INVESTIGATION OF THE ANTIPROLIFERATIVE PROPERTIES OF TUMOUR PROMOTING PHORBOL ESTERS AND RELATED COMPOUNDS

IAN LESTER DALE

Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

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The University of Aston in Birmingham

Investigation of the antiproliferative properties of tumour promoting phorbol esters and related compounds

Ian Lester Dale
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Tumour promoting phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) exert a multitude of biological effects on many cellular systems, many of which are believed to be mediated via the activation of the enzyme protein kinase C (PKC). TPA and other biologically active phorbol esters inhibited the proliferation of the A549 human lung carcinoma cell line. However, after 5-6 days culture in the continued presence of the phorbol ester cells began to proliferate at a rate similar to that of untreated cells. Resistance to TPA was lost following subculturing, although subculture in the presence of 10 nM TPA for more than 9 weeks resulted in a more resistant phenotype. The selection of a TPA-resistant subpopulation was not responsible for the observed resistance.

The antiproliferative properties of other PKC activators were investigated. Mezerein induced the same antiproliferative effects as TPA but synthetic diacylglycerols (DAGs), the presumed physiological ligands of PKC, exerted only a non-specific cytotoxic influence on growth. Bryostatins 1 and 2 were able to induce transient growth arrest of A549 cells in a manner similar to phorbol esters at nanomolar concentrations, but at higher concentrations blocked both their own antiproliferative action and also that of phorbol esters and mezerein. Fourteen compounds synthesized to mimic features of the phorbol ester pharmacophore and/or DAGs did not mimic the antiproliferative properties of TPA in A549 cells and exerted only a DAG-like non-specific cytotoxicity at high concentrations.

The subcellular distribution and activity of PKC was determined following partial purification by non-denaturing polyacrylamide gel electrophoresis. Treatment with TPA, mezerein or bryostatins resulted in a concentration-dependent shift of PKC activity from the cytosol to cellular membranes within 30 min. Significant translocation was not observed on treatment with DAGs. Chronic exposure of cells to TPA caused a time- and concentration dependent down-regulation of functional PKC activity. A complete loss of PKC activity was also observed on treatment with growth-inhibitory concentrations of bryostatins. No PKC activity was detected in cells resistant to the growth-inhibitory influence of TPA.

Measurement of intracellular Ca^{2+} concentrations using A549 cells cultured on Cytodex 1 microcarrier beads revealed that TPA, mezerein and the bryostatins induced a similar rapid rise in intracellular Ca^{2+} levels.

Keywords: growth inhibition; phorbol esters; protein kinase C; bryostatins; A549 human lung carcinoma.

TO MY PARENTS

WITH LOVE

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
[γ-^{32}P]ATP	adenosine 5'-[γ-^{32}P]triphosphate
BCECF	bis(carboxyethyl)carboxyfluorescein
BM 41.440	1-S-hexadecyl-2-methoxymethyl-rac-glycero-3-phosphocholine
cAMP	adenosine 3'5'-cyclic monophosphate
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	cytosolic calcium concentration
$[Ca^{2+}]_e$	extracellular calcium concentration
cDNA	complementary DNA
CFE	colony forming efficiency
Ci	curies
COS	CV-1 Origin-defective SV40
cpm	counts per minute
DAG	diacylglycerol
d H_2O	distilled water
1,2-diC_8	1,2-sn-dioctanoylglycerol
1,3-diC_8	1,3-sn-dioctanoylglycerol
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor

Et-18-OCH_3	1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine
FCS	foetal calf serum
Fig.	figure
Fura-2	1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methyl phenoxy)-ethane-N,N,N',N'-tetraacetic acid
Fura-2/AM	fura-2 pentaacetoxymethyl ester
g	acceleration due to gravity
G protein	guanine nucleotide binding protein
h	hours
Hepes	N-2-hydroxyethyl piperazine-N'-2-ethane-sulphonic acid
H-7	1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine
IC_{50}	concentration which inhibits cell growth by 50%
Ins 1,4,5P_3	inositol 1,4,5-trisphosphate
Ins 1,3,4,5P_4	inositol 1,3,4,5-tetraphosphate
kDa	kilodaltons
NADH	nicotinamide adenine dinucleotide (reduced form)
LC_{50}	concentration which results in 50% cell death
LDH	lactate dehydrogenase
log	logarithm
min	minute
n	number of experiments
OAG	1-oleoyl-2-acetyl-sn-glycerol
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PC	phosphatidylcholine
PDA	phorbol-12,13-diacetate
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-α-phorbol-12,13-didecanoate
PDGF	platelet derived growth factor
PE	phosphatidylethanolamine
PI-PLC	phosphoinositide-specific phospholipase C
PKC	protein kinase C
PLC	phospholipase C
PS	phosphatidylserine
PtdIns	phosphatidylinositol
PtdIns4P	phosphatidylinositol-4-phosphate
PtdIns4,5P_2	phosphatidylinositol-4,5-bisphosphate
Quin-2	(2-[2-bis-[carboxymethyl]amino-5-methyl-phenoxy)methyl]-6-methoxy-8-bis[carboxy-methyl]aminoquinoline
Quin-2/AM	quin-2 tetrakis[acetoxymethyl]ester
RNA	ribonucleic acid
RPA	12-O-retinoylphorbol-13-acetate
rpm	revolutions per minute
R59022	6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]ethyl]-7-methyl-5 H-thiazolo-[3,2-a] pyrimidin-5-one
s	second
SD	standard deviation
SDS	sodium dodecylsulphate
TCA	trichloroacetic acid

SRI 62-834	[(±)-2-{hydroxy[tetrahydro-2-(octadecyloxy)-methylfuran-2-yl]-methoxy}phosphinyloxy-N,N,-N-trimethylethaniminium hydroxide]
[^{3}H]TdR	5-[methyl ^{3}H]-thymidine
TEMED	N,N,N',N'-tetramethylethylenediamine
Ti_8	12-O-tetradeca(tetra-2.4.6.8-en)oylphorbol-13-acetate
TPA	12-O-tetradecanoylphorbol-13-acetate

All other abbreviations refer to SI units

SECTION 1: INTRODUCTION

SECTION 1: INTRODUCTION

1.1. The need for new strategies in cancer chemotherapy

A major goal of current cancer research is to understand the molecular basis of the uncontrolled growth of neoplastic cells. Although malignant cells are derived from host cells, they are highly aberrant both in structure and behaviour. To varying degrees, cancer cells display a lack of differentiative features, an augmented rate of cell proliferation and failure to maintain normal cell position (Ruddon, 1987).

For an anticancer drug to be effective in the clinic, it must kill malignant tumour cells *in vivo* at doses that allow the survival and recovery of the patient. However, the majority of anticancer drugs exert their activity, at least in part, *via* the production of cytotoxic lesions which are not restricted to the malignant cells and often damage normal host tissues. A novel approach to cancer chemotherapy involves the conversion of malignant cells to differentiated mature cell types which do not proliferate. Therapy that induces the differentiation of tumour cells, rather than therapy which destroys both proliferating normal cells and tumour cells, is attractive (Bloch, 1984; Sartorelli, 1985). A number of studies on murine tumour systems and clinical reports have indicated that certain malignant cells have the ability to spontaneously differentiate to mature cell types which have lost the capacity for proliferation (Pierce, 1970; Brinster, 1974). Therefore, at least in some cell types, the malignant state

is not irreversible.

In normal developmental processes, control over proliferation and differentiation is highly coordinated by protein factors (reviewed by Sachs, 1987). In addition to these natural differentiation factors, a number of compounds have been described which have the ability to induce differentiation. Both physiological agents and a number of diverse exogenous compounds can promote the differentiation of various cell types in culture (reviewed by Sartorelli, 1985).

This thesis is concerned with the antiproliferative properties of phorbol esters and related compounds. Phorbol esters are potent tumour promoters in the mouse two-stage carcinogenesis model (reviewed by Yuspa and Poirier, 1988) and also elicit pleiotropic effects, including growth inhibition and differentiation, in numerous biological systems (reviewed by Nishizuka, 1984; Vandenbark and Niedel, 1984). Elucidation of the nature of the growth arresting interactions between phorbol esters and cells could conceivably lead to the rational design of compounds which mimic the growth-inhibitory properties of phorbol esters without being tumour promoters and also the identification of novel biochemical targets in cancer treatment. Particularly, the identification of the enzyme protein kinase C (PKC) as the major phorbol ester receptor has suggested a potential target for therapeutic intervention in malignant disease. A role for PKC in stimulus-response coupling has been shown for the release, secretion and exocytosis of cellular constituents from a variety of

endocrine and exocrine tissues and for the activation of many other cellular functions (reviewed by Nishizuka, 1986), often as a result of investigations which employed phorbol esters as biochemical tools.

