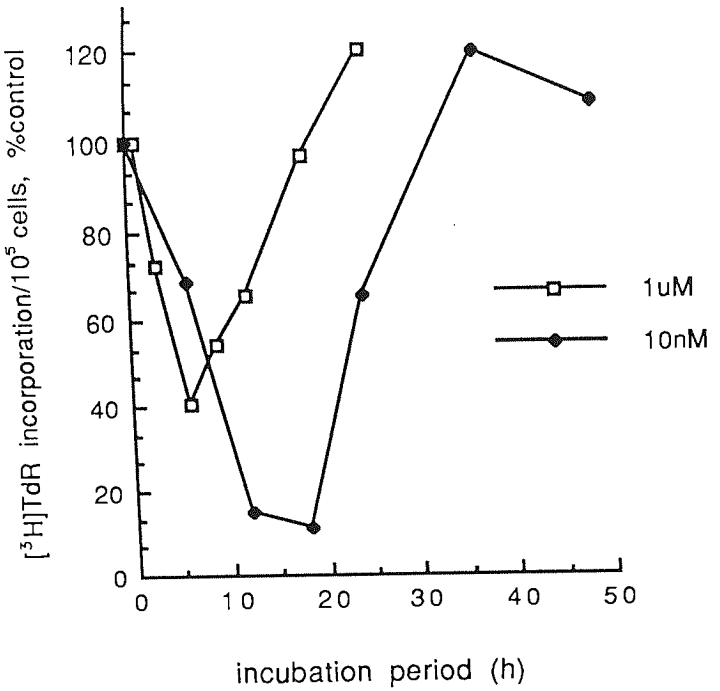
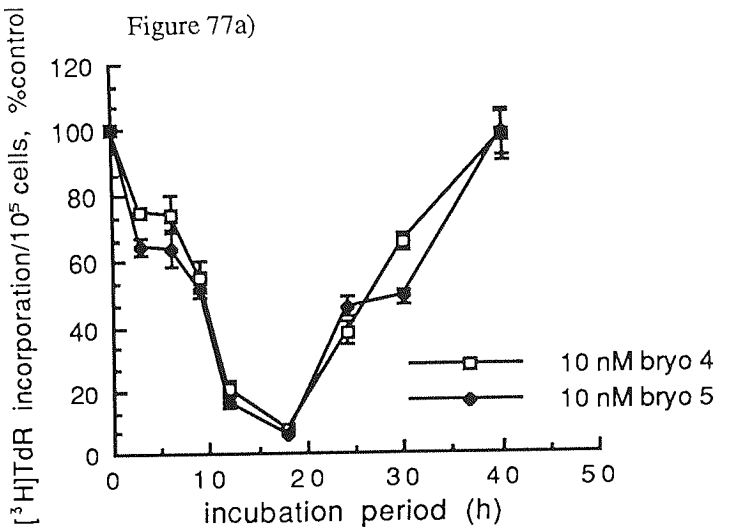


Figure 76. Time course of the inhibition of [^{3}H]TdR incorporation following treatment of A549 cells with -◆- 10nM and -□- 1µM bryo 1.
Cells (10^5 or 2 x 10^5) were exposed to bryo 1 4h after seeding and incubated for various time intervals before measurement of [^{3}H]TdR incorporation (mean ∓ SD, n=9).

Figure 77a)



77b)

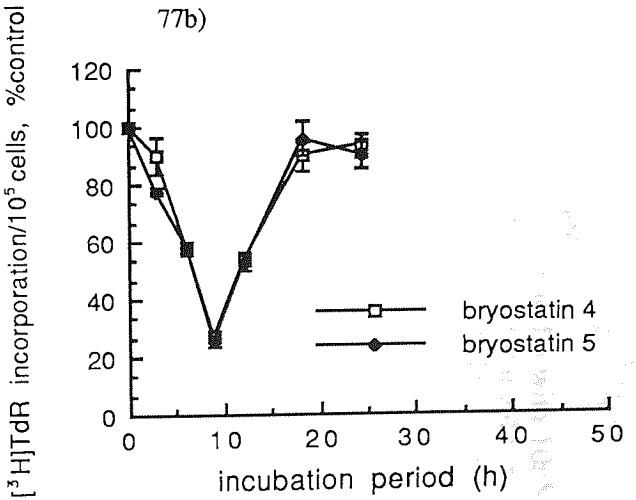


Figure 77. Time course of the inhibition of [^{3}H]TdR incorporation following treatment of A549 cells with a) 10nM and b) 1µM bryo 4 -□- and bryo 5 -◆-.
Cells (10^5 or 2 x 10^5) were exposed to bryo 4h after seeding and incubated for various time intervals before measurement of [^{3}H]TdR incorporation (mean ∓ SD, n=9).

Figure 78. The effect of bryos on A549 cell cycle distribution.
Cells in log phase of growth were treated for 6h (B), 18h (C and D) with 10nM (B and C), 1μM (D) bryo 1 (B and D) or bryo 4(C) before being fixed and stained for cell cycle analysis. Data from 20,000 events were collected with respect to particle size (mean ± SD, n = 3-6).

Figure 78

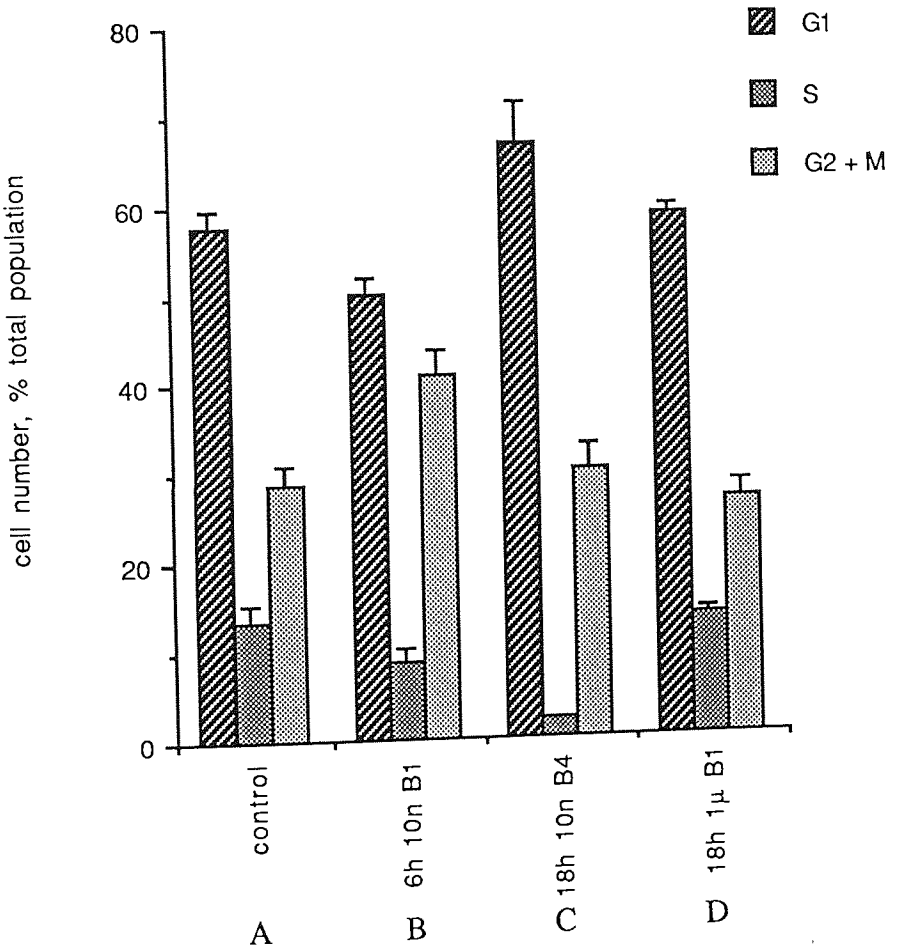
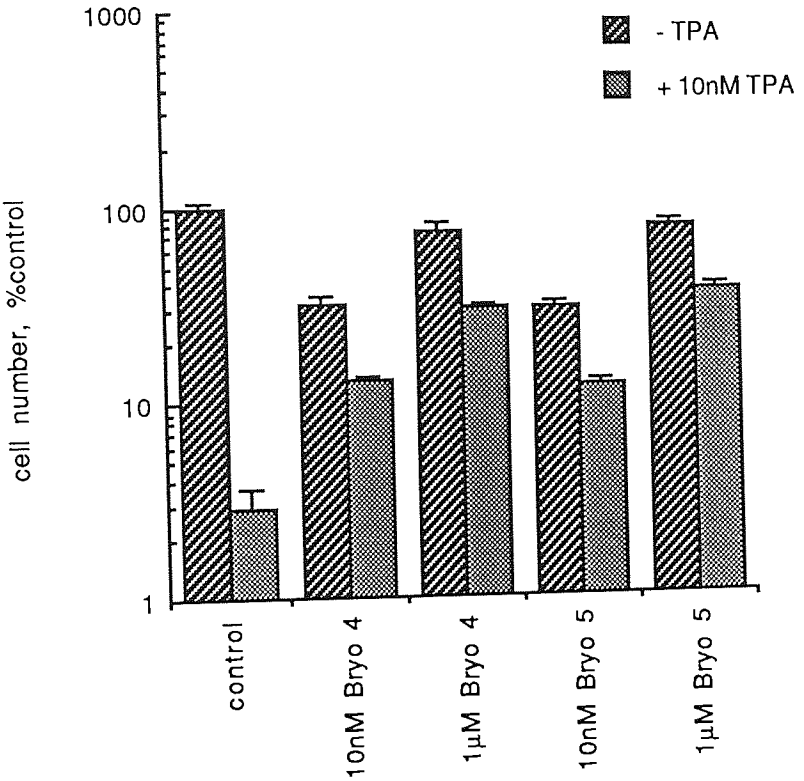
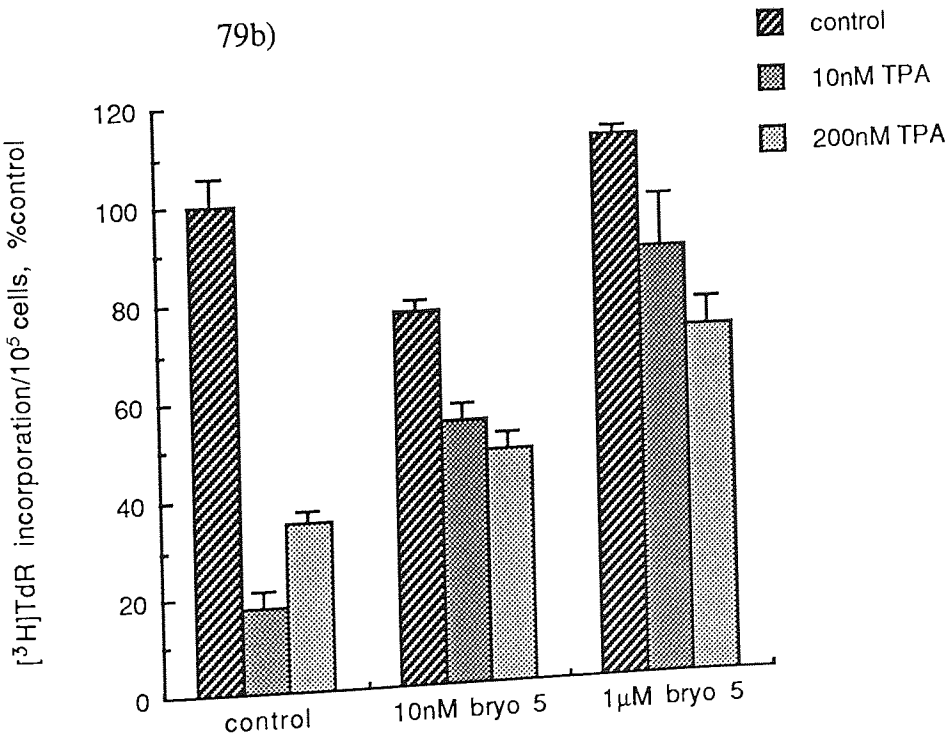


Figure 79. Effect of bryos 4 and 5 on A549 cell growth arrest elicited by TPA.
a) Cells (2 x 10^4) were seeded and treated with 10nM TPA. Certain groups were co-exposed to 10nM or 1μM bryo 4 or bryo 5. Counts were performed following 96h exposure (mean + SD, n=9).
b) Cells (10^5) were seeded. [^{3}H]TdR was measured following 48h exposure to bryo 5 (10nM or 1μM) and TPA (10nM or 200nM) (mean ∓ SD, n=9).

Figure 79a)



79b)



the absence of competitor, by 10nM bryo 1, 2, 4 and 5 was 43.4, 42.4, 58.7 and 61.8% respectively. The equivalent concentration of TPA was able to displace 62.2% [^{3}H]PDBu from the A549 phorbol ester receptor (section 4.2.3). Values calculated to displace 50% [^{3}H]PDBu from receptor sites were 20.6nM, 250.0nM, 6.7nM and 196pM for bryos 1, 2, 4 and 5. The corresponding concentration of TPA which would occupy 50% receptors in the presence of 50nM [^{3}H]PDBu was 4.2nM. These values show that the bryos possess exquisite affinity for the phorbol ester receptors on intact A549 cells at nM concentrations. Interestingly, bryo 5 displayed a superior ability to displace [^{3}H]PDBu which extended to the pM range. Binding of bryos 1 and 2 to intact SH-SY5Y cells suggested binding constants within the nM concentration range (Jalava *et al.*, 1990). These authors report a 10-fold increase in the ability of bryo 1 to compete for [^{3}H]PDBu binding compared to bryo 2. It is likely that the observed difference in binding abilities are a direct consequence of structural differences between the bryos. Also, bryos may bind with varying affinities to PKC isoforms. We observed a correlation between binding affinity and the concentration at which maximum inhibition of DNA synthesis was achieved (Dale and Gescher, 1989; figure 80). This relationship was also observed by Jalava; maximal inhibition of [^{3}H]TdR incorporation by bryo 2 was one order of magnitude larger than that seen with bryo 1. The ability of bryos 1,2,4 and 5 to displace PDBu from its specific receptors at concentrations which evoke growth arrest strongly support the hypothesis that ligand binding to the phorbol ester receptor (PKC) is a prerequisite for the onset of A549 cell growth inhibition.

The 4-α isomer of PDD which was unable to induce cytostasis in A549 cell cultures at non toxic concentrations, was unable to displace PDBu from receptor binding sites (section 4.2.3). Early studies performed by Berkow and Kraft (1985) established the ability of bryo to bind to the phorbol ester receptor, to activate polymorphonuclear leukocytes and to stimulate the phosphorylation of proteins in these cells in a manner almost identical to phorbol esters. In a more recent report, a difference in isozyme specificity between bryo 1 and TPA was demonstrated (Kraft *et al.*, 1988).

In further experiments the hypothesis was tested that picomolar (pM) concentrations of bryo are able to compete for phorbol ester receptor binding or retard A549 cell growth. The formation of this hypothesis was guided by the following observations. The kinetics of ligand release from PKC are thought to differ dramatically between bryos and phorbol esters (Sharkey *et al.*, 1988). The rate of release of phorbol esters from PKC at 37°C is rapid and monotone. In contrast, release of radiolabelled bryo 4 was heterogeneous, and the second phase of release was very slow with a half life of several hours. Hence, bryo-bound PKC may be tightly anchored at the membrane and may contribute to an observed increased affinity and specificity of bryos for the phorbol ester receptor. This hypothesis is referred to as the glue-trap model for bryo action (Blumberg *et al.*, 1989). Direct binding measurements using radioactive bryo 4 (Sharkey *et al.*, 1988) and competition experiments using unlabelled bryos have suggested that the potency of bryos for PKC under appropriate conditions of reconstitution into PS, may reflect a dissociation constant in the pM range rather than the nM dissociation constants typical of phorbol esters. De Vries *et al.* (1988) demonstrated that the pM affinity of bryo 1 for rat brain phorbol ester receptors displayed

little dependence on [^{3}H]PDBu concentration. In contrast, displacement of radioligand by unlabelled phorbol ester was dependent on the concentration of [^{3}H]PDBu. Figure 80b illustrates that in A549 cells, pM concentrations of bryo 5 were capable of displacing 50nM [^{3}H]PDBu from receptors.

In the light of these results, it may be hypothesised that responses to phorbol esters at μM concentrations might be equivalent to responses evoked by certain bryos at nM concentrations. Support for this hypothesis has been provided by Warren *et al.* (1988). Bryo 1 (6nM) was able to phosphorylate two unique 70kDa proteins which were phosphorylated in response to phorbol ester, only at concentrations exceeding 600nM. Certain similarities have emerged from work conducted in our laboratory which may be interpreted as support for this view. Dale and Gescher (1989) and figure 74 demonstrated biphasic concentration-dependent growth responses following exposure of A549-FCS cells to bryos. In cells supplemented with 10% NBCS (figure 22, section 4.1) maximum inhibition of [^{3}H]TdR incorporation was elicited by 10nM TPA, following 24h exposure. Higher concentrations of TPA (100nM and 1μM) evoked weaker growth arrest. One could speculate that toxicity, caused by further increases in TPA concentration prevents a biphasic response from being detected. It may also be possible that at this particular time point the re-emergence of growth may not yet be observed. This is not the case with A549-US cell cultures. These cells exhibit a biphasic dose response to TPA following 24h exposure, and a very transient arrest of growth (section 4.1.3), reminiscent of the growth inhibition induced by bryo. Additionally, fig 79b reveals that 200nM TPA failed to inhibit [^{3}H]TdR incorporation to the same extent as 10nM TPA following 48h incubation. Thus, in A549 cells, experiments were conducted to investigate whether bryo 1 at pM concentrations affects cell growth or [^{3}H]PDBu binding.

The ability of pM concentrations of bryo 1 and TPA to displace binding of [^{3}H]PDBu to intact cell cultures following an incubation period of 30min was examined. Extended incubations (100 min) were performed in the presence of 10pM bryo 1 and TPA only, to investigate whether increased displacement of PDBu could be incurred by bryo 1, arising from a cumulative association with the phorbol ester receptor due to slower dissociation kinetics compared with phorbol ester release. No difference was detected between the affinities of TPA and bryo 1 for the phorbol ester receptor (result not shown). After 30 min, 100pM bryo 1 and TPA were able to displace 16.1% and 17.9% [^{3}H]PDBu from its receptors; 10pM caused negligible displacement. After the extended incubation period, specific binding of both TPA (10pM) and bryo 1 (10pM) had increased by 13.31% and 8.04% respectively, but standard deviations were large ($6.5 < SD < 10$, $n=6$) and the difference was statistically insignificant.

Assays were performed to investigate the binding of bryo 1 and TPA to crude preparations of cytosolic phorbol ester receptor. Steady state binding occurred within 1 min but 10 min reaction time at room temperature was permitted to allow equilibration of the reaction mixture (Hannun and Bell, 1987). Negligible difference in the potential to bind to A549 cytosolic preparations was observed between bryo 1 and TPA at pM concentrations. The results suggests that both TPA and bryo 1 possess higher affinity (at nM concentrations)

for cytosolic phorbol ester receptors than for binding sites on intact cells. IC_{50} values calculated for bryo 1 and TPA were 657pM and 746pM respectively. Bryo 1, at concentrations of 1nM and particularly 10nM incurred significantly greater displacement of PDBu from crude cytosolic A549 receptors than TPA ($p = 0.002$) (Figure 81). It is worth noting that incubations for increased periods of time (100min) yielded very high non-specific binding values. Figure 82a illustrates that pM concentrations of bryo 1 induced negligible decrease in incorporation of labelled thymidine following 18h exposure. However, cell numbers were significantly lower following a 96h incubation period with concentrations of bryo 1 at and above 50pM (figure 82b).