1.2. Phorbol esters as tumour promoters

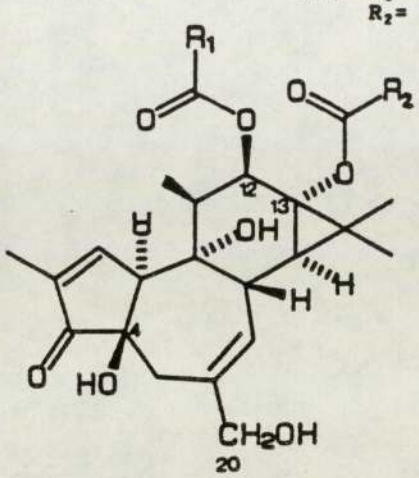
The multistage model of carcinogenesis is now well established as one of the best models available for the investigation of the biochemical and molecular mechanisms involved in carcinogenesis (Blumberg, 1980; Yuspa and Poirier, 1988). The first stage, initiation, comprises an acute subcarcinogenic dose of an "initiator" carcinogen such as 7,12-dimethylbenz[a]anthracene (DMBA). Initiation is rapid, requiring only a single administration of carcinogen (Colburn and Boutwell, 1966). Although no apparent pathological change occurs (Berenblum and Shubik, 1949), the tissue is irreversibly altered (Van Duuren et al, 1975), presumably as the initiator interacts with genomic DNA of normal cells to produce "initiated cells" which are the precursor to the future tumour. Promotion, the second stage, requires the repeated topical application of the promoting compound over several weeks, and results in the appearance of many tumours (Boutwell et al, 1982). Unlike the initiating compound, the promoter is weakly or not carcinogenic when applied to mouse skin alone and its action is reversible, at least in the early stages of promotion (Boutwell et al, 1982). Induction of tumours does not occur if the promoter is administered before the initiating carcinogen (Berenblum and Haran, 1955).

The above protocol constitutes the two-stage, or initiation-promotion, model of tumour formation. The promotion stage has been further subdivided into two different components, Stages I and II, based on differences between the promoting ability of various tumour promoters (Furstenberger et al, 1981; Slaga et al, 1980).

In 1941 Berenblum detected tumour-promoting and irritant properties of croton oil, derived from the plant Croton tiglium, when applied to the skin of mice (Berenblum, 1941). In the 1960s, the laboratories of Hecker and Van Duuren independently reported the isolation and characterisation of various tumour-promoting compounds from croton oil. All of these substances were esters of a diterpene parent compound, phorbol. Phorbol derivatives esterified at positions 12- and 13- were found to be responsible for the tumour promoting, inflammatory and skin irritant properties of croton oil whereas phorbol and phorbol monoesters were inactive (Hecker, 1968; Van Duuren et al, 1965). The structure of the most potent tumour-promoting phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), is shown in Figure 1. There are many other phorbol esters with varying degrees of promoting, inflammatory and irritating properties (Slaga et al, 1978). Studies with these agents have formed the basis for virtually all of the current hypotheses on the molecular events in tumour promotion.

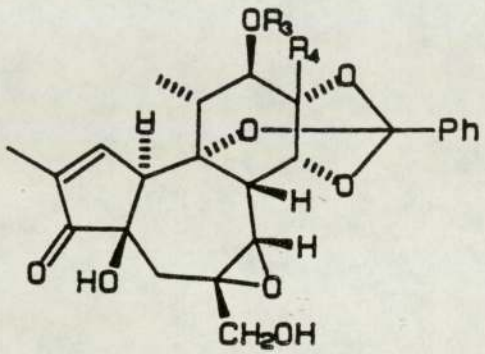
In view of the biological and biochemical pleotropism exhibited by irritant promoters of the diterpene type, such as the phorbol esters, much research effort has been directed towards the investigation of structure-activity

TPA: R_1= $-(CH_2)_{12}CH_3$
R_2= $-CH_3$

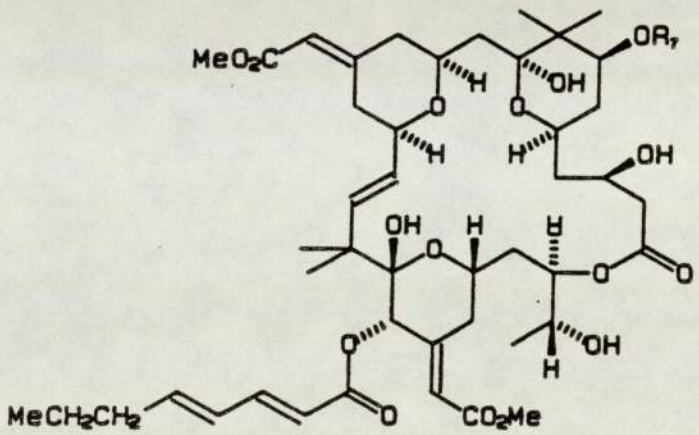


Phorbol ester

R_3= $-(CH=CH)_2Ph$
R_4= $-C(CH_3)=CH_2$



Mezerein

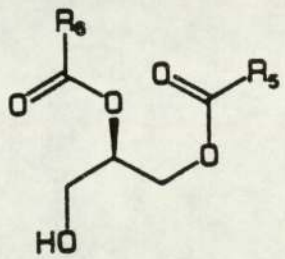


Bryostatin 1: R_7= $-COCH_3$
Bryostatin 2: R_7= $-H$

Bryostatin

OAG: R_5= $-(CH_2)_7CH=CH(CH_2)_7CH_3$
R_6= $-CH_3$

DiC_8: R_5=R_6= $-(CH_2)_6CH_3$



DAG

Figure 1. Structures of PKC activators used in this study.

relationships of these compounds. Changes of the phorbol moiety of TPA usually resulted in greatly reduced irritant and tumour-promoting properties-for example, oxidation of the 20-hydroxyl to an acid, ether formation at the 4-position or any change of the carbon skeleton, including formation of the 4-α-stereoisomer by epimerization. However, other chemical alterations such as oxidation of the 20-hydroxyl to an aldehyde or deoxygenation of the 4-hydroxyl had little effect (Slaga et al, 1978).

The ester moieties at the 12- and 13- positions of phorbol were found to be essential for biological activity. Of the "symmetric" phorbol diesters, in which the same fatty acids are in both positions, irritant, tumour promoting and biological activities increased with increasing length of the saturated fatty acid chain up to the didecanoate derivative. Phorbol esters with ester moieties derived from unsaturated fatty acids, such as Ti_8, displayed increased irritancy but reduced tumour-promoting activity when compared with their saturated analogues. Among "nonsymmetric" phorbol esters, with an acetate group in the 13- position and saturated fatty acids with increasing chain length in the 12- position, TPA was the most potent derivative. TPA was somewhat more active than the most potent symmetric diester, PDD (Driedger and Blumberg, 1980a). Interestingly 12-O-retinoylphorbol-13-acetate (RPA), in which the tetradecanoyl moeity of TPA was replaced by a retinoyl moeity, was found to be almost as potent as TPA in its irritant properties yet was active only as a second stage tumour promoter and not as a first stage

promoter (Furstenberger et al, 1981). To date, structure-activity relationships appear to indicate that both the diterpene moeity and the ester moeity are essential for interaction with the cellular target molecule(s).

It appears that the lipophilicity of diterpene esters is also an important determinant of their biological activity. However, small structural alterations were found to affect biological activity greatly without altering lipophilicity, indicating that other factors are also important.

A large number of irritant diterpene esters which have similar structures to phorbol esters have been identified (reviewed by Hecker, 1985). Mezerein, whose structure is illustrated in Figure 1, induced cellular proliferation and irritation of mouse skin as effectively as TPA (Mufson et al, 1979) but it was only active as a second stage tumour promoter (Slaga et al, 1980). Resiniferatoxin, an ortho ester of resiniferonol was highly irritant but inactive as a mouse skin tumour promoter (Driedger and Blumberg, 1980). Although structurally unrelated to phorbol esters, the indole alkaloids teleocidin (Fujiki et al, 1979) and lyngbyatoxin (Cardellina et al, 1979) and the polyacetate compound aplysiatoxin (Horowitz et al, 1983) have also been found to induce the same spectrum of biological responses as do the phorbol esters.

Computer modelling studies have been used to investigate how such structurally diverse compounds can have essentially the same activity as TPA. In 1986, two models were proposed by superimposing teleocidins and phorbols based on the superposition of atomic positions and chemical structures

(Jeffrey and Liskamp, 1986; Wender et al, 1986). From these studies, a number of structural features for biological activity were predicted. However, a recent study in which molecules were superimposed in terms of physical and chemical properties showed poor agreement with the two previously published models (Itai et al, 1988). It is clear that further information on structure-activity relationships is required before the essential common structural features of these compounds can be identified.

1.3. The phorbol ester receptor

The lipophilic nature of phorbol esters had suggested to investigators that their primary site of action was the cell membrane and several kinetic studies with various cell types supported this theory. The biological potency of phorbol esters, capable of inducing responses at nanomolar concentrations, and the correlation of biological activity with tumour-promoting ability of a series of phorbol ester analogues indicated that the modulation of cellular responses resulted from interaction at specific cellular binding sites (reviewed by Blumberg, 1980; Ashendel, 1985).

Driedger and Blumberg (1980b) developed a specific receptor ligand binding assay using [^{3}H]PDBu, which is a potent tumour promoting phorbol ester but much less lipophilic than TPA. They were able to demonstrate specific phorbol ester receptors in chick embryo fibroblasts in culture (Driedger and Blumberg, 1980b) and in the particulate fraction of mouse skin (Delclos et al, 1980). Typical of receptor-ligand interactions, binding was rapid, saturable,

reversible, of high affinity, and linearly related to the amount of protein in the assay. Binding of [^{3}H]PDBu to the receptor was competitively inhibited by biologically active phorbol esters (Driedger and Blumberg, 1980b), related plant diterpene esters such as mezerein (Shoyab and Todaro, 1980) and teleocidin and aplysiatoxin (Horowitz et al, 1983). The presence of Ca^{2+} increased the affinity of [^{3}H]PDBu for the receptor by a mechanism which did not involve competition for the binding site (Dunphy et al, 1981).

After the demonstration of specific binding of phorbol esters to receptors, studies were undertaken to determine the distribution of the receptor in tissues and among species. In broken cell and tissue preparations the binding activity was predominantly in the particulate fraction (Dunphy et al, 1981). However, an aporeceptor was demonstrated in the cytosol of tissue homogenates which required Ca^{2+} and phosphatidylserine (PS) in order to exhibit binding activity (Ashendel et al, 1983a; Leach et al, 1983).

[^{3}H]PDBu was used to detect specific receptors in virtually all cells and tissues tested, including cells in culture (Blumberg et al, 1982). In the rat, the receptor was found in every tissue tested with the highest level in the brain and was also detected in several animal phyla, for example in primitive organisms such as the earthworm and snail (Kuo et al, 1980) but not in bacteria (Driedger and Blumberg, 1980b). This suggested that the receptor performed a biochemical function basic to multicellular life.

Despite the multitude of investigations into the

interactions of phorbol esters with biological systems, the biochemical nature of the major phorbol ester receptor remained unclear until the early 1980s. However, it is now apparent that many of the effects of tumour promoters are a result of their ability to modulate the activity of the enzyme PKC, a key component in cellular signal transduction induced by the breakdown of polyphosphoinositides.

1.4. The phosphoinositide signalling pathway

A full review of the phosphoinositide signalling pathway is beyond the scope of this thesis, and several recent reviews have dealt comprehensively with this subject (for example, Nishizuka, 1984; Berridge, 1984; Berridge, 1985; Nishizuka, 1986; Kikkawa and Nishizuka, 1986; Berridge, 1987). A brief outline of the phosphoinositide cycle is given below and illustrated in Figure 2.

The phosphoinositides are a quantitatively minor component of the mammalian cell membrane phospholipid. Uniquely among membrane phospholipids, phosphatidylinositol (PtdIns) can be further phosphorylated to phosphatidylinositol-4-phosphate (PtdIns4P) and phosphatidylinositol-4,5-bisphosphate (PtdIns4,5P_2). It is now widely accepted that phosphoinositide breakdown by a wide range of extracellular messengers which activate cellular functions and proliferation represents a major signalling system to the cell, analogous to but distinct from the cAMP-adenylate cyclase system (Nishizuka, 1984; Berridge, 1987).

The signal transduction unit within the plasma membrane consists of (1) a receptor which detects the incoming

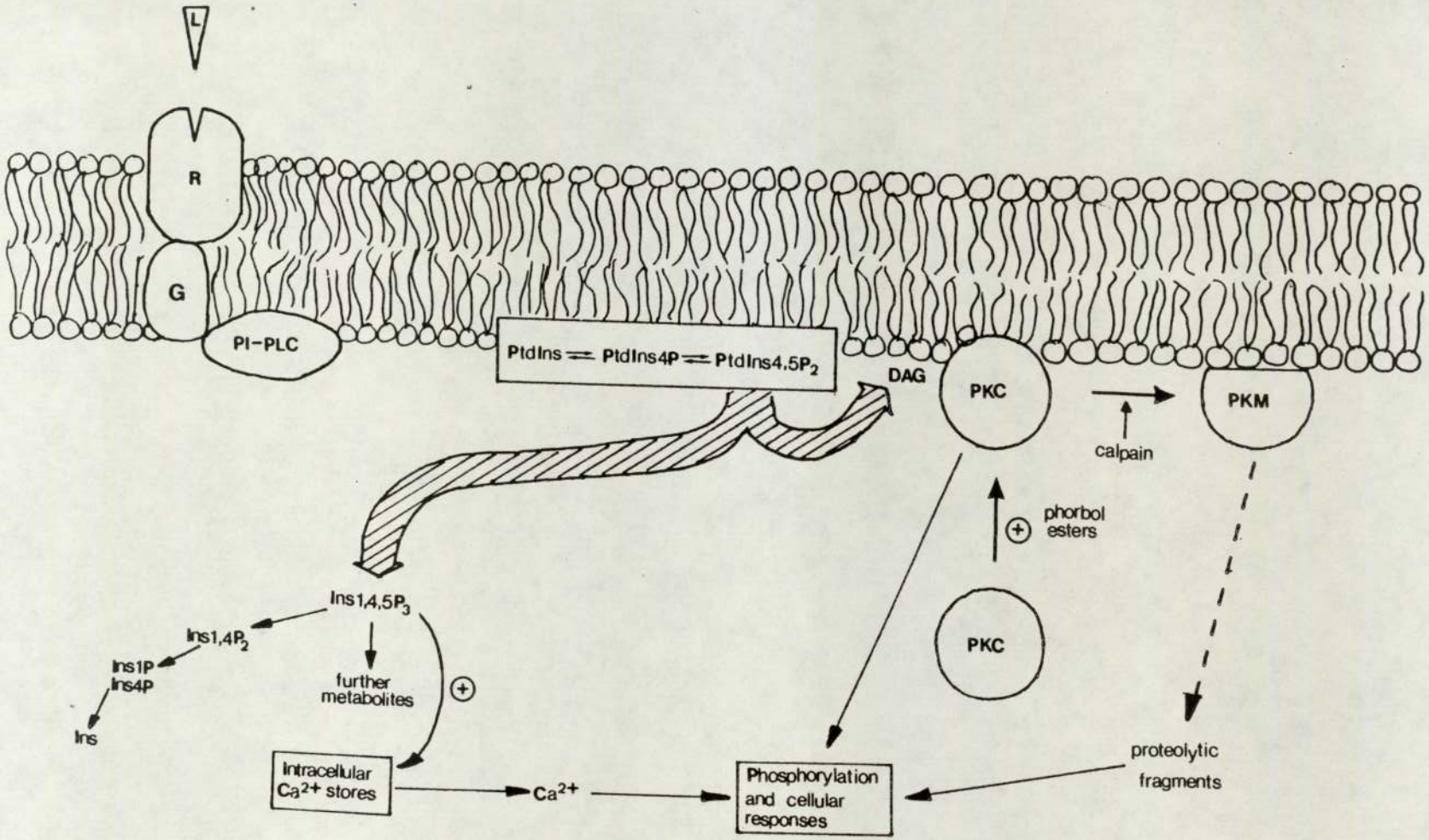


Figure 2. The phosphoinositide signalling pathway. Binding of ligand (L) to its receptor activates a G protein (G) which then stimulates the activity of PI-PLC. Hydrolysis of PtdIns4,5P_2 by PI-PLC results in the formation of Ins 1,4,5P_3, which releases Ca^{2+} from internal stores, and DAG, which activates PKC. See text for further details.

signal, (2) a G protein which couples the receptor to the third component, and (3) a phosphoinositide-specific phospholipase C (PI-PLC) responsible for the hydrolysis of the phosphoinositide (Berridge, 1987). Originally PtdIns was considered to be the important substrate (Hokin and Hokin, 1953) but it now appears that the major target is PtdIns4,5P_2, as an increased rate of hydrolysis of PtdIns4,5P_2 has been demonstrated in many different cell types in response to a variety of external stimuli (Berridge, 1987). Breakdown of PtdIns4,5P_2 yields two second messengers, inositol 1,4,5-trisphosphate (Ins 1,4,5P_3) and diacylglycerol (DAG), thus forming a bifurcating signal pathway for transferring information into the cell.