It has been established that subnanomolar concentrations of bryo 5 possess affinity for the phorbol ester receptor of intact A549 cells (figure 80b). We wished therefore to investigate whether picomolar concentrations of bryo 5 interfere with A549 cell growth. [^{3}H]TdR incorporation following 18h exposure indicated negligible inhibition of DNA synthesis. However, cell counts performed subsequent to exposure for 96h to bryo 5 revealed that concentrations as low as 1pM significantly retarded A549 cell growth (figure 83).

4.6.3 Investigation of mechanisms of action of bryos including examination of the role of PKC in A549 cell growth inhibition.

Introduction.

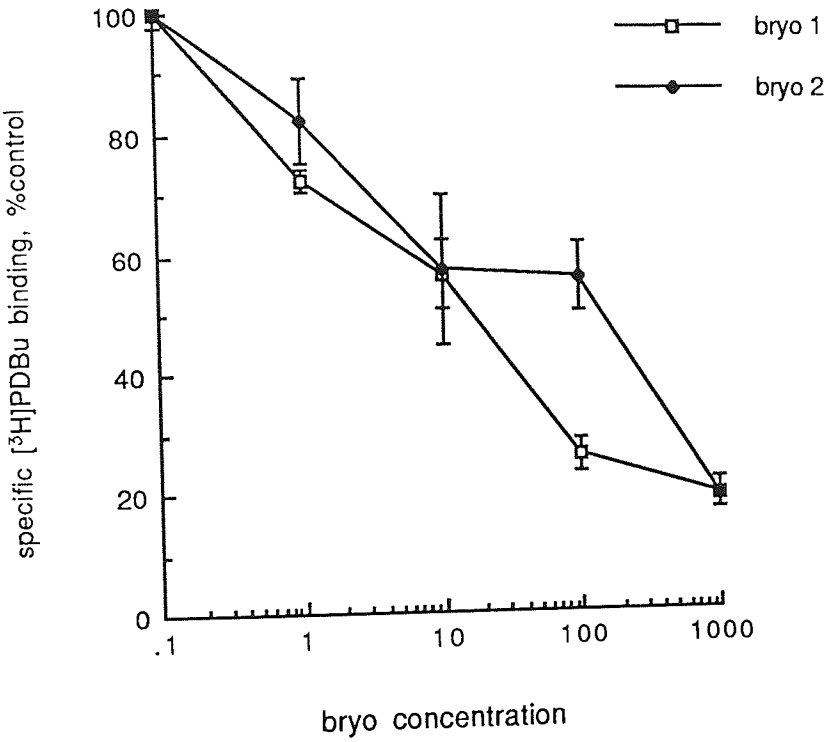
The greater stability of TPA compared to the transient presence of DAG within biological membranes causes protracted stimulation of PKC (section 4.2.1) which may underlie the pleiotropic effects of TPA, including induction of cellular differentiation and promotion of malignant transformation. In order to elucidate mechanisms responsible for the differential effects of bryos, compared with TPA, on A549 cell growth, the ability of bryo to modulate subcellular distribution of PKC was quantified. The effect of bryo 1 on the growth of cells in the presence of STSPN was monitored. Additionally, a preliminary experiment was performed to investigate whether bryo interacts with signal transduction pathways mediated via inhibitory G proteins. Several of the PKC assays described below were carried out in collaboration with Dr. I.L. Dale.

Results and discussion.

Figure 84 demonstrates the effect on the subcellular distribution of PKC in A549-FCS cells following incubation with 10nM bryo 1 and 4 or 1μM bryo 1. Bryos were able to induce a large and rapid translocation of PKC activity from the cytosol to the particulate fraction. The ability of the bryos, like TPA, to induce such rapid subcellular redistribution of PKC strongly supports the hypothesis that translocation and activation of PKC is a crucial event in the mediation of growth arrest. Further evidence in favour of this hypothesis is provided by the inability of 4-α-PDD and synthetic DAG analogues to induce growth arrest or affect subcellular redistribution of PKC (sections 4.2.1 and 4.5). However, the physiological relevance of such a major shift in enzyme subcellular location is in doubt. Evidence suggests that only a minute fraction of total PKC requires activation for an enzyme-dependent response to ensue. Bosca *et al.* (1989) reported full expression of

Figure 80. Displacement of [3H]PDBu from intact A549 cells by various concentrations of a) bryo 1 - - and bryo 2 - - b) bryo 4 - - and bryo 5 - -.
Confluent A549 monolayers were incubated for 30 minutes with 50nM [3H]PDBu and bryo. Specific binding of bryos to cells was determined by subtracting non specific binding, conducted in the presence of excess unlabelled PDBu, from total [3H]PDBu binding (mean ∓ SD, n=9).

Figure 80a)



80b)

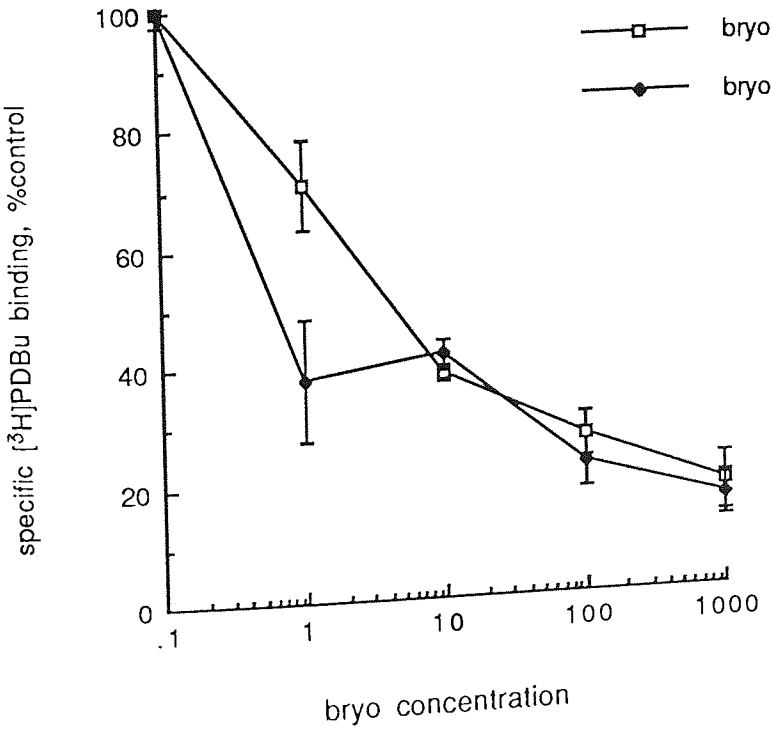


Figure 81. Affinity of bryo 1 and TPA for cytosolic phorbol ester receptors (mean ∓ SD, n=9).

Figure 81

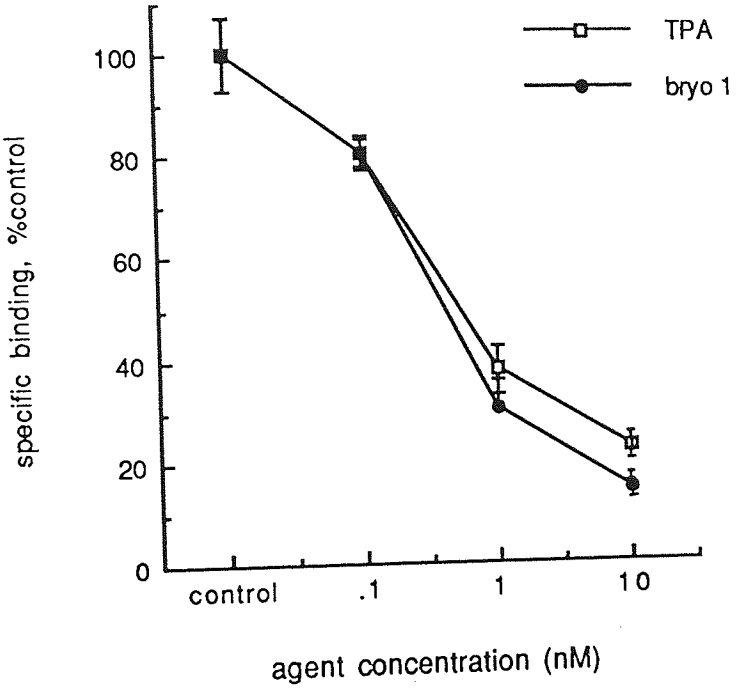
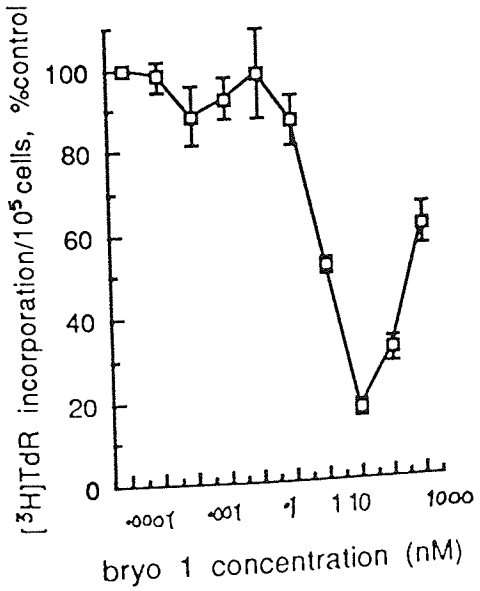


Figure 82. Effect of pM concentrations of bryo 1 on a) A549 cell numbers. Cells were seeded at a density of 2 x 10^4 and counted following 96h exposure (mean + SD, n=9). b) DNA synthesis. Cells were seeded at a concentration of 2 x 10^5, allowed 4h to attach and exposed for 18h to bryo 1before [^{3}H]TdR incorporation was assessed (mean ∓ SD, n=9).

Figure 82a)



82b)

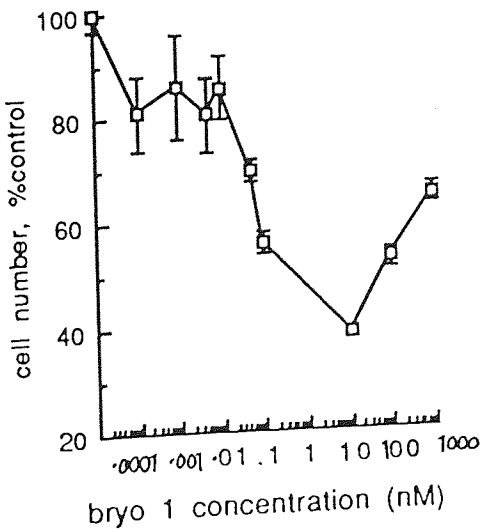
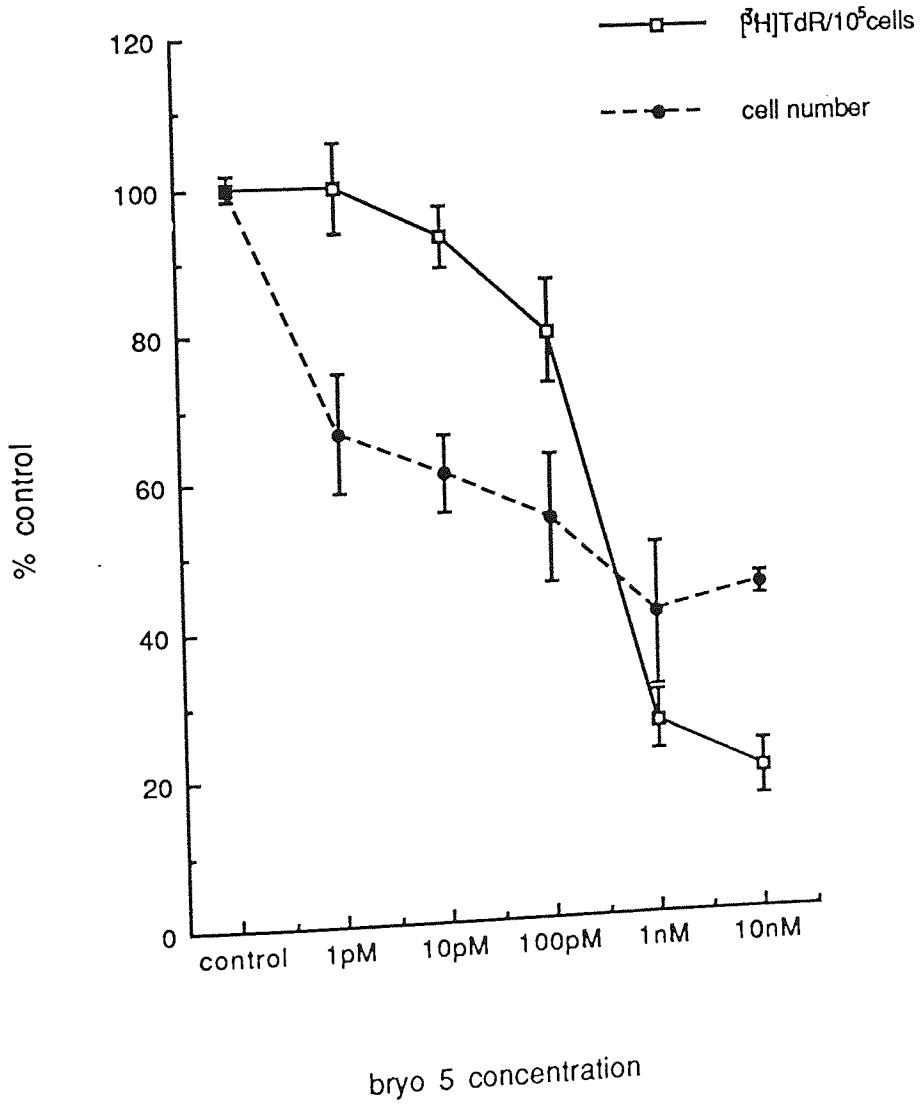


Figure 83. Effect of pM concentrations of bryo 5 on A549 cell growth.
Cells (2×10^4) were seeded and following 96h exposure to bryo 5 were counted (-●-).
Cells (2×10^5) were seeded and allowed 4h to attach. Following 18h exposure to bryo 5 [^{3}H]TdR incorporation was determined (-□-) (mean ± SD, n=9).

Figure 83



certain PKC-dependent biological responses in B cells following activation of less than 5% PKC. Moreover, activation of PKC by platelet activating factor (PAF) has been reported by Pelech *et al.* (1990) to be independent of enzyme translocation; these authors conclude that PAF may directly modulate the activity of membrane-associated PKC without eliciting further recruitment to the membrane. Issandou *et al.* (1988) have observed the occurrence of PKC-dependent protein phosphorylation following 90% enzyme down-regulation. It is noteworthy that bryo does not induce levels of translocation caused by equivalent concentrations of TPA (Dale *et al.*, 1989; figure 37). At no time point investigated during this study did membrane translocation exceed 45% of total PKC content.

Figures 84 and 85 illustrate how prolonged exposure to byros resulted in time- and concentration-dependent enzyme proteolysis and down regulation; an observation common to many cell types *in vitro*, upon continued exposure to bryo (Kraft *et al.*, 1987; Jalava *et al.*, 1990). Thus, like TPA, bryo is able to evoke sustained enzyme activation; in contrast to the transient PKC activity stimulated by DAG (Issandou and Darbon, 1988; Issandou *et al.*, 1988) or hormones (Farrar and Anderson, 1985).This result seems to indicate that bryo, like TPA is stable within biological membranes.

Activation and down-regulation of PKC activity following exposure to bryo has been reported in JB6 mouse epidermal cells (Kraft *et al.*, 1988). In these cells, bryo stimulated a smaller loss of PKC from the cytosol, but a more rapid loss of immunologically detectable PKC than did equivalent concentrations of phorbol ester. Similarly, prolonged exposure of HL60 cells, Friend erythroleukaemia cells, primary mouse epidermal cells and C3H $10T^1/2$ cells to bryo 1 procured a more rapid degradation of measurable PKC activity than comparable concentrations of phorbol ester (Kraft *et al.*, 1987; Blumberg *et al.*, 1989). Implications of the glue-trap model for bryo action, forwarded by Blumberg *et al.* (1989) may be as follows. Whereas the rapid rate of release of phorbol esters from PKC may permit the translocated enzyme to access adjacent membrane compartments, bryo bound PKC may be tightly anchored at the membrane. Such association could conceivably account for the more rapid down-regulation of PKC; as active, membrane-bound PKC is selectively targetted by proteolytic enzymes (Kishimoto *et al.*, 1989). Such results have led these authors to correlate accelerated enzyme degradation with the transient duration of bryo action. Figures 37 and 84 illustrated that following treatment of A549 cells with bryo 1, bryo 4 or TPA (10nM), the onset of down-regulation was evident after 3h. Bryo 1 at a concentration of 1μM appeared to initiate down-regulation after only 30min exposure, as was observed after treatment of cells with 300nM TPA (figure 37; Dale *et al.*, 1989). Hence, in A549 cells, it cannot be concluded that the transient nature of growth arrest by bryo is a consequence of a more rapid rate of enzyme degradation, compared with the down-regulation of PKC induced by TPA. However, in accordance with the results obtained using JB6 cells (Kraft *et al.*, 1987), the observations described above for A549 cells suggest decreased enzyme translocation by bryo 1 compared to TPA.

These phenomena may be a consequence of cell type-specific expression of PKC isozymes. Thus, the decreased translocation observed following bryo treatment compared to TPA may reflect differential sensitivity of isoforms to specific activators. The disparity in

rates of PKC degradation may reflect isozyme-specific sensitivity to down-regulation. Indeed differential sensitivity of isoforms to proteolytic enzymes has been demonstrated (Ase *et al.*, 1988; Huang *et al.*, 1989). Moreover, Adams and Gullick (1989) report cell type specific differences in rates of and sensitivity to PKC degradation.

We wished to investigate the hypothesis that agent-specific stimulation of PKC isozymes may explain the differential modulation of A549-FCS growth. As bryos, at equal concentrations to TPA, induced less enzyme translocation, it was postulated that TPA may preferentially activate an isoform of PKC, possessing reduced sensitivity, or insensitive to stimulation by bryo. Monospecific mouse anti-PKC-α and anti-PKC-ß Abs were obtained and Western blot experiments were performed using cytosolic and membrane fractions from A549 cells following treatment for 30min with of TPA and bryo 1 (100nM). No immunoreactive PKC-ß was detected in A549 cells. Immunodetectable PKC-α was present (figure 51, section 4.2.4). In control cells the majority of PKC-α resided in the cytosolic fraction. Treatment with TPA and bryo 1 caused a shift of immunoreactive PKC-α to the membrane compartment. However, TPA-induced translocation exceeded that induced by bryo considerably (figure 50, 2e and f; 51, 2g and h). These results are in accordance with those obtained measuring PKC activity. Thus it can be concluded that in A549 cells, bryo 1 induces less activation of PKC-α within membrane compartments than does TPA. Isakov *et al.* (1990) have speculated that a change in expression of several cell cycle-related genes may preferentially be regulated by PKC-α. However the possible involvement of PKC-isozymes γ, ζ, δ and ε can not be ruled out. Activation of one or more of these isozymes may contribute to the differential effects observed following treatment of cultures with TPA or the various bryos.

In mouse JB6 cells, differences between bryo 1 and TPA in the modulation of PKC have been reported (Kraft *et al.*, 1988). Bryo 1 and TPA demonstrated identical competition for binding to the α and γ isozymes, but bryo 1 displayed decreased binding to PKC-ß when compared to TPA.