Using permeabilized pancreatic cells, Berridge and coworkers demonstrated that Ins 1,4,5P_3 functions to release Ca^{2+} from the endoplasmic reticulum (Streb et al, 1983; Berridge et al, 1984). An increase in the intracellular level of Ca^{2+} has been implicated in the control of growth of many different cell types (Boynton et al, 1974; Metcalfe et al, 1980). Measurement of intracellular Ca^{2+} levels with fluorescent probes such as quin-2 have suggested that a number of growth factors act, at least in part, by releasing Ca^{2+} from intracellular stores (Moolenaar et al, 1984; Hesketh et al, 1985).

As in many tissues PtdIns and PtdIns4P are also simultaneously hydrolysed in response to external stimuli, it has been proposed that they may play a role in signal transduction. Since PtdIns and PtdIns4P would release

inositol phosphates that do not mobilize Ca^{2+}, this would bias the signal pathway towards the DAG route and away from Ca^{2+} (Berridge, 1987). Recently, the number of inositol phosphates known to exist naturally has proliferated and it now stands at over 20 (reviewed by Michell, 1986; Irvine et al, 1988). It is clear that the metabolism of inositol phosphates is highly complex. Ins 1,4,5P_3 can be further phosphorylated to Ins 1,3,4,5P_4, now believed to modulate the action of Ins 1,4,5P_3, and these two compounds are further metabolized by a number of pathways after their inactivation by a specific 5-phosphatase. In addition, a number of inositol pentakis- and hexakisphosphates have also been reported in animal cells, as have cyclic inositol polyphosphates. The possible physiological significance of most of these inositol phosphates remains unclear (reviewed by Irvine et al, 1988).

DAG, the other second messenger derived from hydrolysis of phosphoinositides, is normally absent from membranes. Typical of a second messenger, DAG is produced only transiently following receptor stimulation as it is rapidly metabolised, either by phosphorylation to phosphatidic acid by DAG kinase before conversion back to inositol phospholipids or by lipase-mediated hydrolysis (reviewed by Nishizuka, 1986). The importance of DAG in signal transduction was only realized with the discovery and characterization of the enzyme PKC.

Interest in the phosphoinositide pathway has been further heightened by the finding that, in addition to the phorbol esters, a number of oncogenes and transforming gene products

appear to act *via* this pathway. For example, it was found that the sis oncogene produced PDGF (Waterfield *et al*, 1983), a potent activator of phosphoinositide hydrolysis (Berridge *et al*, 1984; Habenicht *et al*, 1981). The erb B gene is related to the EGF receptor except that the external EGF binding site is missing (Downward *et al*, 1984). It is believed that the EGF and phosphoinositide signalling pathways operate, at least in part, by activating the same second messengers (reviewed by Berridge *et al*, 1985). The ras gene product might function as an unregulated G protein to couple surface receptors to PI-PLC (Berridge and Irvine, 1984). It has also been proposed that the transforming gene products $pp60^{v\text{-}src}$ and $pp68^{v\text{-}ros}$ possess PtdIns kinase activity and increase phosphoinositide turnover *in vivo* (Macara *et al*, 1984; Sugimoto *et al*, 1984), although this is disputed by other workers (MacDonald *et al*, 1985).

1.5. Protein kinase C

1.5.1. Some biological properties

The importance of protein phosphorylation in the generation and transmission of signals in the cell has long been recognised. Protein kinases and their complementary protein phosphatases are able to modulate the activity of proteins in a rapid and reversible manner (reviewed by Greengard, 1978; Hunter, 1986). With the advent of molecular cloning techniques the number of proven or putative protein kinases has now risen to nearly 100, and the finding that a large number of growth factors and oncogenes, such as the EGF and insulin receptors and src, abl and fps gene products are

protein kinases, indicates the important role that protein phosphorylation plays in the cell (Hunter, 1987).

PKC was first described in 1977 by Nishizuka and coworkers as a proteolytically activated protein kinase (Inoue et al, 1977), but was later demonstrated to be a calcium-activated, phospholipid-dependent protein kinase (Takai et al, 1979a). The enzyme was initially implicated as a mediator of the effects of a wide variety of inositol lipid turnover agonists when it was found that DAG is able to directly activate the enzyme (Takai et al, 1979b; Kishimoto et al, 1980). In the presence of DAG, low Ca^{2+} concentrations and a phospholipid such as phosphatidylserine (PS) the kinase activity of PKC becomes activated. The Ca^{2+} concentrations required are in the micromolar range, which is physiological, although the enzyme can also be activated in the absence of DAG, but in the presence of PS, when the Ca^{2+} levels are millimolar. Thus, the role of DAG is to dramatically increase the affinity of the enzyme for Ca^{2+}, thereby rendering it fully active without a net increase in the Ca^{2+} concentration (Kishimoto et al, 1980; Kaibuchi et al, 1981). However, the enzyme can also be activated by the synergistic action of an increase in Ca^{2+} and the formation of DAG.

The DAG chemical structure is illustrated in Figure 1. In vitro, all DAGs able to bind to and activate the kinase have a 1,2-sn configuration, and neither the sn-2,3-DAG enantiomer nor the 1,3-DAG diastereomer are active (Rando and Young, 1984; Boni and Rando, 1985). The observed stereospecificity has also been demonstrated in vivo (Nomura

et al, 1986), suggesting that a highly specific lipid-protein interaction is needed for the activation of PKC.

A number of studies have investigated the interaction between DAG and PKC by means of structure-activity relationships. Virtually any modification of the glycerol backbone of DAG results in a strong decrease in activity, such as the addition of a single carbon atom in the glycerol backbone (Rando, 1988) or modification of the hydrophilic residues of the DAG, for example the replacement of the 3-hydroxyl group by other moeities (Ganong et al, 1986). In contrast, it seems clear that little specificity is associated with the fatty acyl chains of the DAG, as long as the diacyl chains are sufficiently hydrophobic to allow intercalation into membranes (Mori et al, 1982; Lapetina et al, 1985; Go et al, 1987). The relative positions of the fatty acyl chains does not appear to be important as the 1-acetyl-2-oleoyl-sn-glycerol is as active as 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Mori et al, 1982). The endogenous DAGs that activate the kinase are thought to contain saturated fatty acids at the 1 position and unsaturated fatty acids (usually arachidonic acid) at the 2 position (Kikkawa and Nishizuka, 1986). Synthetic DAGs such as OAG and 1,2-sn-dioctanoylglycerol (1,2-diC_8) (see Figure 1), which are more hydrophilic than physiologically present DAGs but still sufficiently hydrophobic to partition into the cell membrane, are normally employed to probe the link between phosphoinositide breakdown and PKC activation (discussed further in Section 1.5.2).

PS is absolutely required for the enzyme activation; other phospholipids such as phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin are unable to support kinase activation except when mixed with PS to form active complexes (Boni and Rando, 1985). Recent studies seem to indicate that the phospholipid requirement of PKC is almost certainly not based on specific protein-lipid interactions (reviewed by Rando, 1988).

PKC is widely distributed in tissues and organs of mammals and other organisms (Kuo et al, 1980; Minakuchi et al, 1981). In the rat the spleen, brain and vas deferens were found to contain the highest level of enzyme activity, whereas skeletal and cardiac muscle and fat tissue contained the lowest (Kuo et al, 1980). In many tissues PKC activity far exceeds that of cAMP-dependent protein kinase. Human neutrophils and leukaemic cells are also exceptionally rich sources of the enzyme (Helfman et al, 1983a; Helfman et al, 1983b). PKC has been purified to apparent homogeneity from a number of species (Kikkawa et al, 1982; Wise et al, 1982; Shoyab and Boaze, 1984; Kikkawa et al, 1986). The purified protein consists of a single polypeptide chain with a molecular weight of 77-85 kDa and appears to be composed of two separate domains. This holoenzyme can be cleaved by a Ca^{2+}-dependent protease (calpain I) to produce two fragments with molecular weights of 51 and 26 kDa (Kikkawa et al, 1982; Kishimoto et al, 1983). The larger fragment is hydrophilic and contains the catalytic domain, but is active in the absence of Ca^{2+}, phospholipid and DAG (Kikkawa et al, 1982) while the smaller hydrophobic domain possesses the

regulatory and membrane-binding functions (Nishizuka, 1986). PKC localized at the membrane is more susceptible to this limited proteolysis (Kishimoto et al, 1983). The physiological significance of this reaction remains to be established, although it may take part in the down-regulation of PKC itself (see below).

The enzymic reaction catalyzed by PKC is the transfer of the γ-phosphate group of ATP to seryl and threonyl, but not tyrosyl, residues of proteins (Nishizuka, 1986; Kikkawa and Nishizuka, 1986). Like many other protein kinases, the enzyme phosphorylates itself in the presence of Ca^{2+}, phospholipid and DAG, although the significance of this autophosphorylation is unknown. Identification of the important substrates of PKC is essential for an understanding of the mechanism(s) of action of this signalling pathway. However, in vitro studies have shown that many proteins are efficiently phosphorylated by PKC, of which few are likely to be physiologically relevant substrates (reviewed by Ashendel, 1985; Nishizuka, 1986; Kikkawa and Nishizuka, 1986). Identification of biologically important target substrates of PKC requires evidence that they are phosphorylated in vivo under conditions in which PKC becomes activated. Phosphorylation of the epidermal growth factor (EGF) receptor, resulting in a decreased number of high-affinity binding sites and reduced tyrosine-specific protein kinase activity of the receptor, is one of the few examples in which phosphorylation by PKC appears to result directly in an altered biological response (Cochet et al, 1984).

1.5.2. PKC as the major phorbol ester receptor

As characterisation of the phorbol ester receptor by Blumberg and others proceeded in the 1970s (reviewed by Blumberg, 1980) it became increasingly clear that there were marked similarities between the receptor and PKC, which at that time was being independently characterised by Nishizuka et al (reviewed by Nishizuka, 1984). These similarities included tissue distribution, absolute level in the brain, evolutionary conservation, high Ca^{2+} sensitivity and phospholipid association. It is now clear that the phorbol ester binding assays developed by Blumberg and coworkers and the PKC enzymatic assays of Nishizuka and coworkers were measuring different functional activities of the same protein.

A crucial finding was reported in 1982, when it was demonstrated that phorbol esters were able to bind to and activate PKC in a manner similar to DAG, since they also reduced the Ca^{2+} requirement and increased the maximal enzyme activity, but did not further activate PKC which had been maximally activated by DAG. Furthermore, the ability of a series of phorbol esters to activate PKC correlated with their efficacy in binding to the phorbol ester receptor (Castagna et al, 1982). A number of laboratories were subsequently able to copurify, ultimately to homogeneity, phorbol ester binding activity and PKC activity, thus proving that the two activities were mediated by the same protein (Ashendel et al, 1983b; Kikkawa et al, 1983b; Leach et al, 1983; Niedel et al, 1983; Sando and Young, 1983).

An important feature of the currently accepted view of the

mechanism of activation of PKC by agonists is the "mobile-receptor" hypothesis. Treatment of cells with phorbol esters was observed to cause translocation of the enzyme from the cytosol to cellular membranes (Kraft and Anderson, 1983). It has been proposed that cytosolic PKC is inactive but becomes activated following translocation to membranes in the presence of agonist. If PKC is only active when associated with membranes, the implication is that the enzyme can phosphorylate only membrane proteins (Nishizuka, 1984). However,the exact intracellular localization of PKC is unclear because the enzyme, believed to be loosely bound to membranes in the presence of Ca^{2+}, is extracted for assay in the presence of a strong Ca^{2+} chelator in order to prevent Ca^{2+}-dependent proteolysis (Kikkawa and Nishizuka, 1986).

Chronic exposure of phorbol esters to cells leads to the loss or down-regulation of PKC activity (Rodriguez-Pena and Rozengurt, 1984; Blackshear et al, 1985) as the prolonged association of activated PKC at the membrane initiates the proteolytic degradation by calpain of the PKC molecule and its sustained disappearance from the cell (Kishimoto et al, 1983; Young et al, 1987). The physiological relevance of down-regulation of PKC activity is unclear but may play a role in determining cellular responsiveness (see Murray et al, 1987).

The identification of PKC as a major target of phorbol esters generated much scientific interest because it suggested that these compounds were acting through the phosphoinositide pathway, already established as one of the

major signal transduction mechanisms within cells (see Section 1.4). The ability of phorbol esters to directly activate PKC without the stimulation of phosphoinositide breakdown helped to rationalize the ability of these compounds to induce many diverse biological responses within cells (Nishizuka, 1984).

The demonstration by Nishizuka and coworkers that DAGs could stimulate PKC activity (see Section 1.5.1) suggested that DAG could be the endogenous phorbol ester analogue (reviewed by Nishizuka, 1984). Indeed, DAGs were found to be competitive inhibitors of phorbol ester binding to PKC, indicating that they share a common binding site on the kinase (Sharkey et al, 1984; Hannun et al, 1985). However, phorbol esters are much more potent than DAGs as PKC activators. For example, the ability of TPA to bind to PKC is four orders of magnitude greater than that of the analogously substituted DAG glycerol 1-myristate 2-acetate (Sharkey and Blumberg, 1986). When coupled with the fact that, unlike DAGs, phorbol esters are not readily metabolized in biological systems (Welsh and Cabot, 1987), this explains the potency of phorbol esters as agonists of PKC and inducers of physiological effects.

Nishizuka (1984) proposed that phorbol esters such as TPA have a molecular structure that in part is very similar to that of DAG which allows them to mimic DAG in the activation of PKC. However, structurally diverse tumour promoters such as mezerein, teleocidin and aplysiatoxin, which are known to induce many of the biological responses associated with phorbol esters, were also potent activators of PKC (Miyake

et al, 1984; Fujiki et al, 1984). The ability of such structurally diverse molecules to bind to and activate the enzyme suggests that a DAG-like structure is not always essential. Nevertheless, at least for some of the molecules, inspection of molecular models has revealed plausible structural similarities (discussed in Section 1.2).

Direct evidence supporting a second messenger function for DAG in vivo has emerged from studies using cell permeable DAGs such as diC_8 and OAG (reviewed by Ashendel, 1985; Bell, 1986). Thus, for example, in human fibroblasts 1,2-diC_8 stimulates the phosphorylation of the EGF receptor at threonine 654 (Davis et al, 1985), consistent with PKC phosphorylating the same site on the purified EGF receptor in vitro (Cochet et al, 1984; Davis et al, 1985). Other in vivo examples of the ability of DAGs to mimic phorbol ester responses include mitogenesis of 3T3 fibroblasts (Rozengurt et al, 1984), superoxide production by neutrophils (Fujita et al, 1984) and mimicry of some of the biochemical changes associated with tumour promotion by TPA in mouse epidermis, such as the induction of ornithine decarboxylase activity (Smart et al, 1986). Similarly, other methods which elevated DAG levels in cells, such as treatment with bacterial PLC to degrade membrane phospholipids or blockage of DAG metabolism with the DAG kinase inhibitor R59022, led to phorbol ester-like effects (Jeng et al, 1985; de Chaffoy de Courcelles et al, 1985). However, in other systems DAGs were unable to mimic phorbol ester action, such as in the induction of differentiation of the U937 monoblastoid cell

line (Ways et al, 1987). Yamamoto et al (1988) found the growth of the A65T mouse thymic leukaemia cell line to be strictly dependent on the presence of tumour promoters such as TPA and mezerein. However, cells cultured in the presence of the DAGs OAG or 1,2-dicaprylin did not proliferate. Further, TPA and DAG mutually stimulated the phosphorylation of completely different sets of endogenous proteins. Verma (1988) has investigated the potency of 1,2-diC_8 as a tumour promoter in mouse skin. The DAG was found to mimic mezerein and RPA as an incomplete (Stage II) tumour promoter but, unlike TPA, was not a complete nor a Stage I tumour promoter (see Section 1.2). The dissimilar tumour-promoting activities of TPA and 1,2-diC_8 indicate that PKC activation may not be sufficient for tumour promotion by TPA (Verma, 1988).

The hypothesis that DAGs parallel TPA in inducing the monocytic differentiation of the HL-60 human promyelocytic leukaemia cell line has been tested in work reported in several recent papers. In 1985, Bell and coworkers reported that bihourly treatment with 1,2-diC_8 resulted in the differentiation of HL-60 cells to cells with morphological characteristics of macrophages (Ebeling et al, 1985). However, two conflicting reports found that OAG was incapable of inducing differentiation (Kreutter et al, 1985; Yamamoto et al, 1988). More recently, Morin and coworkers were unable to induce differentiation on treatment with 1,2-diC_8 alone, although concurrent exposure of cells to both 1,2-diC_8 and the Ca^{2+} ionophore A32187 resulted in a measurable increase in the fraction of cells expressing a

differentiated phenotype (Morin et al, 1987).