We wished to investigate whether down-regulation of receptors was observed following treatment of cells with bryos 1 and 2 and to determine whether any distinction could be detected between bryo and TPA. [^{3}H]PDBu binding was examined in A549 cells which had previously been exposed to bryo or TPA. The results are represented in figure 86. A rapid decrease in [^{3}H]PDBu binding was obtained which intimates time and concentration dependence. However, residual specific binding of [^{3}H]PDBu to cells, although greatly reduced, still occurred following pretreatment with agent for 24h. Receptor binding was reduced by 75.8%, 64.9%, and 85.1% following exposure to 100nM bryo 2 and 10nM and 1μM bryo 1 respectively; compared to 29.6% by 10nM TPA. These results demonstrate that no apparent significant difference can be observed in down-modulation of receptor binding between bryo 1 and TPA.

The fact that growth arrest by bryo is so transient, and yet measurable PKC activity remains negligible in the continued presence of bryo renders it unlikely that PKC

down-regulation is important in the mediation and maintenance of A549 growth inhibition. In fact one may hypothesize that PKC activation may be necessary for the induction of a growth inhibitory response by bryo. A concentration-dependent correlation was observed between growth arrest and enzyme down-regulation when the time course of these two effects was taken into account. Very preliminary experiments have been conducted in order to address this issue. The possibility that STSPN (section 4.2.5) may inhibit bryo 1-induced growth arrest was examined. Figure 87 illustrates the time course of the effect of 10nM bryo 1 in the presence of 10nM STSPN on the growth of A549 cells, assessed by incorporation of [^{3}H]TdR. Interestingly, the results of this experiment raise more questions than have been answered. STSPN (10nM) itself is a potent inhibitor of A549 cell growth (section 4.2.5). However, during the initial 6-9 hours of exposure, negligible inhibition of DNA synthesis was detected (figure 87). This profile is in contrast to the time dependencies observed with TPA or bryo 1. With the PKC activators, inhibition of [^{3}H]TdR incorporation occurred within 1h of incubation and thence proceeded stepwise. DNA synthesis was inhibited initially by approximately 40% and maintained at this value during the first 6-9h. Potent arrest of growth arose after this time and was maintained for less than 24h (bryo 1) or 5-6 days (TPA). It is known that STSPN binds to the catalytic domain of PKC (Nakadate *et al.*, 1988), induces association of enzyme with membranes (Wolf and Baggiolini, 1988), but inhibits catalytic activity of PKC and thus presumably enzyme down regulation. Bryo binds to a phorbol ester receptor site located within the regulatory domain, and induces enzyme translocation, active catalysis and down regulation of enzyme activity. The experiment showed that during the initial hours of co-incubation, DNA synthetic capacity appeared to be a consequence of competition between cellular responses evoked by bryo and STSPN individually. As STSPN instigated growth arrest, the response observed was additive to that of bryo until such time when, in cells exposed to bryo alone, proliferative potential was regained. However, incubation of cells with STSPN and 10nM bryo together led to no such restoration of proliferative capacity, and inhibition of [^{3}H]TdR incorporation was retained at levels more potent than those induced by STSPN alone (figure 87a). This observation is consistent with a competitive relationship. It has been established that STSPN at nM concentrations does not prevent the binding of phorbol esters and phospholipids to PKC (Tamaoki *et al.*, 1986). Moreover STSPN antagonized PKC-catalysed protein phosphorylation in a concentration-dependent manner (Davis *et al.*, 1989). On the assumption that by inhibiting the activation of PKC, STSPN inhibits enzyme down-regulation one could argue as follows: continued competition for PKC by these agents may ensue, leading to protracted activation of PKC by bryo 1; the consequence of which may be the observed potent arrest of DNA synthesis. Experiments will be conducted to test the validity of this hypothesis. It is noteworthy that compared to 10nM TPA in the presence of 10nM STSPN, 10nM bryo 1 together with STSPN was able to maintain inhibition of [^{3}H]TdR incorporation to a greater degree (figure 56, section 4.2.5). This may be a consequence of the glue-trap hypothesis describing the dynamics of bryo dissociation kinetics from the phorbol ester receptor.

Exposure to 1μM bryo 1 rendered cells quickly devoid of measurable PKC activity, thus it

would appear that the proreplicative effect of bryos is independent of PKC activity. Further work supports this view. Cells maintained in the continued presence of 10nM TPA, which possess no measurable PKC activity, demonstrated cross-resisitance to growth inhibition by bryo 1 (section 4.2.1). However introduction of bryo 1, 10nM and 1μM into these cultures in the presence of 10nM TPA increased DNA synthesis 1.8 and 1.5-fold (figure 88). It appears that in PKC-depleted cells, the presence of bryo is able to abolish inhibition of DNA synthesis induced by TPA. This evidence offers support for the proposal that the absence of active PKC is necessary for the pro-replicative response of bryo to take effect.

It has been reported by van Corven *et al* (1989) that a mitogenic or proreplicative response is mediated *via* the activation of a pertussis toxin (PTX) sensitive, adenylate cyclase inhibitory G protein (Gi). PKC, following activation has been reported to possess a regulatory role in rendering the α subunit of Gi to be in the inactive state (Crouch and Lapetina, 1988). Bryo 1 has been shown to lead to the induction of mitogenesis in density arrested fibroblasts (Smith *et al.*, 1985). Lysophosphatidic acid, highly mitogenic for quiescent fibroblasts was able to activate PKC *via* phosphoinositide hydrolysis, arachidonic acid release and a Gi protein. It was activation of the latter that was necessary and sufficient for cell division to begin. We wished to investigate whether bryo, *via* intervention with this mechanism following down-regulation of measureable PKC activity, was able to mediate its proreplicative response. Cells were exposed for 48h to TPA (10nM), bryo 1 (10nM) or these agents combined, in the absence or presence of 10μg/ml PTX before incorporation of [^{3}H]TdR was measured. Figure 89 demonstrates that the presence of PTX alone after this period of time increases the proliferative potential of cells. Cultures incubated with bryo 1, or bryo 1 and TPA in the presence of PTX also possessed a slightly increased rate of DNA synthesis, however PTX was unable to diminish the potent arrest of [^{3}H]TdR uptake in cultures treated with TPA. Moreover, inhibition of Gi by PTX did not abolish the proreplicative effect of bryo1, thus the hypothesis suggesting a role for a PTX-sensitive Gi protein has to be rejected.

4.6.4 Effect of bryo 1 on the growth of A549-US cells.

Introduction.

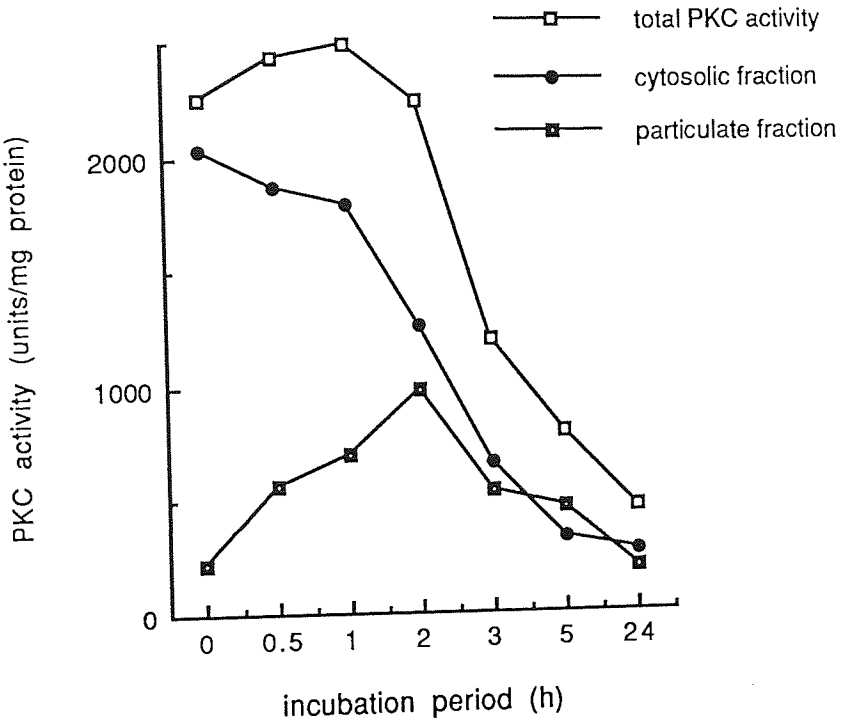
It has been established that unlike TPA, bryo is able only to elicit very short term growth arrest in A549-FCS cultures. However TPA, in A549-US populations, was unable to elicit maintained growth arrest, and inhibition of [^{3}H]TdR incorporation was transient (section 4.1.3). Possible underlying mechanisms, responsible for such differential growth responses to the same compounds, by the same cell type, in different culture environments have been discussed (sections 4.1.5 and 4.2). In this section, the effect of bryo 1 on the growth of A549-US cells was examined.

Results and discussion.

Figure 90a demonstrates that a biphasic dose response curve was obtained, though less pronounced than that elicited by bryo 1 in A549-FCS (Dale and Gescher, 1989) Growth arrest was also weaker than the arrest of growth induced by TPA in A549-US cultures

Figure 84. Translocation and down-regulation of PKC activity following treatment of A549 cells with A) 10nM, B) 1μM bryo 1 and C) 10nM bryo 4.
Total cellular PKC activity -□- and activity restricted to the cytosolic -●- and particulate -▣- fraction of A549 cells was measured following various incubation periods and partial purification by non-denaturing PAGE. Values represent mean of 2 or 3 experiments (SD < 15%).

Figure 84a)



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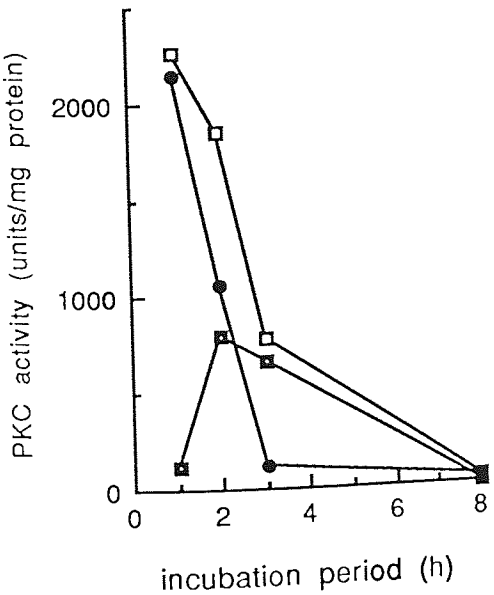


84c)

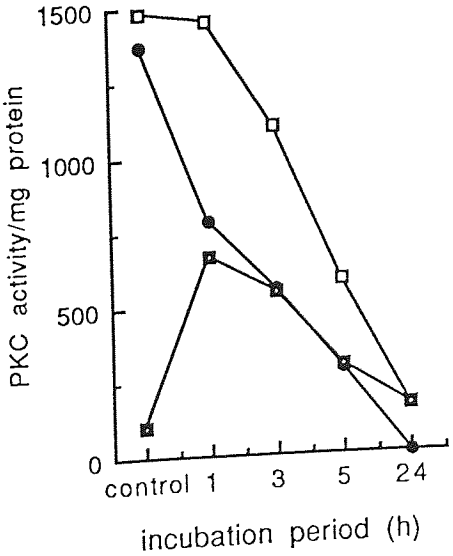
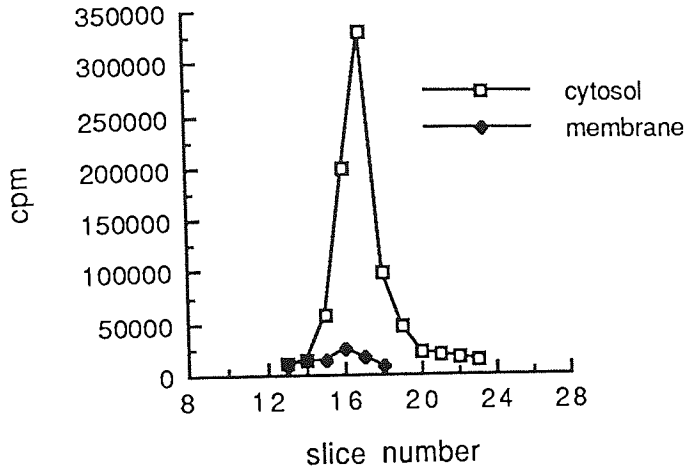


Figure 85. Resolution and subcellular distribution of PKC activity.
Cytosolic and particulate enzyme content was partially purified by non-denaturing PAGE following treatment of cells with a) vehicle only and 1μM bryo 1 for b) 30min, c) 1h, d) 24h. PKC was eluted from gel slices and activity assayed using protamine sulphate substrate.

Figure 85a)



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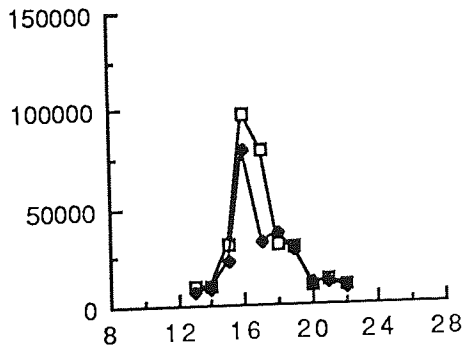

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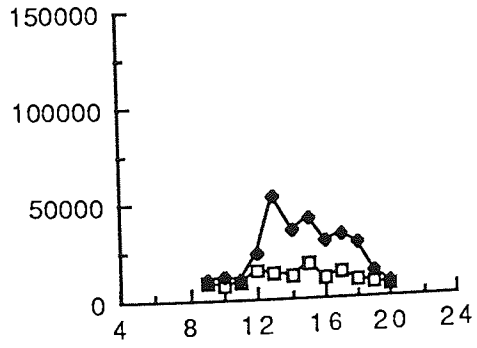

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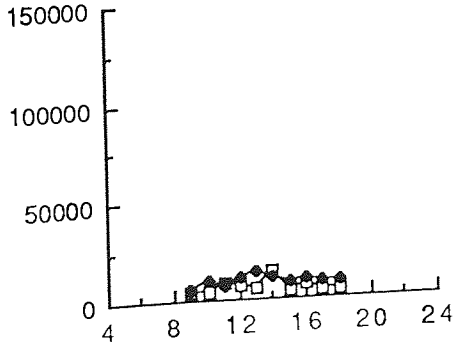
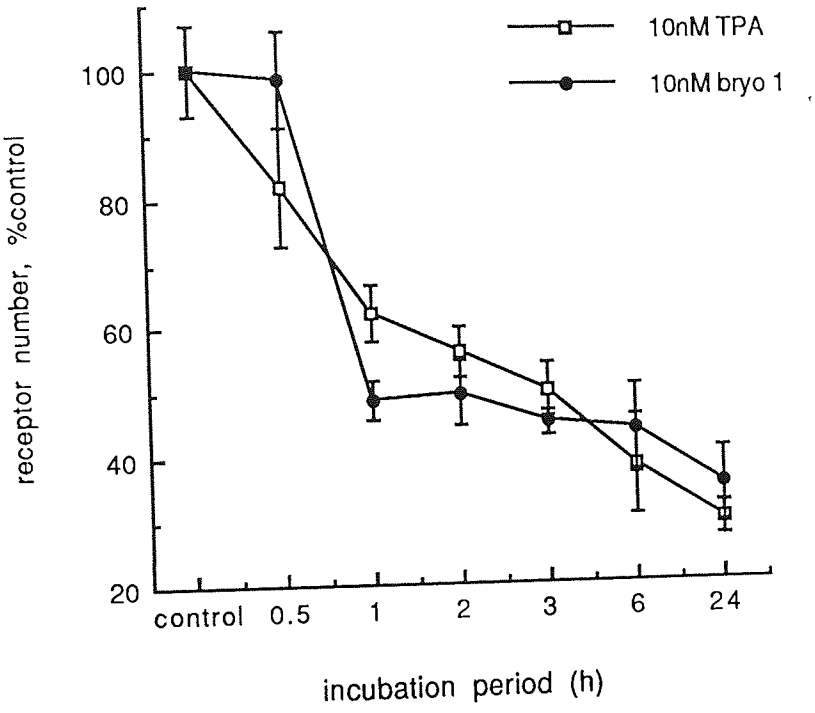
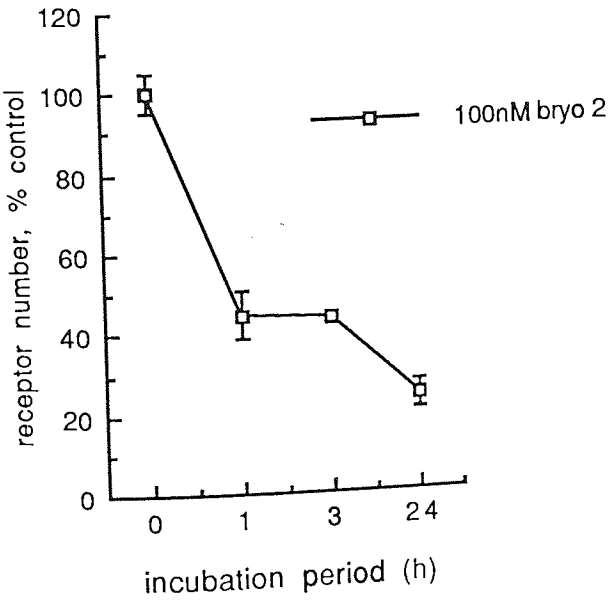


Figure 86. Measurement of [^{3}H]PDBu binding potential on A549 monolayer cultures following individual treatments of varying lengths with a) 10nM TPA and 10nM bryo 1, b) 100nM bryo 2 and c) 1μM bryo 1 (mean ± SD, n=9).

Figure 86a)



86b)



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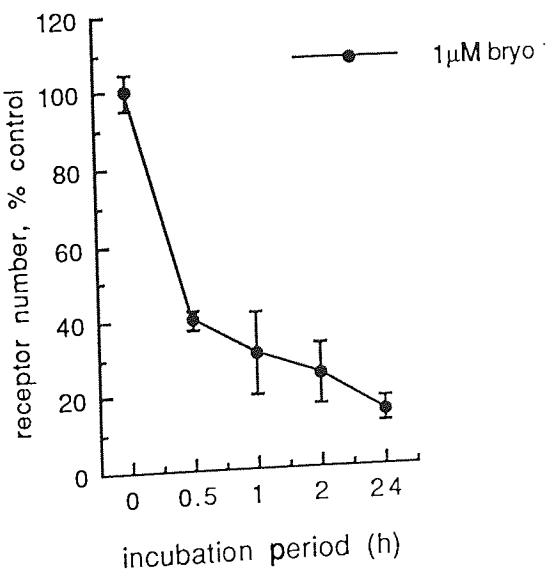


Figure 87a. Effect of STSPN and bryo 1 on A549 DNA synthesis.
Cells (2 x 10^5) were seeded and treated for varying lengths of time with 10nM bryo 1 and/or 10nM STSPN before [^{3}H]TdR incorporation was determined (mean $\pm$ SD, n=3).