Although evidence supporting the role of PKC as the major phorbol ester receptor is very strong, there is no proof at present that the enzyme is the sole target of tumour promoters. The heterogeneity of responses of phorbol esters, other tumour promoters and DAGs in biological systems renders it unlikely that all their activities can be explained by a single, homogeneous class of receptors. It is possible that the recent demonstration of PKC to be a family of discrete proteins can account, at least in part, for the observed varied responses (reviewed by Nishizuka, 1988).

1.5.3. Molecular heterogeneity of PKC

Although once thought to be a single entity, it is now apparent that PKC is in fact a family of enzymes with several subspecies having closely related structures (reviewed by Kikkawa et al, 1988; Nishizuka, 1988; Parker et al, 1988).

The complete primary sequences of four homologous subspecies, α, β_I, β_{II} and γ, initially emerged from sequence analysis studies of the cDNAs isolated from bovine (Coussens et al, 1986; Parker et al, 1986), rat (Housey et al, 1987; Knopf et al, 1986; Ono et al, 1986), rabbit (Ohno et al, 1987) and human (Coussens et al, 1986) brain libraries. The β_I and β_{II} subspecies, which differ from each other only in a short sequence in their carboxy-terminal regions, were shown to be derived from the alternative splicing of a single RNA transcript (Ono et al, 1987b). A remarkable feature of the sequences of each

subspecies in different animals is the high degree of conservation.

The α, β_I, β_{II} and γ subspecies consist of a single polypeptide chain with four conserved (C_1-C_4) and five variable (V_1-V_5) regions. The conserved regions C_1 and C_2 in the amino-terminal region of the protein are believed to be the regulatory domain which interact with DAG, Ca^{2+} and phospholipid. The region C_1 contains a cysteine-rich repeat sequence similar to the "zinc finger" found in many metalloproteins and DNA-binding proteins (Berg, 1986). Although there is no evidence that PKC can bind to DNA, a recent report has indicated that zinc can both activate and contribute to the binding of PKC to plasma membranes (Csermely et al, 1988). The conserved regions C_3 and C_4 located in the carboxy-terminal region of the molecule appear to be essential for catalytic activity, and region C_3 contains an ATP-binding sequence which has been found in many other protein kinases (Edelman et al, 1987; Hunter and Cooper, 1985).

Very recently, Nishizuka's group have isolated a further three members of the family, termed δ, ε and ζ, from a rat brain library. These subspecies are related to, but clearly distinct from, the other four subspecies (Ono et al, 1987a). These three subspecies also contain the putative "zinc finger" region, although the ζ subspecies has only one such sequence, but do not retain the conserved region C_2 (Nishizuka, 1988).

Ohno et al (1988) have reported the characterisation of a subspecies from rabbit brain, termed nPKC, which is

regulated by phospholipid, DAG and phorbol ester but independent of Ca^{2+}. nPKC exhibits almost complete sequence homology with the ε subspecies from rat brain characterised by Nishizuka's group (Ono et al, 1987a) and would appear to be the corresponding rabbit brain ε subspecies.

Upon chromatography on an hydroxyapatite column, PKC from brain tissue could be resolved into three subfractions, types I, II and III (Huang et al, 1986; Kikkawa et al, 1987; Ono et al, 1987b). Comparison of the subspecies expressed in mammalian COS (CV-1 Origin-defective SV40) cells that were transfected by the respective cDNAs allowed the identification of the relationship between the enzyme subfractions and cDNAs. Type I corresponds to the γ-subspecies; Type II is an unequal mixture of β_I and β_{II} subspecies; and Type III corresponds to α-cDNA (Kikkawa et al, 1987; Ono et al, 1987b). Rather less is known about the new additional members of the family, and the way in which these subspecies fractionate from hydroxyapatite columns remains to be determined.

The existence of a number of PKCs suggests that they may play different biological roles. One would therefore expect to be able to detect differences in their enzymatic properties. Indeed, it is already apparent that the enzyme types I, II and III (that is, γ, β_I + β_{II} and α) display certain differences in their modes of activation, kinetic properties and substrate specificities Nishizuka, 1988; Kikkawa et al, 1988). For example, the α, β_I and β_{II} subspecies are strongly activated by 1-stearoyl-2-arachidonylglycerol, the major endogenous DAG

derived from phosphoinositide breakdown, whereas synthetic DAGs such as 1,2-diC_8 are more effective activators of the γ subspecies than the α and β subspecies. Both β subspecies show substantial activity in the absence of Ca^{2+} but are poorly activated by free arachidonic acid. The γ form, on the other hand, is significantly activated by micromolar concentrations of arachidonic acid and the α subspecies responds to higher concentrations, but only in the presence of high Ca^{2+} (Sekiguchi et al, 1987). Interestingly, activation by free arachidonic acid does not require phospholipids.

In addition to the subspecies described above, a number of structurally undefined enzymes from tissues such as lung and platelets respond to the phospholipid, DAG and Ca^{2+} cofactors in different ways (Kosaka et al, 1988), indicating that other members of the PKC family remain to be identified.

With the use of a combination of biochemical, immunological and cytochemical methods using subspecies-specific antibodies, the tissue distribution and relative activity of multiple PKC subspecies, particularly α, β and γ, have been assessed in rat brain and several other tissues (Kikkawa et al, 1987; Kitano et al, 1987; Shearman et al, 1987). In the rat the γ subspecies is found only in the brain and spinal cord, with the highest specific activity being expressed in the cerebral cortex and hippocampus (Shearman et al, 1987). The β_I and β_{II} subspecies are expressed in many tissues such as liver, kidney and spleen in variable ratios, β_{II} expression usually being much greater than that of β_I

Nishizuka, 1988). The α subspecies is the most widely distributed form and has been detected in many tissues and cells (Ido et al, 1987a; Kikkawa et al, 1987; McCaffrey et al, 1987). The remaining subspecies have not been characterised to the same extent, but δ and γ appear to be expressed in many tissues whereas ξ is possibly localized in brain tissue (Nishizuka, 1988). Most cell types contain more than one subspecies of PKC.

In addition to the differing distributions of the various PKC isozymes, the expression of the subspecies in rat tissues appear to be differently controlled during development (Hashimoto et al, 1987; Yoshida et al, 1988).

It is worth noting that several forms of PI-PLC have also been purified that are immunologically unrelated and that differ in their molecular properties (Ryu et al, 1987a, 1987b). Like PKC, PI-PLC lies at a critical point in the bifurcating phosphoinositide signalling pathway, and there is consequently much interest in the nature of this enzyme(s) and the means by which it is coupled to receptor activation. Recent reports describing the complete primary structures of several forms of the enzyme have suggested that there is significant sequence homology between PI-PLC and the products of various tyrosine kinase-related oncogenes, raising the possibility that PI-PLC and cytoplasmic tyrosine kinases are modulated by common cellular component(s) (Katan et al, 1988; Stahl et al, 1988; Suh et al, 1988). It seems clear that the molecular heterogeneity of both PKC and PI-PLC is important in the generation of the many diverse cellular responses induced by

phosphoinositide turnover.

1.6. Growth-inhibitory properties of phorbol esters

1.6.1. Modulation of growth by phorbol esters

Although phorbol esters were initially the subject of study as a result of their potency as tumour promoters in mouse skin, the observation that they produce a number of biological and biochemical changes in many cultured cells (reviewed by Blumberg, 1980; Diamond et al, 1980) led to much research into their biological mechanism(s) of action. However, as this thesis is focused on the growth-inhibitory properties of these compounds, this section will review only the influence of phorbol esters on cell growth. In many cultured cell lines treatment with TPA results in profound changes in growth, such as the induction of mitogenesis (reviewed by Diamond et al, 1980), induction or inhibition of differentiation (reviewed by Vandenbark and Niedel, 1984) and inhibition of growth (reviewed by Gescher, 1985).

In this thesis the A549 cell line has been used as a model system in the investigation of the growth-inhibitory properties of phorbol esters.

1.6.2. The A549 human lung carcinoma cell line

The A549 human lung carcinoma cell line was initiated in 1972 from explant culture of an alveolar cell carcinoma (Girard et al, 1973). The cell line has been proposed as a type II alveolar cell model (Lieber et al, 1976), the lung cell type which produces, stores and secretes pulmonary surfactant, the phospholipid-rich material which lines the alveolar surface (reviewed by Rooney, 1985). However, doubt

as to the suitability of the A549 cell line as a type II cell model (Mason and Williams, 1980; Rooney et al, 1977) and the advent of procedures for the successful isolation of type II pneumocytes mean that use of the A549 cell line model in studies of pulmonary physiology has been largely superceded (see Rooney, 1985).