Figure 87a

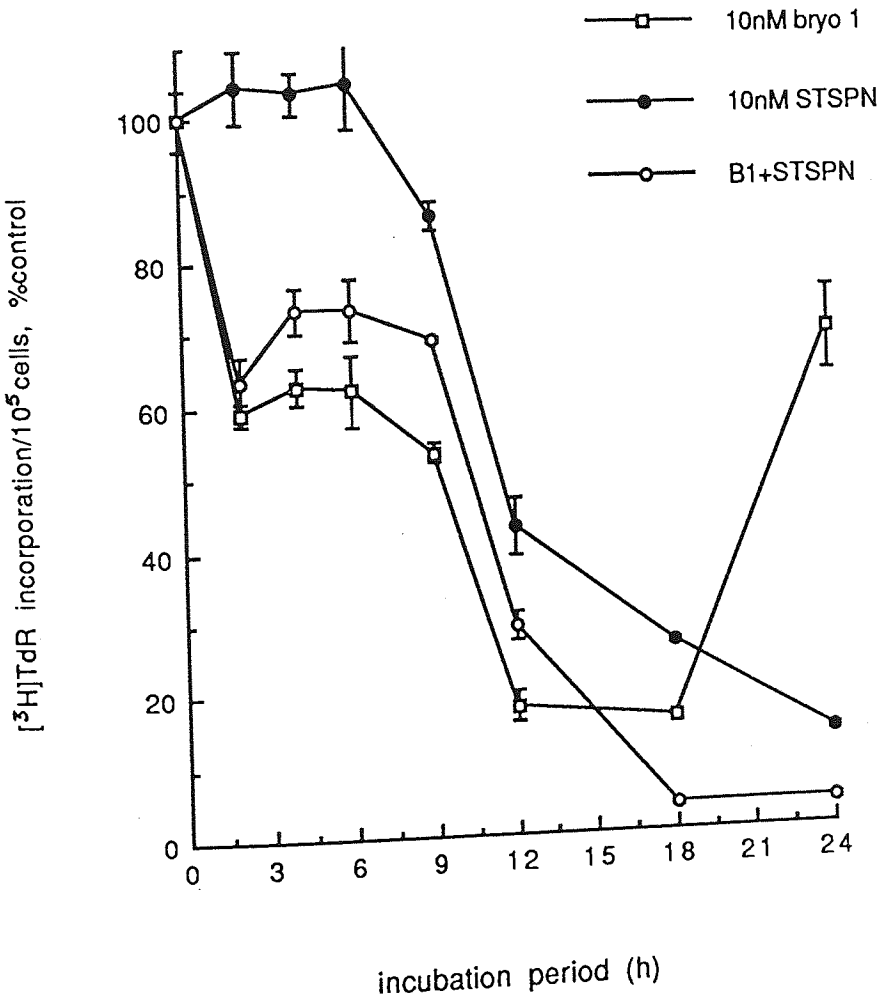
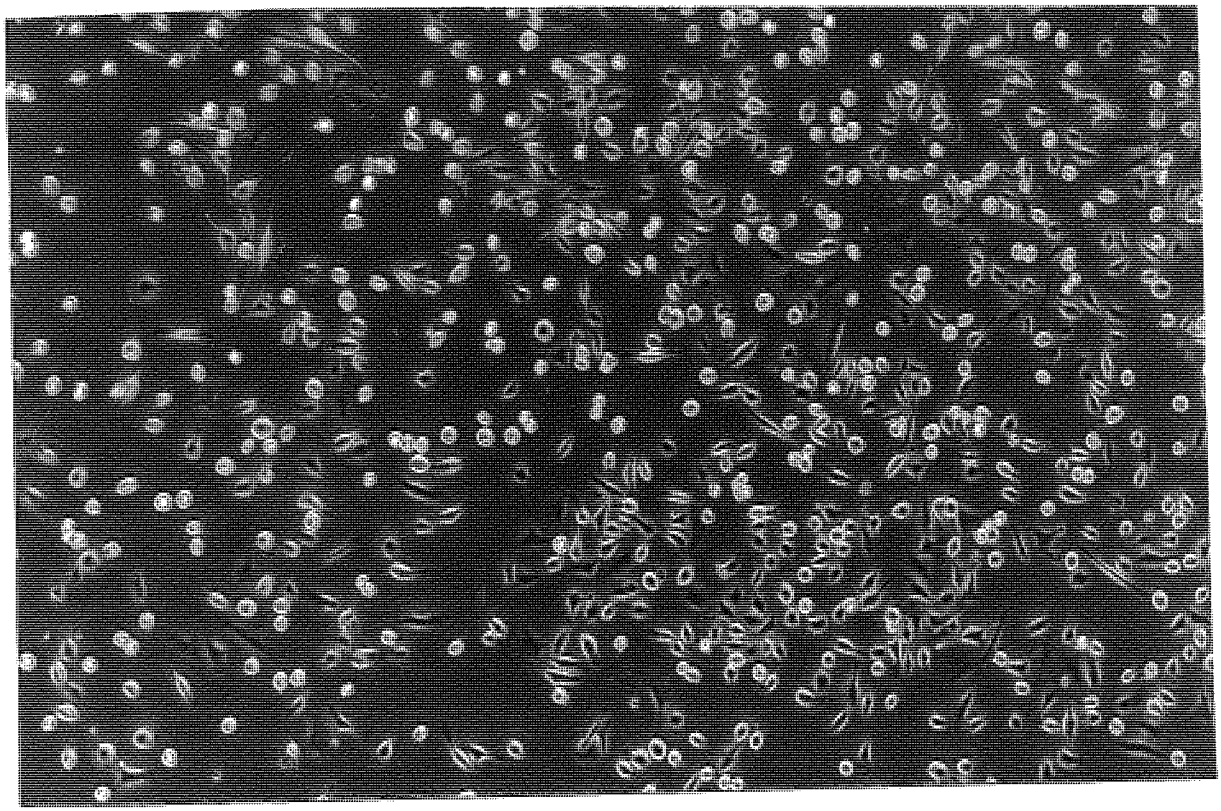


Figure 87b. Phase contrast micrographs of cells exposed to bryo 1 or STSPN and bryo 1. Cells (2 x 10^5) were exposedto i) bryo 1 (10nM) alone or ii) STSPN (10nM) and bryo 1 (10nM) for 12h before photographs were taken (magnification x 20). Cells exposed to STSPN only are demonstrated in figure 55.

i



ii

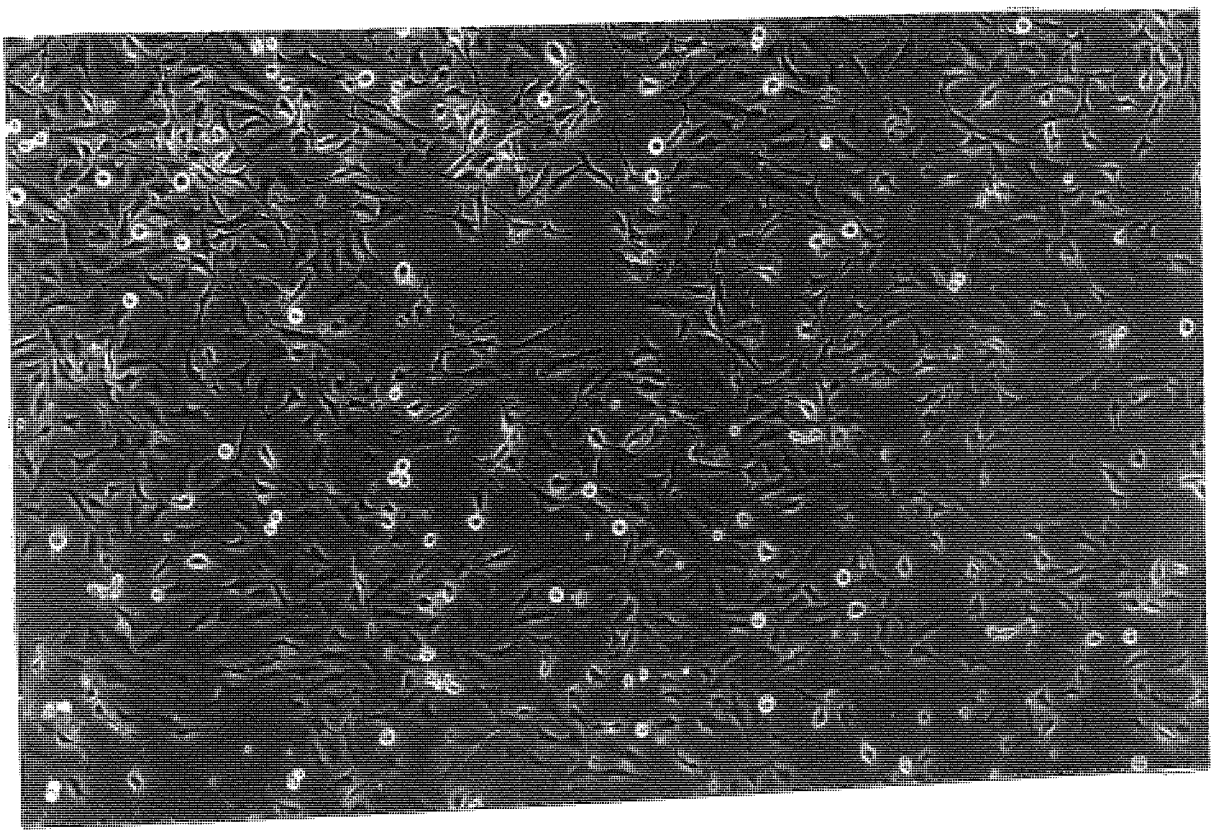


Figure 88. Effect of bryo 1 on the growth of PKC-depleted, A549-TPA cells.
Cells maintained in the permanent presence of 10nM TPA were seeded at a concentration of 2 x 10 and exposed to 10nM or 1 μM bryo 1 for 24h before DNA synthesis was assessed (mean ± SD, n=6-9).

Figure 88

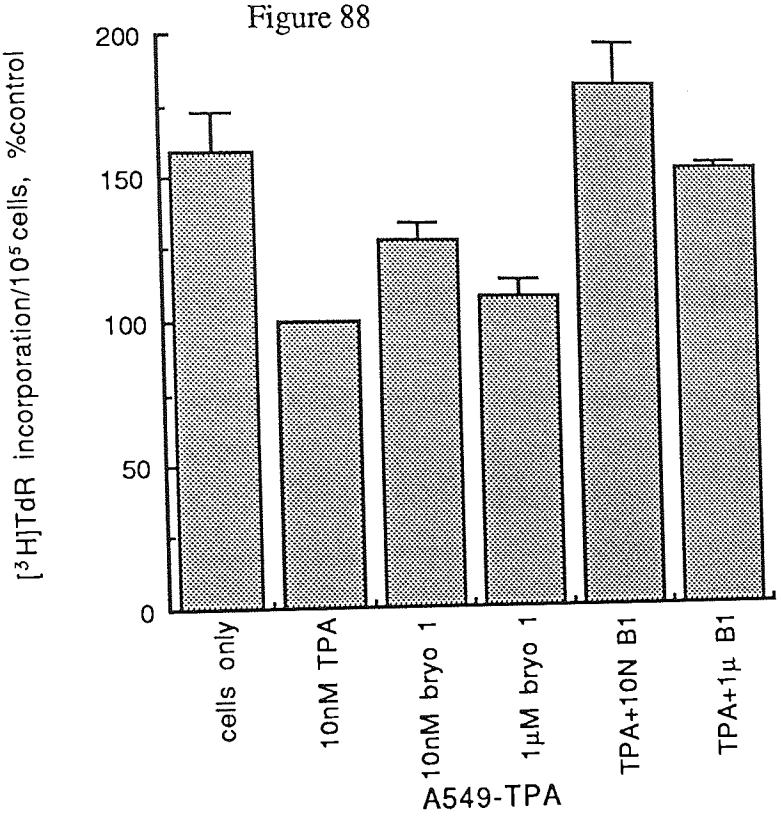


Figure 89

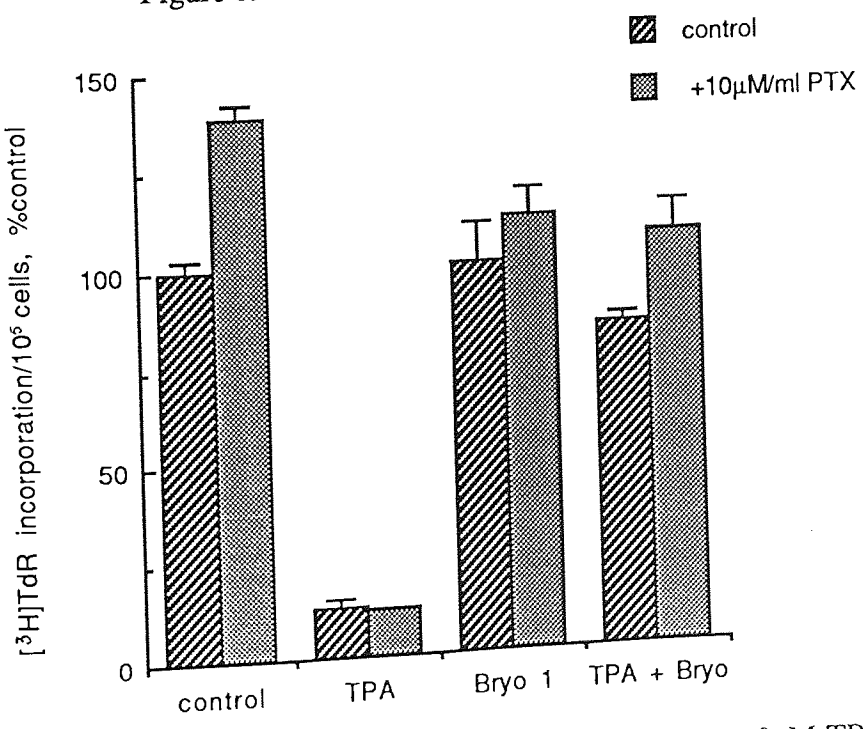


Figure 89. [3H]TdR incorporation following 48h exposure to 10nM TPA and/or 10nM bryo 1 in the presence and absence of 10μg/ml PTX.
Cells (10^5) were seeded 4h before treatment began (mean ± SD, n=6).

(section 4.1.3). Bryo 1 at a concentration of 10nM elicited the most potent response; inhibiting cell growth by 48.51%, as assessed by counting cultures following 96h exposure. Figure 90b illustrates the very transient nature of growth arrest. Incorporation of radiolabelled thymidine into cultures incubated in the presence of 1nM, 10nM and 1μM bryo 1 was similarly inhibited. Maximum inhibition of [^{3}H]TdR incorporation occurred after 15h (79.3%), 12h (85.3%) and 9h (73.5%) with 1nM, 10nM and 1μM respectively. After 24h incubation, proliferative capacity was again equal to that of control cultures.

Assays to measure the displacement of PDBu from cytosolic receptors of A549-US populations by TPA and bryo 1 were conducted. The results are illustrated in figure 91. They demonstrate that specific phorbol ester binding was 36.8% + 1.6% of phorbol ester binding to cytosolic phorbol ester receptors in A549-FCS cells. TPA, the more potent inhibitor of growth, and bryo 1 exhibited similar affinities for receptors: 1nM and 10nM TPA displaced 42% and 38.3% receptor-bound [^{3}H]PDBu; 1nM and 10nM bryo 1 displaced 31.8% and 36.7% receptor-bound [^{3}H]PDBu. Bryo 1 and TPA were substantially less able to compete for [^{3}H]PDBu cytosolic receptors derived from A549-US cells than to receptors obtained from A549-FCS cells. Concentrations of PKC activators which would occupy 50% receptor sites could not be calculated.

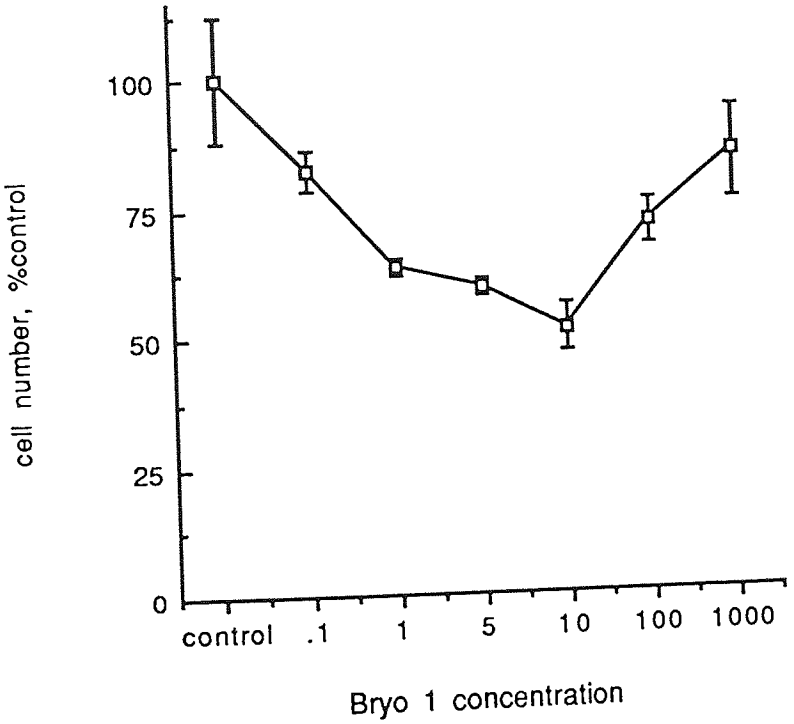
The hypothesis under examination in section 4.1.5 was that serum factors, absent in the US medium supplement, may act in synergy with TPA to augment the maintained growth inhibition induced by nM concentrations of this phorbol ester. It was established that fetuin, upon introduction into serum free media, was able to restore some of the growth arrest induced by TPA in A549 cells (section 4.1.5.5). The effect of bryo 1 on the growth of A549-US cells was examined in the presence of fetuin. As demonstrated in figure 92a) and b), fetuin was unable to influence the transient nature of growth arrest induced by bryo 1. Moreover, bryo1 demonstrated partial ability to block the TPA-induced growth inhibition restored by the presence of fetuin in A549-US cells. This observation supports the hypothesis that TPA but not bryo initiates events sequential to PKC activation, in order to elicit certain responses in cells in culture, and that these events are dependent on the presence of serum factors and can be blocked by bryo 1.

4.6.4 General discussion.

Several hypotheses have been advanced in an attempt to explain the heterogeneous actions elicited by bryos, yet, precise understanding of the biochemical mechanisms involved remains elusive. Generally, different phorbol esters induce similar maximal responses with different potencies. Bryos however differ in the maximal extent to which they induce various responses or block actions induced by phorbol esters. In A549-FCS cells we observed very brief but potent inhibition of DNA synthesis by bryos and varying abilities to block the extended growth arrest induced by phorbol esters (section 4.6.1; Dale and Gescher, 1989). Transient responses evoked by bryos, which mimic the action of phorbol esters, followed by antagonistic effects have been observed in other laboratories. Pasti *et al.* (1988) established that treatment of mouse primary epidermal cells with tumour

Figure 90. Effect of bryo 1 on A549-US cell growth.
a) A549-US cells (2 x 10^4) were seeded, allowed 4h to attach and exposed to increasing concentrations of bryo 1 for 96h before cell counts were performed (mean + SD, n=9).
b) A549-US cells (2 x 10^5) were seeded and allowed 4h to attach before treatments commenced. Cells were exposed to 1nM, 10nM and 1μM bryo 1 for varying periods of time before [^{3}H]TdR incorporation was measured (mean ∓ SD, n=9).

Figure 90a)



90b)

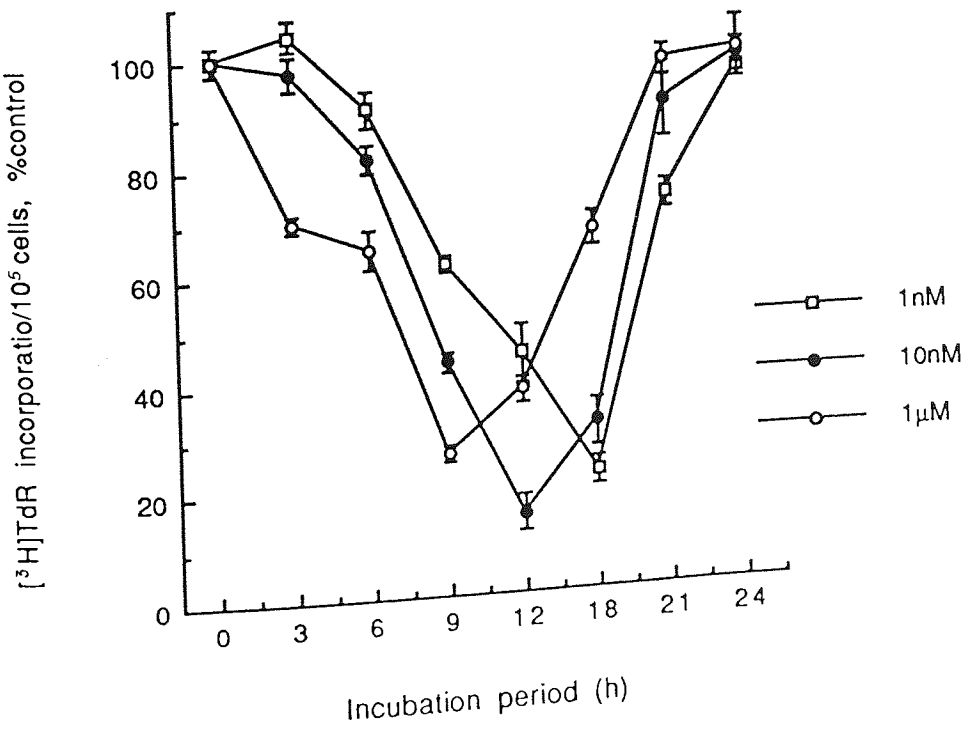


Figure 91. Displacement of [^{3}H]PDBu by TPA and bryo 1 from cytosolic receptors prepared from A549-FCS and A549-US cells (mean ± SD, n=9).

Figure 91

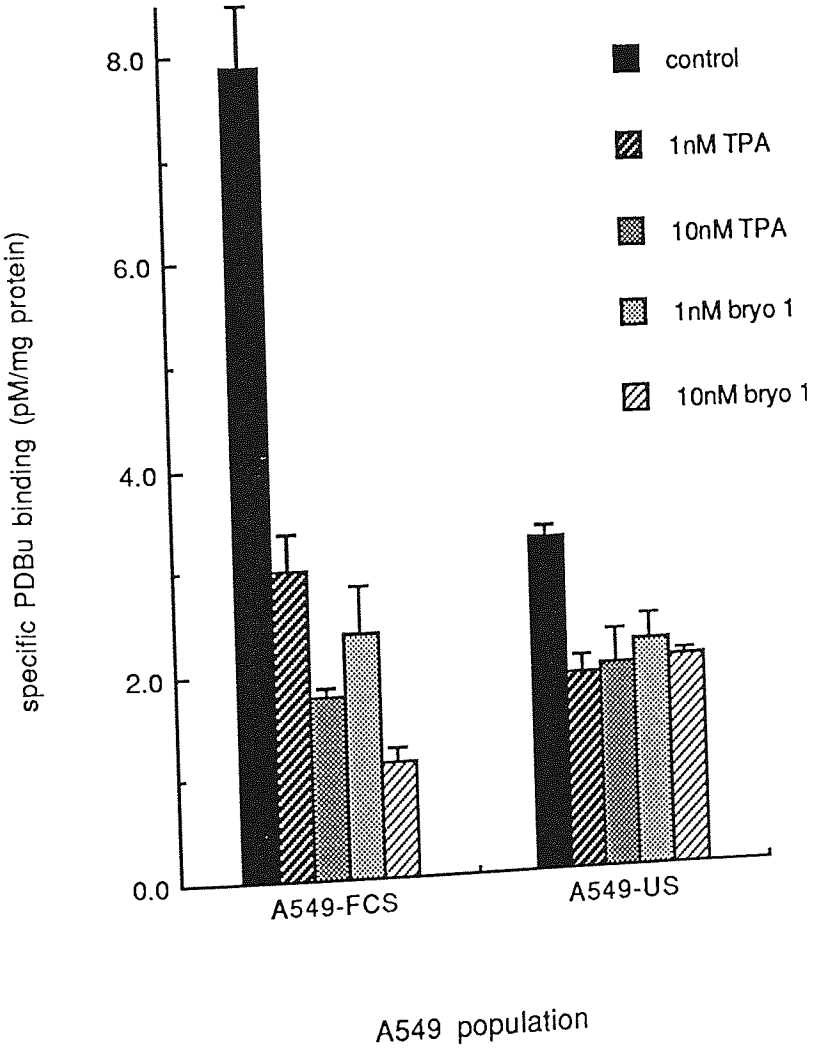
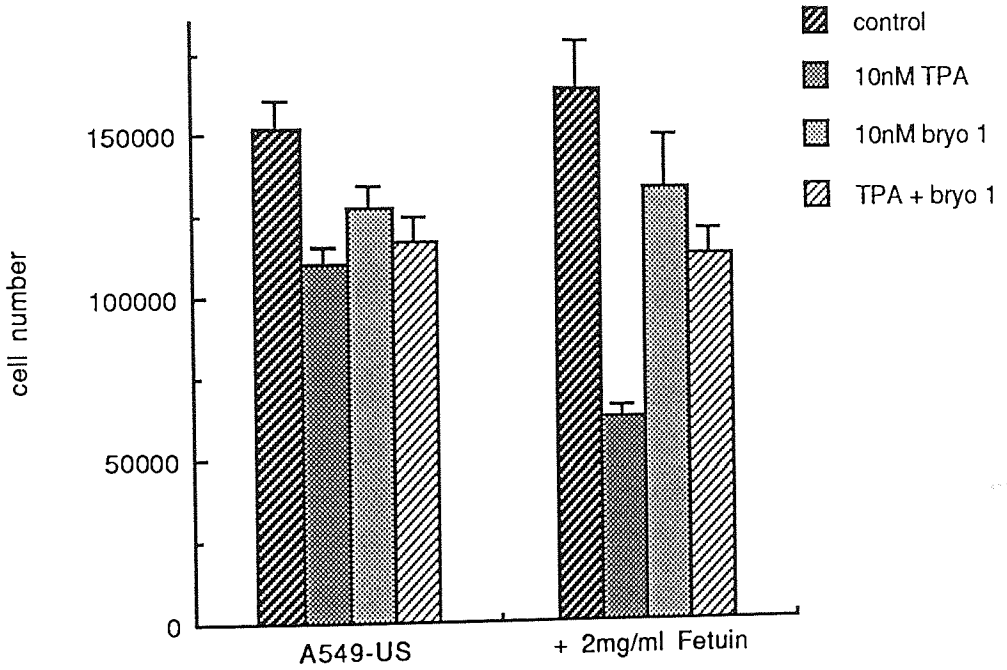
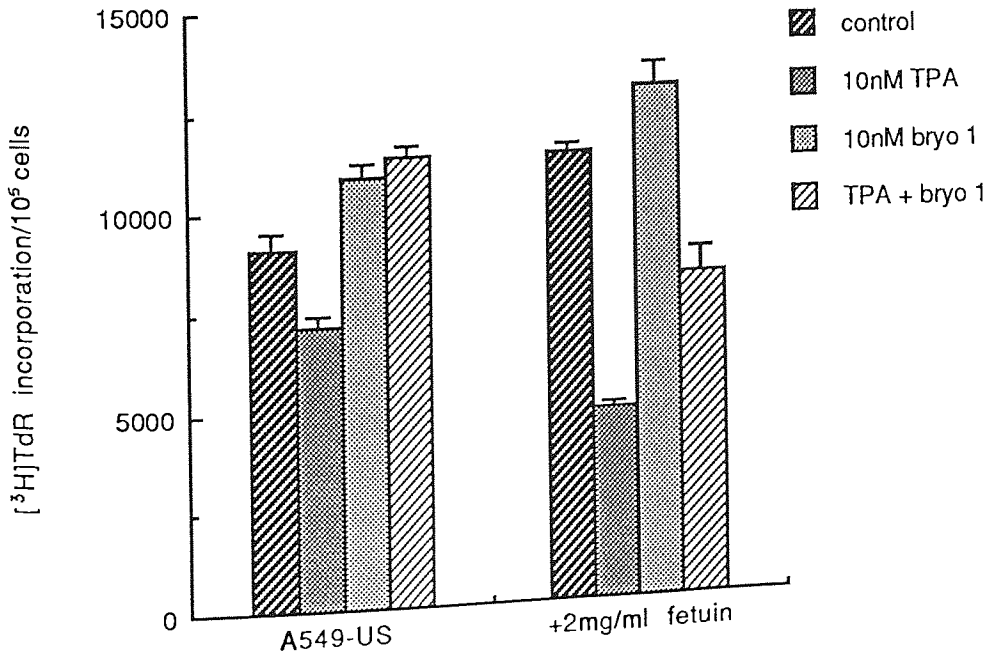


Figure 92. Effect of bryo 1 and TPA on the growth of A549-US cells in the presence or absence of 2mg/ml fetuin.
a) Cells (2 x 10^4) were seeded and exposed to 10nM TPA and/or 10nM bryo 1 for 96h before cell counts were performed (mean ± SD, n=6-9).
b) Cells were seeded (2 x 10^5), exposed to 10nM TPA and/or 10nM bryo 1 for 24h before [H]TdR incorporation was assessed (mean ± SD, n=6-9).

Figure 92a)



92b)



promoting phorbol esters led to disruption of intercellular communication. In control cells, microinjection of Lucifer yellow dye into a single cell led to the spread of dye to 30-40 cells during the subsequent 10min incubation period. Phorbol ester treatment rapidly suppressed such transfer and maintained suppression for 12-24h. Bryo 1 caused an initial rapid suppression of Lucifer yellow transfer (90min), after which time dye transfer returned to normal. At this time bryo was also able to block the inhibition of dye transfer induced by phorbol ester. These authors suggested that one difference between the mechanisms of bryo and phorbol ester action is that the duration of action for bryos is shorter than that for phorbol esters. It has been proposed that a more transient duration of action for bryos may be associated with accelerated breakdown of PKC (Blumberg *et al.*, 1989). This does not appear to be so in A549 cells (figures 37 and 84). Moreover, Dell `Aquila *et al.* (1988) have demonstrated that while bryos are able to mimic certain short term responses of phorbol esters they can at the same time antagonize responses which they have not induced. During the initial 30 min of exposure, both bryo 1 and phorbol ester initiated a rapid decrease in EGF binding in C3H $10T^1/2$ cells, but bryo 1 also antagonized the release of arachidonic acid metabolites. This finding argues against a clear cut ability of bryos to differentiate between short term and long term responses. Thus, an alternative hypothesis has to be sought.

Sako *et al.* (1987) proposed that bryos may stimulate proliferative responses to phorbol esters, but antagonize responses which pursue differentiation. Certain populations of primary mouse epidermal cells proliferated when exposed to phorbol esters, accompanied by the stimulation of ornithine decarboxylase. In other populations of primary mouse epidermal cells phorbol esters induced differentiation. Markers of this pathway are the formation of epidermal transglutaminase and a cornified envelope. Bryo 1 at nM concentrations induced neither transglutaminase activity nor cornified envelope formation, but suppressed both these responses elicited following phorbol ester treatment. However, ornithine decarboxylase activity was stimulated by bryo 1 (Sako *et al.*, 1987). These results suggest that bryo 1 may distinguish between phorbol ester responses related to proliferation and those related to differentiation. Interference with the differentiation pathway by bryo has been reported previously (Kraft *et al.*, 1986; Dell`Aquila *et al.*, 1987). Dell`Aquila *et al.* reported the ability of bryo 1 to restore differentiation blocked by phorbol ester in Friend erythroleukaemia cells induced to differentiate by treatment with hexamethylene bisacetamide. Yuspa *et al.* (1987) postulated that the induction of differentiation is a critical component in the process which leads ultimately to tumour promotion. They predicted an inability of bryo to evoke tumour promotion. In accordance with this prediction, experiments using Sencar mice demonstrated that bryo 1 itself is not a complete tumour promoter and possesses only weak activity relative to mezerein as a second stage promoter. Moreover, co-application of bryo 1 and TPA led to a 50% suppression of tumour response relative to TPA treated controls (Hennings *et al.*, 1987). It is noteworthy however that ornithine decarboxylase stimulation was an early response (3h) and the markers of differentiation; epidermal transglutaminase activity and cornified envelope formation, occurred as late events following PKC activation (9h and 24h respectively). It should also

be noted that bryo 1 was able to promote the clonal growth of JB6 cells in soft agar; an *in vitro* model for tumour promotion (Kraft *et al.*, 1988).

Events downstream of PKC activation, triggered by TPA but not by bryo may explain the paradoxical aspects of bryo action. As discussed previously, the phorbol ester-induced differentiation of HL60 cells was inhibited in the presence of bryo 1 (Kraft *et al.*, 1987). These authors reported that treatment of cells with bryo mimicked the effect of phorbol esters on protein phosphorylation; both agents induced the phosphorylation of the transferrin receptor and 5 cytoplasmic proteins, Mr 17-43kDa, during the same time course. PKC translocation, and down-regulation of active enzyme occurred in both cases. However, bryo treatment didnot affect RNA levels of the c-*myc* oncogene when used over a 1-100nM concentration range and for varying lengths of time, whereas exposure to phorbol ester led to a rapid decrease in c-*myc* mRNA levels. It was concluded that the phosphorylation events induced by the activation of PKC, and enzyme down-regulation were not sufficient for differentiation to occur indicating the need for additional events which may include the down-regulation of c-*myc* . A mutant HL60 cell line displaying phorbol ester tolerance has been developed which underwent transient growth inhibition but not differentiation upon exposure to TPA. The defect was not due to the absence of TPA-induced phosphorylation. However, TPA had no effect on the c-*myc* protein content of the phorbol ester-tolerant cells, whereas naïve cultures lost the c-*myc* protein antigen within 4h of TPA treatment, as detected by Western blotting (Gailani *et al.*, 1989). These authors concluded that the phorbol ester-tolerant cells possess a defect in the mechanism *via* which PKC regulates c-*myc* transcription and that a reduction in c-*myc* expression is necessary for differentiation to occur in HL60 cells. Larsson *et al.* (1988) conducted experiments to investigate specifically whether reduction of c-*myc* is a prerequisite for terminal differentiation. Constitutive expression of introduced v-*myc* oncogene in U937 monoblastic cells did not interfere with the onset of TPA-induced differentiation. However after 24h the process was aborted and reversed with full recovery of proliferative potential and re-expression of the immature phenotype. The authors concluded that down-regulation of *myc* expression was essential for differentiation and concommittant growth arrest (Larsson *et al.*, 1988). TPA, in the latter two examples behaved as bryo alone, thus supporting the hypothesis that the failure of bryo to manipulate c-*myc* expression may be linked to an inability to evoke differentiation. Evidence to support a contrary view has recently emerged (Jalava *et al.*, 1990). SH-SY5Y, a human neuroblastoma line was induced to differentiate to mature ganglion cells by TPA. Bryos 1 and 2, like TPA, were able to translocate and down-regulate PKC activity and evoke down-regulation of c-*myc* mRNA expression, but they were unable to stimulate the differentiation of these cells. Transient growth arrest accompanied by transient morphology changes were observed upon exposure to bryos. Thus in this cell line, it appears that neither reduction in c-*myc* mRNA expression nor activation and down-regulation of PKC activity was sufficient to induce differentiation.

A cytosolic target other than PKC has been demonstrated for TPA. Hashimoto and Shudo (1990) described the existence of a cytosolic-nuclear tumour promoter-specific binding

protein (CN-TPBP) in HL60 cells. Whether bryos are able to interfere with this protein remains to be tested. However much of the work examining the action of bryos has led investigators to speculate upon the existence of a class of targets which do not recognize phorbol esters at nM concentrations (Dell`Aquila *et al.*, 1987; Blumberg *et al.*, 1989). The phosphorylation of 2 proteins following treatment of HL60 cells with either nM concentrations of bryo 1 or μM concentrations of PDBu lends further support to this hypothesis (Warren *et al.*, 1988). Ligand-specific modulation of activated PKC has been forwarded as an explanation for the divergent effects of TPA and Bryo on cell behaviour. For example, the extent of histone phosphorylation caused by bryo was decreased when compared to TPA at equivalent concentrations (Kraft *et al.*, 1988). Possibly of greater physiological relevance, differential activation of PKC at the nuclear level has been reported. Treatment of HL60 cells with bryo 1 alone or in combination with PDBu but not PDBu alone led to the specific translocation of activated PKC to the nuclear envelope where lamin B was rapidly and specifically phosphorylated (Fields *et al.*, 1988). Similarly, bryo 1 and IL-3, both mitogenic towards FDC-P1 haematopoietic, cells mediated rapid serine-specific phosphorylation of several nuclear envelope peptides including lamin B, the extent of which correlated with the mitogenic response (Fields *et al.*, 1989). Phosphorylation of lamin B is involved in nuclear lamina depolymerization at the time of mitosis. Very recent investigations, conducted to elucidate mechanisms which may underlie the differential responses of HL-60 cells to PDBu and bryo 1, demonstrated that PKC-α and -β_{II} differ with respect to activator responsiveness, intracellular distribution and substrate specificity (Hocevar and Fields, 1990). These authors found selective activation of PKC-β_{II} at the nucleus by bryo 1 and concluded that this may affect the resulting cellular response.

Gschwendt *et al.*. (1988) noted that Ca^{2+} dependence is a common factor in all the phorbol ester antagonistic effects induced by bryos. However, similar end points induced *via* the activation of different pathways were not, or were inefficiently inhibited by bryo. Thus, transglutaminase activity, the marker of differentiation, successfully blocked during coincubation with TPA and bryo 1 (Sako *et al.*, 1987), was not inhibited when induced by Ca^{2+}. With relevance to the differential effects on A549 cell growth elicited by TPA and the bryos, it has been noted earlier (section 4.2.4) that the presence and activation of PKC isozymes γ, δ, ζ and ε cannot be ruled out. PKC-ε is of considerable interest as its Ca^{2+}-independence implies that this isozyme may become selectively activated following the production of DAG in the absence of Ca^{2+} fluxes (Schaap and Parker, 1990). Thus it is interesting to speculate that the activation of PKC-ε may be responsible for phorbol ester mimetic cellular responses elicited by bryos.

Interestingly, a difference in phospholipid metabolism induced by bryo 1 or TPA in the presence of 0.5% ethanol has been reported (Tettenborn and Mueller, 1987). The production of phosphoethanol following treatment with bryo 1 was only 25% of that produced in the presence of TPA. Thus it may be postulated that bryo is not able to efficiently activate PLD and therefore may not possess the same potential for PC

breakdown as phorbol esters.

It can be concluded that efforts to elucidate the biochemical basis for the heterogeneity of the action of bryos in different cell systems are still in the early and largely hypothetical stages. Nevertheless mechanistic differences between bryos and phorbol esters have been identified which may contribute to this heterogeneity. Bryo 1 has demonstrated antineoplastic (Pettit *et al.*, 1970) and antileukaemic (Lilly *et al.*, 1990) properties *in vitro* and potential as a biological response modifier, possessing potent ability to stimulate haematopoietic progenitor cells (May *et al.*, 1987). *In vivo*, bryo 1 exhibited antimetastatic action (Schuchter *et al.*, 1989) possibly *via* utilization of its properties as an immunoenhancer. Such results have led to the consideration of bryo 1 as a potential anticancer agent and to its introduction into phase 1 clinical trial.