In 1985 Gescher and Reed reported that treatment of A549 cells with phorbol esters resulted in potent inhibition of growth (Gescher and Reed, 1985). Incubation of cultures with 10 nM TPA caused complete inhibition of cell growth for 5-6 days, after which cells began to grow at a rate similar to that of control cells. Treatment with TPA also induced a dramatic change in cell morphology, from flat and spread out to rounded and protruding. When treated cells were trypsinised and reseeded sensitivity to TPA was regained. However, when the cells were subcultured in the continuous presence of 10 nM TPA for several weeks they gradually became completely refractory to the growth-inhibitory properties of the phorbol ester such that after 9 weeks continued exposure TPA was unable to slow cell growth and cells were still resistant after trypsinisation and reseeding. This cell variant was termed A549-TPA. The phorbol esters PDBu and PDA were also found to induce less potently the same growth-inhibitory and morphological responses as TPA.

The potential role of PKC in the observed inhibition of A549 cell growth was investigated using the ligand [^{3}H]PDBu to detect specific phorbol ester receptors. Exposure of cells to 100 nM PDBu prior to the receptor binding assay led

to a rapid loss of receptor binding such that after 24 h pretreatment binding was reduced to 38% of controls. However, in cells desensitized towards phorbol ester-induced growth inhibition by treatment with 100 nM PDBu for 6 days, receptor binding capacity was 75% of controls. Also, in the A549-TPA cells more permanently resistant to growth inhibition, the ability of [^{3}H]PDBu to bind to receptors was only decreased little by pretreatment with 100 nM PDBu for 24 h, to 77% of binding in untreated cells. It was proposed that the decrease in receptor binding on exposure to phorbol esters did not appear to cause the refractoriness of the cells towards the effect of TPA and that this down-regulation was more likely to be part of the events by which phorbol esters caused growth inhibition.

1.6.3. Growth-inhibitory properties of phorbol esters in other cell lines.

The inhibition of proliferation of the A549 cell line by phorbol esters is by no means unique and has been observed in a number of other cultured cell lines (reviewed by Gescher, 1985). This subsection briefly outlines the growth-inhibitory properties of phorbol esters in some of these cell types.

In many cases the growth-inhibitory influence of phorbol esters is due to the loss of the proliferative potential of cells as a result of the induction of terminal differentiation (reviewed by Vandenbark and Niedel, 1984). For example, the effects of phorbol esters on leukaemia cell differentiation has been an area of intense study. The HL-60 promyelocytic leukaemia cell line, a much used model

in studies of differentiation, differentiated into cells with macrophage characteristics when treated with TPA (Huberman and Callaham, 1979; Rovera et al, 1979). Treatment of the M5076 murine reticulum sarcoma cell line led to the cessation of cell division, presumed to be the result of the induction of terminal differentiation (Talmadge and Hart, 1984; Talmadge et al, 1982). Cells in which TPA has induced terminal differentiation, such as the HL-60 leukaemia cells, ultimately die. Similarly, in the EBV-negative BJAB and Ramos cell lines growth inhibition by TPA is followed by cell death (Béchet and Guetard, 1983).

As a result of their identity as potent tumour promoters in mouse skin, the effects of phorbol esters on epidermal cells in vitro have also been much investigated. Phorbol esters act differentially on epidermal cell subpopulations, inducing differentiation in one cell class and stimulating cell proliferation in another (Reiners and Slaga, 1983; Yuspa et al, 1982). It has therefore been postulated that the resistance of initiated cells to TPA-induced carcinogenesis leads to the selective clonal expansion of the initiated cell population at the expense of the surrounding normal tissue (Yuspa et al, 1982). Much experimental evidence appears to support this hypothesis. For example, it has been observed that normal mouse epidermal cells terminally differentiate in response to phorbol esters, while initiated epidermal cells and cells from benign skin tumours do not (Hartley et al, 1985; Yuspa et al, 1986). A similar differential response has been observed among normal and neoplastic human epidermal cells

(Parkinson et al, 1984). Also, cultured normal human bronchial epithelial cells differentiate when treated with phorbol esters whereas several lung carcinomas are resistant (Sanchez et al, 1987; Willey et al, 1984).

Treatment of RH-SCL-L11 squamous-cell and RH-SCC-L10 small-cell lung carcinoma cell lines with TPA resulted in the loss of proliferative capacity in vitro and tumorigenicity in vivo and also profound changes in morphology (Olsson et al, 1985). A number of human breast cancer cell lines have been also been reported to be sensitive to the growth-inhibitory influence of phorbol esters. Osborne et al (1981) demonstrated that growth of the MCF-7, ZR75-1 and MDA-MB-231 cell lines was inhibited by TPA. In MCF-7 cells, TPA-induced growth inhibition was accompanied by the stimulation of protein synthesis resulting in hypertrophied cells, and after 10-12 days of exposure to TPA death and detachment of cells from the culture dishes was observed. However, growth inhibition was reversible upon removal of the phorbol ester (Osborne et al, 1981).

TPA is also able to cause non-toxic growth arrest of the A431 human epidermoid carcinoma (Smith et al, 1983) and SVK 14 (McKay et al, 1983) cell lines. The SVK 14 line, derived by SV40 transformation of human foreskin keratinocytes, appears similar to the A549 cell line in that the inhibition of cell proliferation is only transient and cells become insensitive towards TPA after several days exposure (McKay et al, 1983).

A number of studies which have investigated the potential

role of PKC in the induction of growth inhibition by phorbol esters are described in Section 4.

1.7. Bryostatins-a novel class of PKC activator

One of the most intriguing findings of the past few years in the field of phorbol ester and PKC research has been the discovery and characterisation of a novel class of PKC activator, the bryostatins.

In 1970 it was reported that certain marine bryozoans, colonial plankton feeders known as false corals or as sea mosses, contained potent antineoplastic components (Pettit et al, 1970). On the basis of antineoplastic activity against the murine P388 lymphocytic leukaemia system, bryostatin 1 was isolated from the bryozoan Bugula neritina and its structure elucidated (Pettit et al, 1982). A family of 17 bryostatins have since been extracted and purified from Bugula neritina which exhibit similar activity against the P388 system and the structures of many have been determined (Pettit et al, 1983; Pettit et al, 1984; Pettit et al, 1985; Pettit et al, 1986; Pettit et al, 1987a, 1987b).

Initial study of the biological properties of the bryostatins seemed to indicate that they were simply another class of PKC agonist. For example, they activated PKC, blocked phorbol ester binding and induced biological responses typical of the phorbol esters such as the activation of human polymorphonuclear leucocytes and mitogenesis of quiescent 3T3 fibroblasts (Berkow and Kraft, 1985; Smith et al, 1985). However, further investigations

revealed that, in many cell types, bryostatins did not mimic and indeed often antagonized the influence of phorbol esters.

In 1986 Kraft and coworkers reported that bryostatin 1, although able to activate PKC and induce enzyme translocation, failed to mimic phorbol ester-induced monocytic differentiation of HL-60 promyelocytic leukaemia cells and also blocked the differentiation induced by TPA in these cells (Kraft et al, 1986). Paradoxically, a more recent paper appears to demonstrate that bryostatin 1 is able to induce the differentiation of HL-60 cells (Stone et al, 1988), suggesting that different HL-60 sublines might respond differently to bryostatin 1. In other cell systems the biological effects of bryostatins are not so ambiguous. For example, bryostatins restored the differentiation response in Friend erythroleukaemia cells in which drug-induced differentiation was suppressed by PDBu (Dell'Aquila et al, 1987) and blocked phorbol ester-induced differentiation of human colon cancer cells (McBain et al, 1988). Gschwendt et al (1988) have reported the ability of bryostatin 1 to mimic as well as inhibit biological effects of TPA in both in vivo and in vitro systems.

The ability of bryostatins 1, 2, 3, 4, 10 and several of their derivatives to cause arachidonic acid metabolite release and to block phorbol ester action has been investigated in the murine fibroblast CH3 10T½ cell line (Dell'Aquila et al, 1988). In contrast to phorbol esters derivatives, which are often able to induce similar maximal biological responses although differing in potency, the

bryostatins differed in the maximal extents to which they could induce arachidonic acid metabolite release or block phorbol ester-induced release.

Different cell types respond in different ways following exposure to bryostatins. Thus, inhibition by bryostatin 1 of EGF binding in mouse primary epidermal cells is transient (Sako et al, 1987) whereas it is of a much longer duration in the CH3 10T½ murine fibroblast cell line (Dell'Aquila et al, 1988). Sako et al (1987) have studied the effects of bryostatin 1 on mouse primary epidermal cells. This system has previously been shown to be susceptible to both proliferative and differentiative responses caused by phorbol esters in distinct cell subpopulations (Yuspa et al, 1982). At nanomolar concentrations bryostatin 1 induced ornithine decarboxylase activity, a marker of proliferation, though not to the same extent as PDBu. In contrast, it did not induce epidermal transglutaminase activity or cornified envelope production, both markers of differentiation induced by PDBu. Combined treatment with bryostatin 1 and PDBu gave similar results to treatment with the bryostatin alone. As induction of differentiation has been proposed to be an important element of phorbol ester-induced tumour promotion (Yuspa et al, 1982), it was proposed that the bryostatins may be antipromoters. Indeed, bryostatin 1 has been found to be inactive as a complete tumour promoter and only a very weak stage II tumour promoter (Hennings et al, 1987). Furthermore, simultaneous administration of bryostatin 1 with TPA in a complete tumour promotion protocol suppressed the promoting ability of the phorbol ester (Hennings et al,

1987).