4.7. Effect of activators of PKC on the growth of A549 tumour xenografts and on phorbol ester receptor binding *in vivo*.

Introduction.

It has been established that bryo 1 is a potent activator of PKC (Berkow and Kraft, 1985; section 4.6), but not a tumour promoter in the Sencar mouse model for tumour promotion (Hennings *et al.*, 1987). Bryo 1 is reported to possess antineoplastic properties in animals, for example against the P388, L1210 and Walker carcinomas (Pettit *et al.*, 1982). In section 4.6, the transitory growth arrest of A549 human lung carcinoma cells *in vitro* by bryos has been described. The preclinical toxicology of bryo 1 has now been completed and this compound is about to enter phase 1 clinical trials. Toxicities in mice included a reduction in platelet numbers, necrotic changes in the Kuppfer cells of the liver, lymphocyte necrosis in the thymus and lymph nodes, and bone marrow congestion. The purpose of the work described in this section was to compare the effect of bryo 1 on the xenograft growth of A549 cells with the growth of tumours in mice unexposed to drug and in mice which had received PDBu. Additionally, cytosolic phorbol ester binding has been monitored in various tissues following exposure of mice to PDBu, in order to test the hypothesis that similar biochemical changes occur *in vivo* , as those observed in cultured cells *in vitro* (sections 4.2.2 and 4.5). Such an investigation may be of value in the light of the uncertainty concerning the effects of agents such as bryo in patients.

Results and discussion.

Dosing of mice and evaluation of tumour growth was performed by Dr. Simon Langdon at the Medical Oncology unit, ICRF Laboratories, Edinburgh.

Figures 93a) and b) illustrate the rate of tumour growth in the absence and presence of 10μg/kg and 50μg/kg of either bryo 1 or PDBu. Animals possessing palpable tumours received 3 i.p. injections of drug on days 0, 2 and 4. Xenograft volume was calculated on days 6, 8, 11, 20, 25 and 28. Inhibition of tumour growth was not observed in mice following treatment with either bryo 1 or PDBu. There was no statistical difference between control tumour volume and tumour volume in animals in the treated groups. Standard deviations are very large, this reflects the nature of xenograft growth, which is always less reproducible than rodent tumour growth. A 20% death rate was observed in mice receiving 50μg/kg bryo 1. This concentration therefore, appears to exceed the maximum tolerated dose for this schedule.

In a previous study, it has been demonstrated that whilst tumour promoting phorbol esters were able to inhibit proliferation and induce differentiation of a myeloblastic leukaemia cell line *in vitro*; *in vivo*, no such response was observed (Weinberg and Misukonis, 1984). TPA was administered intravenously and leukaemic cells continued to proliferate in the spleen and peritoneal cavity of experimental animals. Schuchter *et al.* (1989), upon evaluation of *in vivo* antitumour activity of bryo 1 against B16 melanoma pulmonary metastases, demonstrated profound antimetastatic potential, in a clearly concentration-dependent manner. However, in a thymidine incorporation assay *in vitro*, B16 cells were found to be insensitive to the effects of bryo. It was concluded that the significant

antimelanoma activity *in vivo*, exerted by bryo 1 may be a consequence of its immunoenhancing properties. It was found in this study that the optimal antitumour dose of bryo 1 (administered i.p.) was 100μg/kg. All animals exposed to 200μg/kg bryo 1 died within 2 days of treatment.

Experiments were conducted at the Aston laboratories to determine cytosolic phorbol ester binding in tumour, lung, brain and liver, following isolation of tissues by Dr. Simon Langdon. Tissues were transported to Aston on cardice. During a minimum interim period, prior to evaluation of phorbol ester binding, samples were stored at -70^{o}C. Preliminary experiments were performed in order to examine the effect of tissue storage on phorbol ester binding. Following excision of lungs and brain from male NMRI mice, homogenization and storage at -70^{o}C for a specific period of time, crude cytosolic extracts were prepared and phorbol ester binding evaluated to ascertain the rate of decay of phorbol ester binding sites. A gradual decline in PDBu binding with increasing storage time is demonstrated in figure 94, which is likely to reflect loss in enzyme activity (see section 4.2). Homogenates of brain cytosol possessed a significantly higher capacity to bind PDBu, reflecting abundant PKC activity in the CNS (Nishizuka, 1988).

Figure 95 illustrates specific PDBu binding in lung, tumour, and liver tissues of tumour bearing nude mice. Animals had received 3 treatments of PDBu at 48 hourly intervals and had been sacrificed 72h after the final dose. Interestingly, the cytosol of tumour and lung tissues from animals which had received PDBu, consistently displayed significantly increased capacity for phorbol ester binding compared to tumour and lung tissues from control animals. Significance levels for the difference in tumour cytosolic binding was $p < .001$. For lung specimens, p values representing levels of significance were $< .001$ and $p = .013$ for 10μg/kg and 50μg/kg PDBu treatments respectively, compared to samples from untreated mice. Cytosolic phorbol ester binding was examined *in vitro* in cells which had been exposed to PDBu for 24h, followed by a recovery period of 24, 48 and 72h in drug-free medium. The results are discussed in section 4.2.2 and reveal that specific PDBu binding in cells following a 72h recovery period was greater than the cytosolic binding potential of untreated cultures. Mixed micelle assays performed using cytosol from brain samples demonstrated a high degree of inter-experimental variability. The results suggest that specific binding sites were preserved during storage, but to highly variable extents in individual brain specimens (values between 16 and 43 pMoles/mg protein specific PDBu bound). Thus, it was difficult to establish a trend in phorbol ester binding potential following exposure of animals to PDBu. However, in liver samples from treated animals, a consistent and significant decrease in cytosolic PDBu binding potential was seen when compared to control specimens ($p < .001$, figure 95). Mean losses of specific cytosolic binding of 42.5% and 57.1% were obtained in mice 72h after the final injection of 10μg/kg and 50μg/kg PDBu respectively. This data indicates that PDBu may elicit down-regulation of PKC activity at this site. It is conceivable that PDBu, rapidly cleared from the circulation, may accumulate in the liver and fat tissues, where, as a result of its metabolic stability, partial down-regulation of active PKC and loss of phorbol ester binding occurs.

In an alternative protocol, animals received a single application of 50µg/kg PDBu and were sacrificed 6h later. The hypothesis was investigated that administration of phorbol ester may cause immediate down-regulation of cytosolic phorbol ester binding potential in tissues *in vivo* . Lung, tumour, brain and liver tissues were examined for phorbol ester binding ability. Cytosolic preparations of lung from control and treated animals demonstrated negligible specific PDBu binding potential, suggesting loss of enzyme activity between the times of isolation and experimentation. In the liver, negligible loss of specific phorbol ester binding sites was observed in specimens from the treated group. Crude cytosolic fractions prepared from brain tissue of treated animals demonstrated a mean increase of 30.5% + 4.7% (n = 3) in PDBu binding potential, compared to untreated mice. Down-regulation of cytosolic phorbol ester binding was observed in tumour samples from animals exposed to 50µg/kg PDBu. Specific binding was consistently low suggesting a uniform loss in enzyme activity during the time between tumour isolation and assay; however, a mean loss of 80.8% + 6.5 (n = 3) phorbol ester binding sites was revealed in samples from treated animals compared to untreated controls (results not shown).

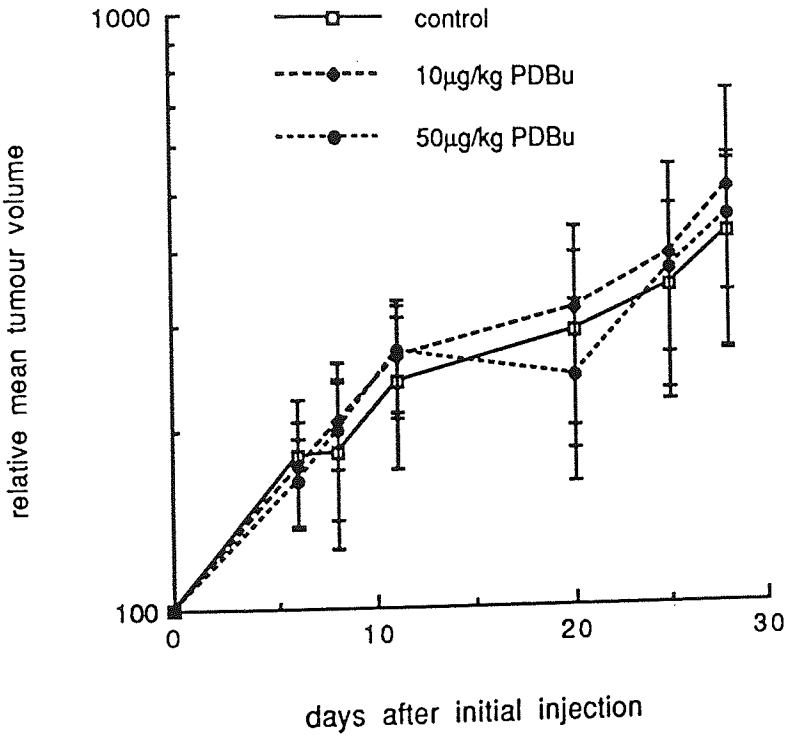
Fournier and Murray (1987), have established that a single application of TPA to mouse skin may lead to a rapid and complete loss of PKC activity, associated with a loss of immunologically detectable PKC and the accumulation of a smaller protein, detectable by antibodies recognizing the regulatory domain of PKC. Loss in enzyme activity was maintained for 3 days; however, skin extracts prepared 8 days after TPA administration yielded PKC activity profiles indistinguishable from controls.

The present protocol for investigating phorbol ester binding properties of tissue samples relies upon the presence of active enzyme and thus is not ideal. Too many variables have been introduced which may compound data. Greater confidence could be conferred upon the results if assays were performed on fresh tissue samples, this would entail experiments being performed at one location.

Experiments investigating the effect of bryo 1 on cytosolic phorbol ester binding potential remain to be performed. Preliminary results described above demonstrate a lack of inhibition of A549 tumour growth by bryo 1 or PDBu. However, bryo may elicit other beneficial antineoplastic effects, undetected in a simple growth curve of one tumour type, following only one schedule for drug administration. In view of the promising immunomodulatory effects of bryo 1, described by Schuchter *et al.* (1989), bryo 1 may show further antineoplastic properties in the forthcoming clinical trials.

Figure 93. A549 tumour xenograft growth.
a) The effect of 10μg/kg and 50μg/kg bryo 1 on the growth of A549 tumour xenografts.
b) The effect of 10μg/kg and 50μg/kg PDBu on the growth of A549 tumour xenografts.
3 IP injections were administered to animals at 48 hourly intervals. Animals were sacrificed on the day indicated and tumour volume calculated (mean ∓ SD, n=7).

Figure 93a)



93b)

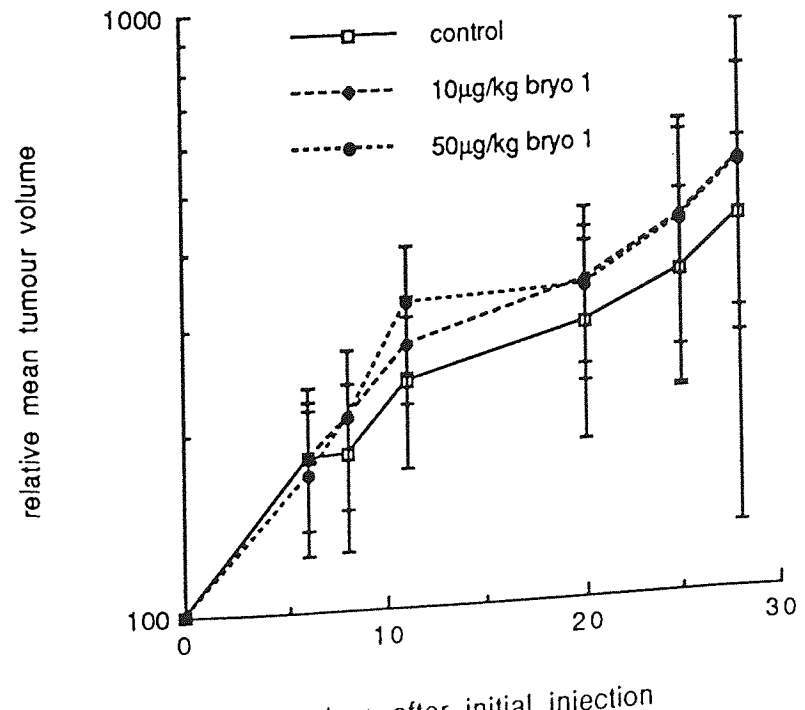


Figure 94. Cytosolic phorbol ester binding in brain and lung homogenates.
The brain and lungs were isolated from male NMRI mice. Crude cytosolic extracts were prepared and samples stored at -70^{0}C until the mixed micelle assay was performed. * pMoles specific PDBu bound for brain samples X10 (mean ± SD, n=3).

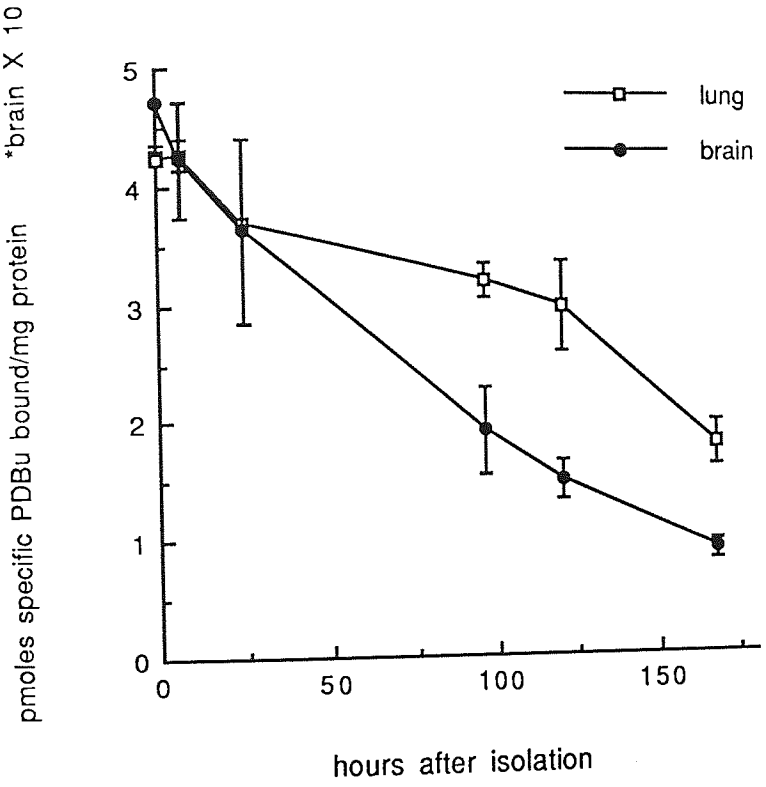
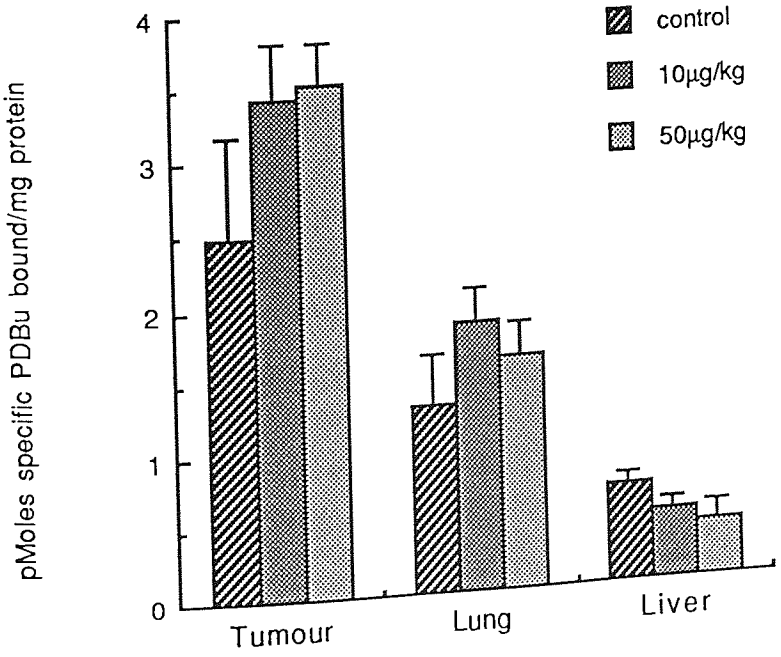


Figure 95. Phorbol ester binding in tumour, lung and liver cytosol preparations from tumour bearing athymic mice.
3 IP injections of 10µg/kg or 50µg/kg PDBu were administered at 48 hourly intervals. Animals were sacrificed 72h later. Tissues were isolated and homogenized, transported to Aston on dry ice and stored at -70^{0}C until the mixed micelle assays were performed (mean ± SD, n = 9-15).



4.8 Analysis of fluorescently tagged phorbol ester binding to PKC using multiparameter flow cytometry.

Introduction.

Certain methods adopted during this study for measuring phorbol ester binding and evaluating PKC activity require prior disruption of cells, crude cytosol preparation or partial enzyme purification. Thus cells are non-viable, and conditions are non-physiological. When phorbol ester binding was examined on cultured cell monolayers, discrepancies arose which are discussed in section 4.2. A novel flow cytometric technique has been developed to quantify phorbol ester binding to PKC in individual viable cells utilising the novel fluorescent phorbol ester Bodipy-3-propionyl-13-acetate (Bod-3-PE). The structure of this fluorophore is illustrated in figure 96.

The analysis of Bod-3-PE binding using FCM requires cells to be in suspension. Thus, preliminary experiments were conducted to evaluate specific phorbol ester binding in suspended A549 cell populations using the ligand [^{3}H]PDBu.

Results and discussion.

Figure 97a) demonstrates specific PDBu binding to A549-FCS and A549-US cell populations, in suspension. In contrast to the results obtained following assay for PKC activity, Western blotting procedures and mixed micelle binding assays, the latter population displayed increased specific binding, by 20.4% compared to A549-FCS cells. Binding assays performed on monolayer cultures generated data in accordance with this finding; specific binding sites were increased by 38% in A549-US cells compared to those cells cultured in an environment fortified with serum (figure 46, section 4.2.3). The same figure illustrates specific binding in A549-FCS cells pre-exposed to 10nM TPA for 24h, and cells designated more permanently resistant to the growth inhibitory properties of TPA. Interestingly, contrary to the detected binding sites of cells in monolayer culture, negligible specific binding was revealed in A549-TPA populations. This finding supports the notion that trypsin may affect membrane-bound proteins. Pre-incubation of A549-FCS cells with 10nM TPA for 24h was unable to abolish entirely specific PDBu binding, but decreased it by 62.4% compared to untreated control cultures. Displacement of 50nM [^{3}H]PDBu following co-incubation of cells with increasing concentrations of TPA is demonstrated in figure 97b). The concentration of TPA, calculated as able to displace 50% PDBu is 47nM.

All binding experiments using Bod-3-PE and FCM were conducted in collaboration with Dr. Caroline Dive. Initial FCM analysis was performed following incubation of cell suspensions for 10min with increasing concentrations of Bod-3-PE. Figure 98 demonstrates the single parameter Gaussian distributions acquired when cells are incubated with 1nM Bod-3-PE. The x axis represents Bod-3-PE (green) fluorescence, the y axis represents cell number. Non-specific binding was determined in the presence of 5μM PDBu. Two dimensional frequency plots representing total and non-specific binding of 1nM Bod-3-PE are demonstrated in figure 99. Relative cell size may also be determined from such plots, following analysis of light scatter. No apparent difference in cell size was distinguished following incubations with TPA.

Increasing concentrations of Bod-3-PE yielded increased total fluorescence, but also generated higher non specific Bod-3-PE fluorescence (not shown). For this reason and for reasons of economy, examination of fluorescently tagged phorbol ester binding to A549 cells was pursued using a concentration of 10nM Bod-3-PE.

Green fluorescence in populations of A549 cells was examined to quantify Bod-3-PE binding in A549-FCS and A549-US cell populations cultured in the permanent presence or absence of 10nM TPA. The data generated displayed similarity to results obtained from the mixed micelle assay for phorbol ester binding and those measuring subcellular PKC activity (section 4.2). Cell populations cultured under conditions of serum deprivation displayed 47% specific Bod-3-PE fluorescence of A549-FCS cells. Greatly reduced specific green fluorescence was observed in A549-TPA populations when compared to untreated control cells (figure 100). Certain differences have been detected in the data obtained during evaluation by selected techniques of phorbol ester receptor binding, PKC activity or protein presence. Mechanisms which underlie such nonconformity, and the functional significance remain to be elucidated. Hypotheses, forwarded to explain the discrepancies are discussed in section 4.2.

The hypothesis has been explored that prior incubation of A549-FCS cells for 24h with 10nM TPA induces down-regulation of Bod-3-PE binding. Compared to untreated A549-FCS cells, a significant decrease of Bod-3-PE fluorescence by 81.% was observed in cell populations pre-exposed to 10nM TPA.

Concentration-dependent displacement of Bod-3-PE specific binding was observed following co-incubation with TPA, bryo 1 and bryo 5 (figure 101). Bryo 5 exhibited higher affinity for the Bod-3-PE receptor, consistent with binding assays performed on intact cell monolayers (figure 80, section 4.6). The data suggests that 50% of receptors would be occupied by <0.1nM bryo 5, 5.8nM TPA and <10nM bryo 1.

It should be emphasized that the above data represent preliminary results and future experiments are planned. Further manipulation of the experimental procedure may be conducted to refine the technique and optimize conditions for maximum sensitivity in analysis of Bod-3-PE binding. A particular observation was that non specific binding was high. An attempt could be made to reduce this by using unlabelled propionyl acetate in place of PDBu to determine non specific binding. Additionally, the introduction of medium only wash stages between incubation with fluorophore and FCM analysis may increase the accuracy of data generated. During preliminary examination to measure Bod-3-PE binding and competition for the Bod-3-PE receptor, the fixed conditions of 10nM Bod-3-PE and 10min incubation period were adopted, in accordance with the conditions employed in other studies (Marrone *et al.*, 1990 and Pershadsingh *et al.*, 1988). Further detailed investigations are warranted in which the concentration of Bod-3-PE is varied. Additionally, the analysis of Bod-3-PE binding may be conducted following different incubation periods. Following 30 min exposure, FCM results may be compared directly with the evaluation of binding of [^{3}H]PDBu to monolayer cultures or cells in suspension. The assay of Bod-3-PE binding by FCM is rapid and potentially very sensitive, it allows analysis of phorbol ester binding in individual viable cells and permits exclusion from

evaluation of non-viable cells. It will reveal heterogeneity in phorbol ester binding within mixed populations. Analysis performed to date has been evaluated upon a linear scale where Bod-3-PE binding appeared homogeneous. Log scale analysis would reveal decisively the presence or absence of heterogeneous cell populations. Heterogeneity may reflect the presence of more than one PKC isozyme, displaying differential affinities for the Bod-3-PE receptor. To date however, PKC-α only has been detected in A549 cells (section 4.2.4). Importantly, this technique may be applied to detect agents potentially capable of competing for the phorbol ester receptor. Moreover, FCM will allow the simultaneous evaluation of multiple cellular parameters. For example, the relationship between phorbol ester binding and intracellular Ca^{2+} flux could be investigated. In addition, using microscopy techniques, fluorescently labelled PKC activators such as Bod-3-PE may be employed to locate the destination of enzyme translocation or to aid detection of functional aberations in transformed cells. With respect to the latter point, Hyatt *et al* . (1990) have established by immunofluorescence that PKC-α was localized in focal contacts in normal but not transformed fibroblasts.

Figure 96. Structure of Bodipy-3-propionyl-13-acetate.

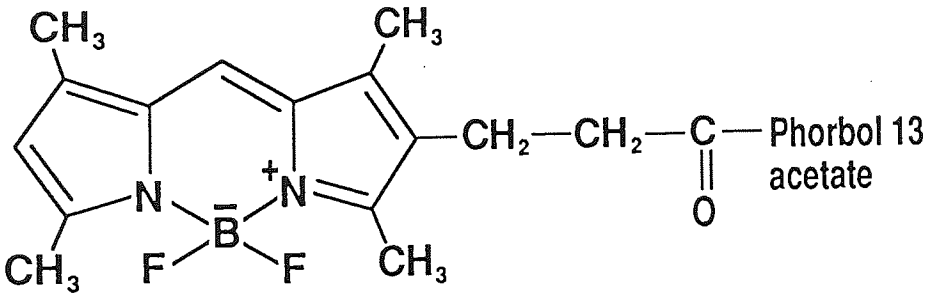
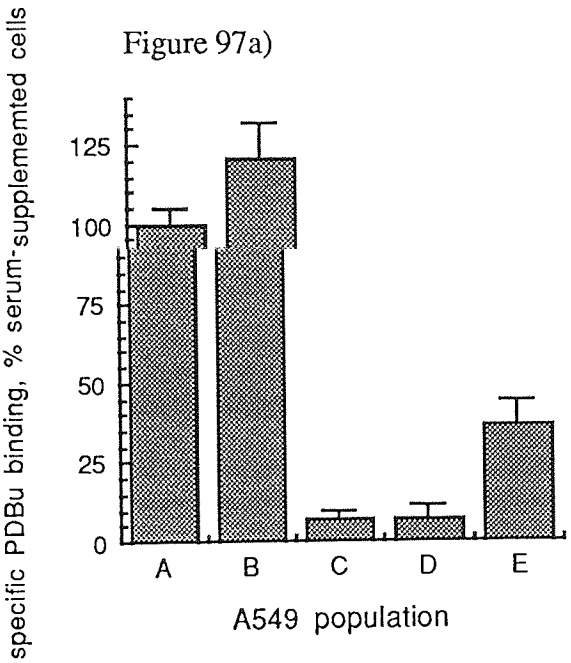


Figure 97. Specific binding of 50nM [^{3}H]PDBu to whole A549 cells in suspension.
a) A549 populations were trypsinized, incubated for 30mins at 37, under constant shaking in presence or absence of 5μM unlabelled PDBu. Washing procedures were performed at 4C, before total and non-specific binding determined.
A. Cells cultured in medium supplemented with 10% FCS.
B. Cells cultured in medium supplemented with 2% US.
C. Serum-supplemented cells grown in in medium fortified with10nM TPA.
D. Serum-deprived cells grown in medium fortified with 10nM TPA.
E. Serum-supplemented cells treated for 24h immediately prior to analysis with 100nM TPA (mean ∓ SD, n=9).
b) Displacement of [^{3}H]PDBu by TPA (mean ∓ SD, n=7-9).



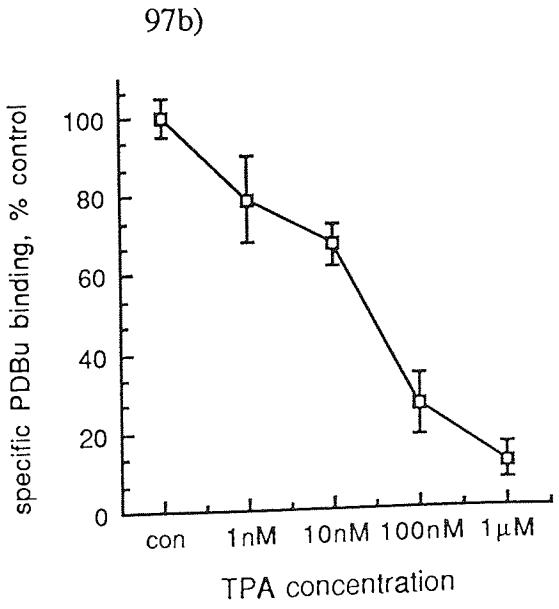
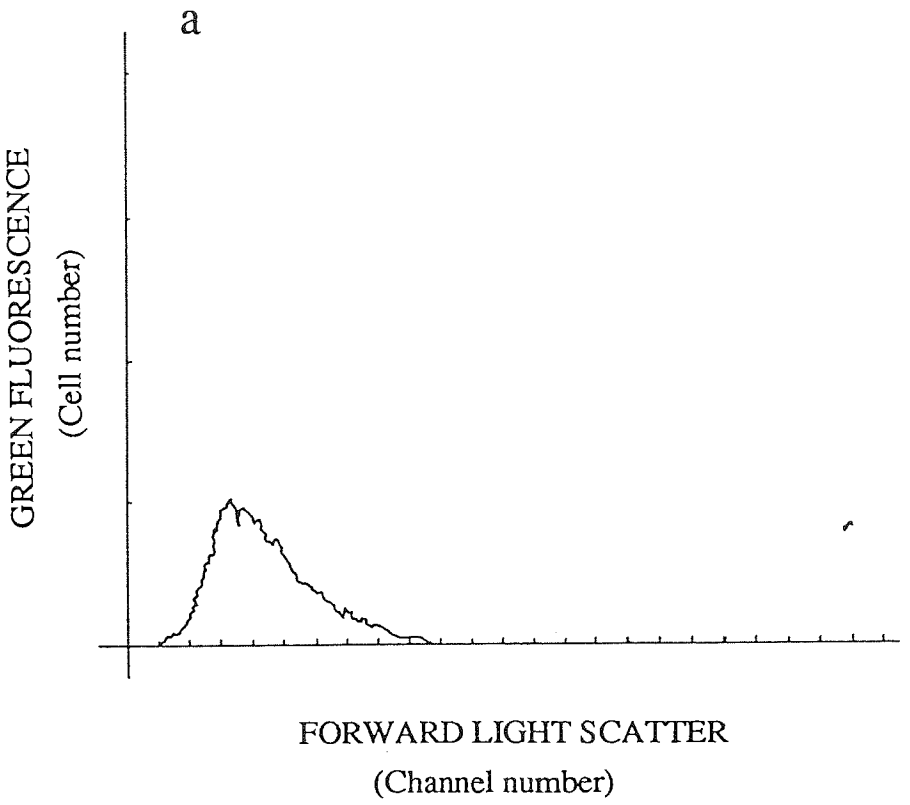


Figure 98. Single parameter Gaussian distribution following incubation of cells with 1nM Bod-3-PE in the absence a) or presence b) of excess unlabelled phorbol ester. a) mean channel number: 16.5, b) mean channel number: 40.7.



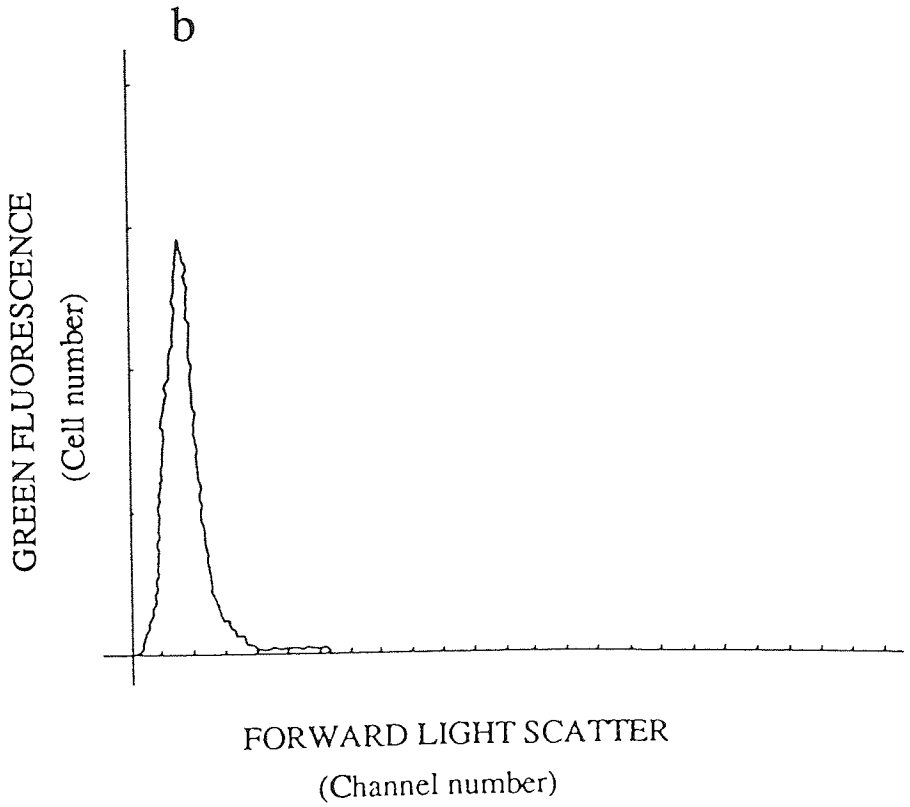


Figure 99. 2 dimensional frequency contour plots illustrating the green fluorescence exhibited by individual cells in the absence (a,c) and presence (b,d) of unlabelled PDBu following incubation with Bod-3-PE only (a,b) co-incubation with Bod-3-PE and 1nM TPA (c,d).

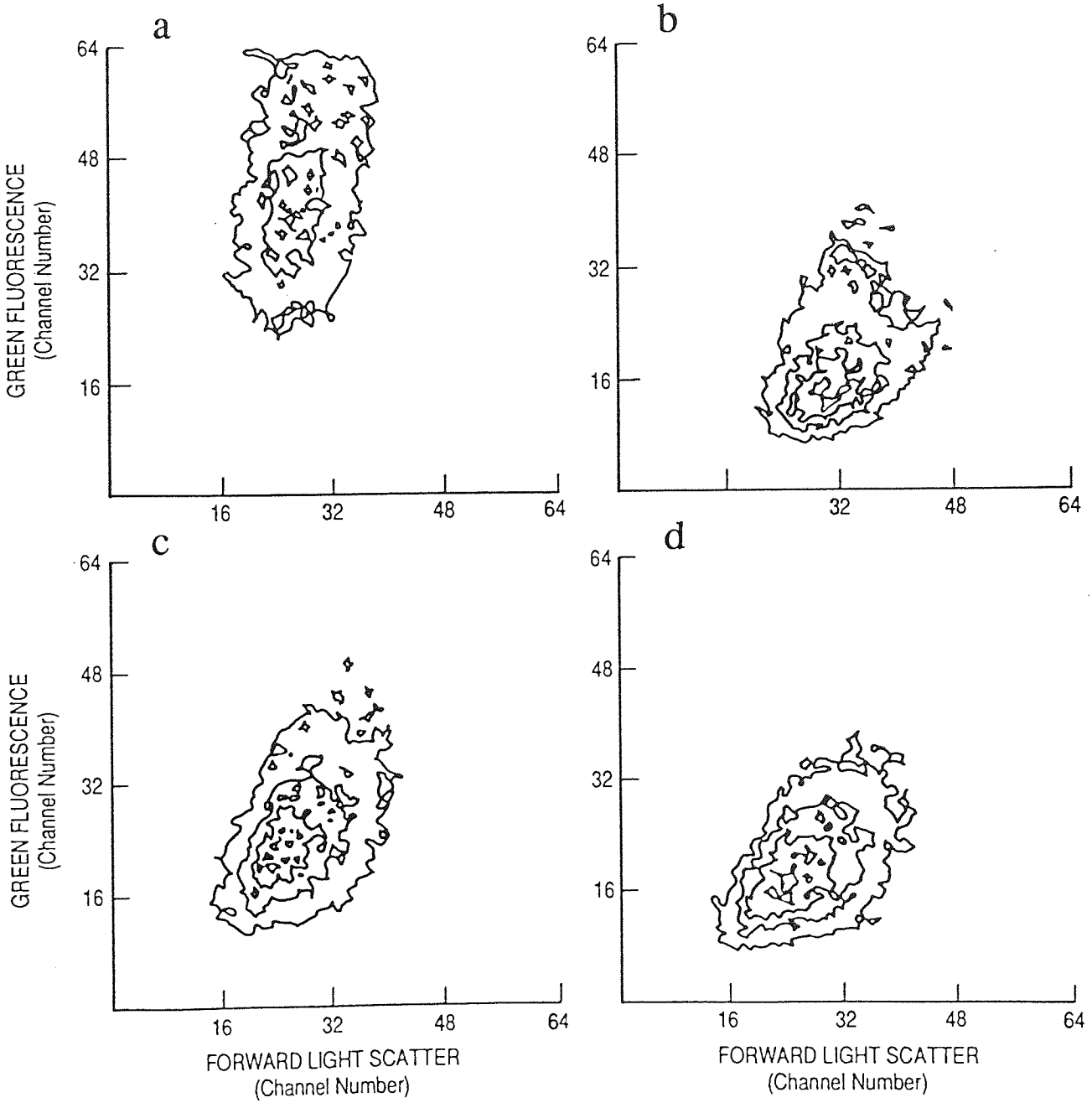


Figure 100. Specific mean green fluorescence exhibited by A549 populations.
A. Cells cultured in medium supplemented with 10% FCS.
B. Cells cultured in medium supplemented with 2% US.
C. Serum-supplemented cells grown in medium fortified with10nM TPA.
D. Serum-deprived cells grown in medium fortified with 10nM TPA.
E. Serum-supplemented cells treated for 24h immediately prior to analysis with 10nM TPA (mean + SD, n=3).

Figure 100

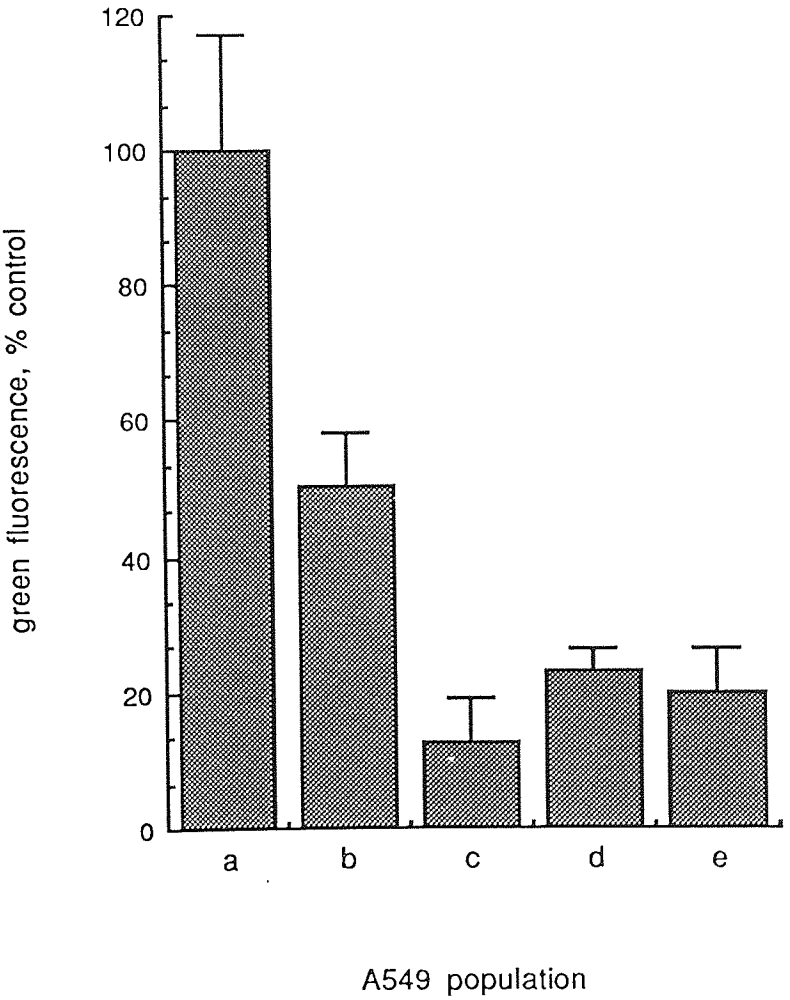
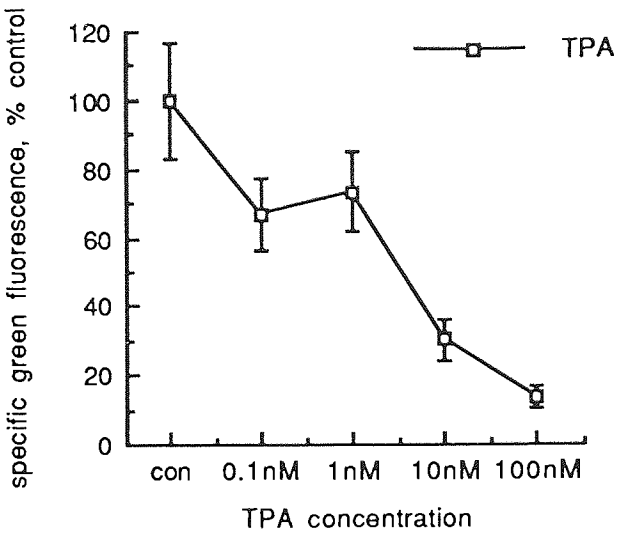
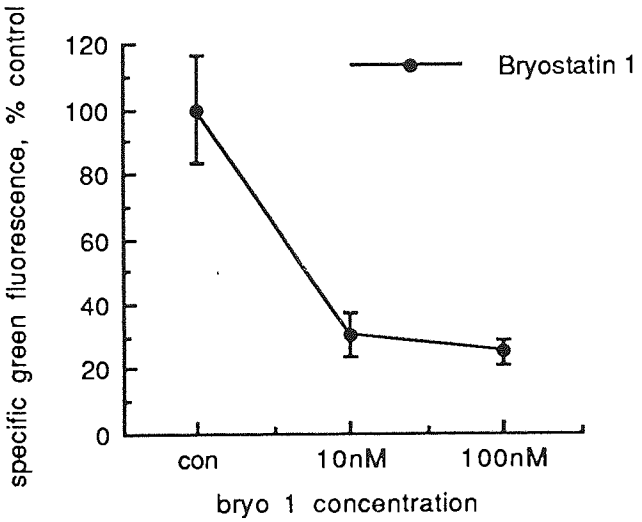


Figure 101a)



101b)



101c)

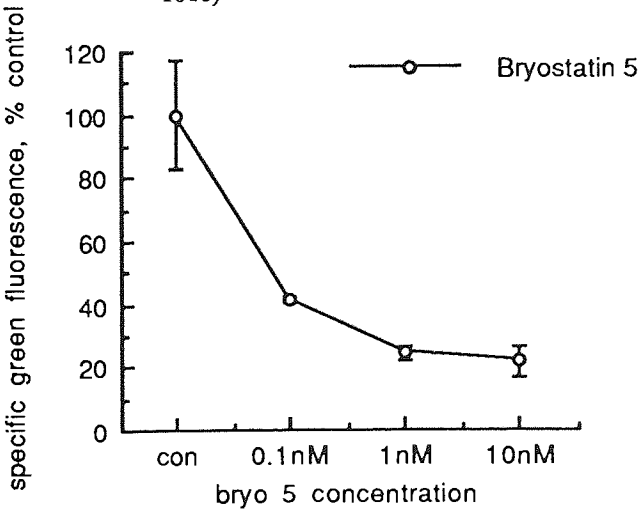


Figure 101. Decreasing specific green fluorescence, representing the displacement of Bodipy-3-PE by increasing concentrations of a) TPA, b) Bryostatin 1 and c) Bryostatin 5 (mean ∓ SD, n=3).

SECTION 5: GENERAL DISCUSSION.

A major goal of the research described in this thesis has been the elucidation of mechanisms crucial for the mediation of growth inhibition elicited by tumour promoting phorbol esters in A549 human lung carcinoma cells. The family of isoenzymes comprising PKC is considered to be the major phorbol ester receptor. Moreover, PKC serves as receptor for bryos, antineoplastic marine compounds. Evidence presented herein supports a role for the activation of PKC-α in the induction of A549 growth arrest by TPA and bryos. These agents displayed exquisite affinity, within the subnM range, for the PDBu receptor. Synthetic DAGs and DAG analogues, unable to cause cytostasis without a cytocidal effect, induced no change in PKC activity and demonstrated only modest competition for PDBu binding sites, at μM concentrations, following the preparation of crude cytosolic extracts of phorbol ester receptor.

However, a clear understanding of mechanisms preceding the cellular responses elicited by tumour promoting phorbol esters appears remote. Cellular responses are generated sequential to the activation of a complex signalling network, receiving multiple inputs (section 1.5). It has become evident that events distal to PKC activation are crucial in the maintenance of phorbol ester-induced growth inhibition. Components of foetal calf serum proved important in the maintenance of TPA-induced growth arrest (section 4.1), factors which had no effect on responses elicited by bryos. Indeed, that bryos displayed the ability to abrogate phorbol ester-evoked growth inhibition implies the involvement of other molecular mechanisms as yet undiscovered; although hypotheses to explain this phenomenon have been discussed in section 4.6. There are reports of novel phorbol ester receptors (Ahmed *et al.*, 1990), for example N-chimaerin, whose mRNA has been located in human brain and certain neuroblastoma and tumour cell lines. A TPA-specific protein, unrelated to PKC has been reported in the cytosol of HL-60 cells which responds to phorbol ester treatment by translocating to the nucleus (Hashimoto and Shudo, 1990). Construction of a series of deletion and truncation mutants of PKC has led to the definition of two distinct [^{3}H]PDBu binding domains, one within each of the two cysteine rich sequences contained within the first conserved region (C1) of PKC. Of the two, it was clearly demonstrated that the second cysteine rich sequence bound [^{3}H]PDBu with higher affinity (Burns and Bell, 1990). This fact has interesting implications in the light of PKC-ζ, which possesses the first cysteine rich region only. Moreover, in yeast an isozyme of PKC has been discovered which was insensitive to activation by phorbol esters (Fredholm and Abbott, 1990).

A main objective of cancer therapeutics has been to develop treatments which possess selective antitumour action. To date, cellular targets that may provide such unique specificity have not been uncovered. Current chemotherapy relies upon the cytotoxic eradication of proliferating neoplastic cells, *via* drug-induced damage to genetic material. Membrane signal transduction offers multiple sites for intervention and over the past decade, the plasma membrane has received considerable attention as a potential locus for

therapeutic intervention. PKC plays a pivotal role in the transduction of cellular signals originating from hormones, growth factors, and oncogenes and is responsible for phosphorylation events regulating cellular function. A wealth of evidence has emerged indicating that PKC appears central to oncological problems (see sections 1.7). Many reports have provided direct evidence suggesting a critical role for specific isoforms in growth regulation and carcinogenesis. Normal fibroblasts, induced to overexpress PKC-β_I or -γ or a mutant PKC-α, displayed a combination of the following characteristics: uncontrolled growth, enhanced tumourigenicity, cell transformation, and tumour growth in athymic mice (Housey *et al.*, 1988; Krauss *et al.*, 1989; Persons *et al.*, 1988; Megidish and Mazurek, 1989). In contrast, overexpression of PKC-β_I in HT29 colon cancer cells caused diminished tumourigenicity (Choi *et al.*, 1990). These authors speculate that in certain tumours, PKC may act as a growth suppressor gene. The presence of TPA (10nM) induced cells to become refractile and round up, moreover, cell growth was inhibited. Thus, it would be intriguing, following complete characterization of A549 isozyme content to compare the ratio and content of PKC subspecies with that of normal type II alveolar cells. To date it has been established that A549 cells used in this study possessed PKC-α protein, PKC-ß was not detected (section 4.2.4). An additional finding was that following manipulation of growth conditions, PKC-α protein content, cytosolic phorbol ester binding, PKC activity and extended sensitivity to the growth inhibitory properties of phorbol esters diminished considerably. However a direct correlation between these phenomena has not been established. Evidence has been provided linking PKC indirectly with the transformed state (Hyatt *et al.*, 1990). PKC levels, subcellular distribution and phorbol ester receptor content in normal and SV40 transformed rat embryo fibroblasts were indistinguishable. However PKC-α, in normal cells was tightly associated with the cytoskeleton and organized in focal centres. Transformed cells displayed irregular localization of PKC-α correlating with a loss of two PKC-binding proteins.

Certain observations have led to the attribution of specific cellular functions to specific subspecies of PKC. For example, a role for PKC-α as an inhibitor of colonic Cl^{-1} secretion has been demonstrated in a human carcinoma cell line (van den Berghe *et al.*, 1990). Martinson *et al.* (1990) concluded that PKC-α activation is critical for responses to muscarinic acetylcholine receptor stimulation to take effect in astrocytoma cells which exclusively express PKC-α. However, it is becoming clear that the biological effect resulting from the activation of a specific PKC isozyme is specific to the cell type in which it is expressed. Thus, if it transpires that A549 human lung carcinoma cells possess exclusively PKC-α it may be possible to correlate with a little more confidence, PKC-α activation with the growth arrest induced by bryos or tumour promoting phorbol esters in this cell line only. Experiments investigating protein phosphorylation following activation of PKC may identify substrates of PKC-α in A549 cells. It may be hypothesized that subspecies of PKC may generate unique biological responses in each cell type in which

they are expressed, partly, as a consequence of the substrate availability within each cell or cellular compartment. A future goal of this project is to characterize PKC-dependent phosphorylation events in A549 cells and to determine whether activator-dependent protein phosphorylation occurs. Such a phenomenon has been demonstrated by Yamamoto *et al.* (1988). The results of such investigations may offer an explanation for the disparate effects of TPA and bryos upon A549 growth. To the same end, a comparison of PKC substrates in A549-FCS and A549-US, TPA-sensitive and `resistant` cell populations may offer insight into mechanisms of resistance and processes preceding release from growth arrest.

Laboratories worldwide have devoted energy and effort to the discovery and development of modulators of PKC. Enzyme activators such as tumour promoting phorbol esters have been shown to suppress the growth of many malignant cell lines (reviewed by Gescher, 1985). Obviously, tumour promoters may not be employed as anticancer drugs, but as classic activators of PKC they have been utilized as tools to aid analysis of the phorbol ester pharmacophore to generate scientific insight and as a guide to the rational design of novel phorbol and DAG analogues. During the course of this study, a series of compounds, designed and synthesized as stable analogues of DAG, have been screened for their ability to bind to the phorbol ester receptor and to exhibit biological activity against A549 tumour cell growth. Negligible specific biological activity was detected (section 4.5). Wender *et al.*, (1986) have performed analysis of the phorbol ester pharmacophore on PKC and have created a series of biochemically successful compounds, as judged by their ability to compete for phorbol ester binding and to elicit biologic activity. Bryos, potent activators of PKC have displayed antineoplastic activity in a number of established tumour cell lines, stimulated the growth of normal human bone marrow and erythroid progenitor cells (May *et al.*, 1987), and demonstrated suppression of phorbol ester induced tumour promotion (Hennings *et al.*, 1987). Wender *et al.* (1988) have extended their work, modelling the bryostatins to the key structural elements of the phorbol ester pharmacophore.

Inhibitors of PKC may possess pharmacological potential, but to date, they have not progressed past the initial stages of drug development. Preliminary studies have been undertaken in this project (sections 4.2.5 and 4.6) examining the effect of STSPN on A549 tumour cell growth and employing this agent to aid elucidation of the role of PKC activation in A549 growth arrest induced by phorbol esters and bryos. A potent cytostatic effect was elicited by this agent. STSPN was reported to be cytotoxic at very low concentrations in tumour cells *in vitro*. This agent inhibits PKC by binding to the ATP-binding site (Tamaoki *et al.*, 1986), hence is able to inhibit most kinases as the ATP-binding regions are all related. This raises the possibility that the cytotoxic potency of STSPN may be too high to be explained by the single mechanism of PKC inhibition. Inhibitors have been employed universally to aid the elucidation of the role of PKC in cellular processes such as differentiation, proliferation and tumourigenesis, often following enzyme activation elicited by such phorbol esters as TPA. However, recently, two functional sites in phorbol esters have been demonstrated which possess distinct activities (Leli *et al.*, 1990). As well as the DAG-like area (region 1), an additional functional site has

been located within the terpene ring area (region 2) where the biologically active ß-phorbols and the inactive α-phorbols differ. It has been suggested that STSPN possesses a region similar to region 2 of phorbol, a hypothesis which has been confirmed experimentally. TPA and STSPN both induced the incorporation of ^{32}P into PC, and the phosphorylation of a 15kDa cytoplasmic protein. STSPN, however was also active as protein kinase inhibitor as it inhibited the TPA-induced phosphorylation of other proteins in the cytoplasm and of a 35kDa membrane protein. Thus, it was concluded that certain effects of phorbol esters may be mediated by region 2, which is also present in STSPN. Such multiplicity of action may complicate interpretation of experimental data. It was suggested that different isozymes of PKC may be selectively activated by one or other of the two regions and may be involved in the observed effects.

The PKC inhibitor sphingosine, cytotoxic to cultured cells (Bell *et al.*, 1988) was able to inhibit the ability of phorbols to induce cellular differentiation (Merrill *et al.*, 1986). It can be appreciated that such contrasting properties may be antagonistic in cancer treatment depending on whether one is attempting to eradicate malignant cells *via* cytotoxic means or by causing terminal differentiation. The anticancer agent adriamycin is able both to activate and inhibit PKC. Enzyme activation derives from the ability to stimulate PI turnover, producing the endogenous activator DAG. Inhibition of PKC, at higher drug concentrations may be a consequence of the ability of adriamycin to alter membrane structure (Tritton and Hickman, 1990).

Thus, accumulating reports demonstrate that it is far from evident whether activation or inhibition of PKC would be therapeutically advantageous. If one considers the multifactorial nature of regulation and transmission of cellular signals, it is unclear whether the manipulation of a single enzyme will be sufficient to disrupt events critical for cell growth. Indeed, we observed only temporary A549 cell growth arrest and subsequent recovery of proliferative potential following interference with normal PKC function. One may speculate that enzyme modulation and modification of the tumour environment could operate in combination to suppress the malignant phenotype. Alternatively, one may argue that modulation of the properties of such a crucial enzyme may not be pharmacologically selective because normal as well as diseased cells could be susceptible to treatment. Such a possibility has been explored during the course of this study. Indeed, samples of liver tissue appeared to have degraded phorbol ester receptor binding sites following treatment of experimental mice with PDBU (discussed in section 4.7).

However, as biological knowledge expands regarding the mode of individual isozyme activation and inhibition, and as subspecies-dependent phosphorylation patterns are deciphered, so one may advance towards enhanced selectivity. The differential inhibition of PKC subspecies has been shown by Pelosin *et al.* (1990). Preincubation of PKC-ß and -γ with PS resulted in loss of activity within 4min. The activity of PKC-α however was not affected by such treatment, suggesting that the molecular organization of the phospholipid sensitive domain of PKC-α may differ from other PKC isotypes. This in turn may confer specificity on the mechanism of action of PKC-α.

Thus, the multiplicity of sites for interaction within PKC and the existence of multiple isoenzymes, displaying subtly unique biological properties and differential tissue expression, offers opportunity for differentially sensitive or specific isozyme modulation and generates potential for a higher degree of selectivity for drug development in the future. Moreover, refined technology which enables a rapid determination of genetic characteristics of individual tumours and allows expansion of knowledge concerning the molecular biology of neoplasia will hopefully secure the potential for greater success in treatment of malignant disease.

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Appendix 1. Contents of serum-supplement: Ultroser G (US).

The serum relacement medium supplement ultroser G was used at a concentration of 2% for the routine subculture of A549 human lung carcinoma cells. A functional breakdown of its contents are given below.

Bovine serum albumin: lipid, hormone and mineral transport: Provides osmotic pressure and buffering capacity.

Cholesterol: membrane biosynthesis.

EGF (impurity): mitogen.

Folic acid.

Free amino acids: cell proliferation.

Glutamine.

Heavy metals: Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, SeO_3^{2+}, Co^{2+}, VO_3^-, $Mo_7O_4^{6-}$ eg enzyme activation.

IGF.

Insulin: promotes uptake of glucose and amino acids by cells.

Lipidic acids: eg linoleic acid, membrane biosynthesis.

Putrescine: polyamine for cell proliferation.

Ribonucleosides (trace).

Steroids.

Thymidine.

Thyroxine: O_2 consumption, energy metabolism, promotes growth and differentiation.

Transferrin: binds Fe^{2+}.

TGF (trace).

Vitamins.

Xanthine.

Appendix 2. Publications.

Abstracts.

Bradshaw, T.D., Dale, I.L., Gescher, A. and Pettit, G.R. Effects of bryostatins (bryos) on protein kinase C (PKC) in A549 human lung carcinoma cells. British Association of Cancer Research: 30th Annual Meeting. Glasgow, 1989.

Dale, I.L., Bradshaw, T.D. and Gescher, A. Comparison of the effects of bryostatins and phorbol esters on protein kinase C activity in A549 human lung carcinoma cells. 6th NCI-EORTC symposium on new drugs in cancer therapy. Amsterdam, 1989.

Dale, I.L., Bradshaw, T.D., Gescher, A. and Pettit, G.R. Effects of bryostatins (bryos) 1 and 2 on protein kinase C activity in A549 human lung carcinoma cells. American Association for Cancer Research: 80th Annual Meeting. San Fransisco, 1989.

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Bradshaw, T.D., and Gescher, A. Effect of serum on modulation of growth by activators of protein kinase C (PKC) in human A549 lung carcinoma cells. American Association for Cancer Research: 81st Annual Meeting. Washington, 1990.

Anderson, L., Cummings, J., Bradshaw, T.D. and Smythe, J.F. Involvement of protein kinase C (PKC) and inositol phosphates (IPs) in sensitive (2780) and resistant (2780^{DOX}) ovarian cell lines. American Association for Cancer Research: 81st Annual Meeting, Washington. 1990.

Papers.

Dale, I.L., Bradshaw, T.D., Gescher, A., and Pettit, G.R. Comparison of effects of bryostatins 1 and 2 and 12-O-tetradecanoylphorbol-13-acetate on protein kinase C activity in A549 human lung carcinoma cells. Cancer Res. 49: 3242-3245, 1989.

Laughton, C.A., Bradshaw, T.D., Gescher, A. Sterically hindered analogues of diacylglycerols. Synthesis, binding to the phorbol ester receptor and metabolism in A549 human lung carcinoma cells. Int. J. Cancer: 44: 320-324, 1989.