A possible explanation for the often dissimilar biological influences of phorbol esters and bryostatins is the differing modulation of PKC activity by these compounds. Treatment of whole HL-60 cells with bryostatin 1, but not PDBu, led to specific translocation of activated PKC to the nuclear envelope (Fields et al, 1988). In an in vitro reconstitution system the bryostatin, but not the phorbol ester, was able to activate purified PKC to directly phosphorylate lamin B, a prominent nuclear envelope protein (Fields et al, 1988).

Kraft and coworkers have demonstrated that bryostatin 1 is less active than phorbol esters in inducing the irreversible transformation and anchorage-independent growth of the JB6 murine epidermal cell line, and at high concentrations blocks nonadherent growth induced by TPA (Kraft et al, 1988). Using partially purified JB6 PKC, bryostatin 1 was found to stimulate less phosphorylation of histone substrate and also was less potent in inducing enzyme translocation. Using purified rat brain PKC isozymes, bryostatin demonstrated identical competition with TPA for binding to the α and γ subforms but decreased binding to the β isozyme, which was found to be the predominant PKC subspecies in JB6 cells. In addition, bryostatin appeared to induce a more rapid down-regulation of PKC than TPA (Kraft et al, 1988).

The above examples of the differential modulation of PKC activity by bryostatins and phorbol esters may explain in part the differing biological effects of these compounds.

1.8. Aims of this study

It is widely believed that many of the diverse biological effects induced by phorbol esters are due to their ability to activate the enzyme PKC, which has emerged as a pivotal enzyme in the receptor-mediated signal transduction associated with phosphoinositide breakdown (Nishizuka, 1984; Nishizuka 1986). Although phorbol esters are potent tumour promoters in mouse skin, they inhibit the growth of a number of malignant cell lines *in vitro*, often as a result of the induction of terminal differentiation (Gescher, 1985). Investigation of the mechanism(s) by which phorbol esters inhibit the growth of cancer cells could conceivably lead to the design of novel anticancer drugs which mimic the antiproliferative properties of phorbol esters without being tumour promoters. As the major phorbol ester receptor, PKC therefore represents a potential target for therapeutic intervention in malignant disease.

Growth of the A549 human lung carcinoma cell line is inhibited by treatment with nanomolar concentrations of TPA, although cells rapidly recover their proliferative potential even when incubated in the continued presence of the phorbol ester (Gescher and Reed, 1985). In view of this rather unusual growth response towards phorbol esters, the A549 cell line has been utilised in the present study as a model system in order to investigate the mechanism(s) by which phorbol esters and related compounds exert their growth-inhibitory effects.

The aims of this study are:

(1) Further characterisation of the nature of the growth

inhibition exerted by phorbol esters in A549 cells.

(2) Characterisation of the growth-inhibitory influence on A549 cells of compounds other than phorbol esters, particularly the PKC-activating compounds mezerein, DAGs and bryostatins, and comparison with phorbol ester-induced growth inhibition. The biological influences of the bryostatins, which induce only a subset of typical phorbol ester responses in a number of cell types and moreover block many phorbol ester responses which they do not themselves induce, were of special interest in these investigations.

(3) Correlation of changes in the proliferative potential of A549 cells induced by the above compounds with alterations in the subcellular distribution and activity of PKC determined by a quantitative non-denaturing PAGE method.

(4) Evaluation of the growth-inhibitory properties in A549 cells of compounds synthesized to mimic regions of the phorbol ester pharmacophore and/or DAG, in order to help identify important elements of the phorbol ester pharmacophore responsible for their growth-inhibitory effects in this cell line.

(5) Development of a method for the culture of A549 cells on microcarrier beads in order to measure cellular Ca^{2+} fluxes and their modulation by PKC activators.

SECTION 2: MATERIALS

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2.1. Sources of chemicals

Sigma Chemical Company Limited, Poole, Dorset, England

Adenosine 5'-triphosphate (ATP) (sodium salt), aprotinin, citric acid, crystal violet, Cytodex 1 microcarrier beads, 1,2-sn-dioctanoylglycerol (1,2-diC_8), diolein, disodium ethylenediamine tetraacetic acid, (Na_2EDTA), ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), glucose, glycerol, histone H1, H-7, magnesium nitrate, mezerein, N,N,N',N'-tetramethylethylenediamine (TEMED), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes), nicotinamide adenine dinucleotide, reduced form (NADH), Nonidet NP-40, 1-oleoyl-2-acetyl-sn-glycerol (OAG), phorbol 12,13-diacetate (PDA), phorbol 12,13-dibutyrate (PDBu), 4-α-phorbol 12,13-didecanoate (PDD), phosphatidylserine (PS), potassium persulphate, quin-2/AM, riboflavin, sodium azide, sodium pyruvate, 12-O-tetradecanoylphorbol-13-acetate (TPA), Triton X-100, Tris base (Trizma), trypan blue.

Fisons PLC, Loughborough, Leicestershire, England

Dimethylsulphoxide (DMSO), disodium hydrogen orthophosphate, glacial acetic acid, glutaraldehyde, glycine, hydrochloric acid, Optiphase MP scintillation fluid, magnesium chloride, magnesium sulphate, methanol, phosphoric acid, potassium chloride, potassium dihydrogen orthophosphate, sodium cacodylate, sodium chloride, sodium hydrogencarbonate, sodium hydroxide.

BDH Chemicals Limited, Poole, Dorset, England

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

Gibco Limited, Paisley, Glasgow, Scotland

FCS, Hams F-12 Nutrient medium, L-glutamine, penicillin, streptomycin, trypsin, EGF (tissue culture grade).

Fluka Chemicals Limited, Glossop, Derbyshire, England

Acrylamide (electrophoresis pure), N,N' methylenebisacrylamide (electrophoresis pure), sodium dihydrogen orthophosphate.

Aldrich Chemical Company, Gillingham, Dorset, England

Trichloroacetic acid.

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

Bradford dye reagent, bovine serum albumin protein standard.

Whatman Labsales Ltd., Croydon, Surrey, England

17Chr chromatography paper.

Millipore UK Ltd., Harrow, Middlesex, England

Millipore 0.2 µM and 0.45 µM filters.

Peptide Synthesis Inc., Japan, via Scientific Marketing associates, London, England

Leupeptin

Janssen Scientific Products, Janssen Pharmaceutical Ltd., Wantage, Oxford, England

R59022.

Calbiochem AG, Lucerne, Switzerland

Fura-2/AM.

Oxoid Laboratories Ltd., Basingstoke, England

Phosphate buffered saline (PBS) tablets.

Boehringer Corporation Limited, Lewes, Sussex, England

Microtitre plates, 96-well, U form.

IBF Biotechnics, Villeneuve-la-Garenne, France

Ultrogel AcA 202.

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

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

Amersham International Plc., Amersham, Buckinghamshire, UK

5-[methyl ^{3}H]-thymidine (specific activity 5 Ci/mmol).

Other chemicals

Bryostatins 1 and 2 were generously supplied by Dr. G.R. Pettit of Arizona State University, Tempe, Arizona, USA. SRI 62-834 was provided by Dr. W.J. Houlihan of the Sandoz Research Institute, New Hanover, New Jersey, USA. 1,3-sn-dioctanoylglycerol (1,3-diC_8) and all synthesized phorbol ester/DAG analogues (compounds 1-14) were synthesized by Dr. C.A. Laughton of the Cancer Research Campaign Medicinal Chemistry Laboratories, Aston University, Birmingham, UK.

All other chemicals were purchased as described in the methods or were of analytical grade.

Tissue culture materials

50 ml, 250 ml and 500 ml culture flasks, 35 mm six well multidishes, 55 mm petri dishes (Nunclon, Denmark), 50 mm Petriperm dishes (Heraeus Equipment Ltd., Brentwood, Essex, England), class II Gelair BSB 3 microbiological

safety cabinet with unidirectional laminar downflow, gassing incubator (Flow Laboratories, Irvine, Scotland), 140 mm petri dishes, 30 ml sterile universals, 7 ml bijou bottles, sterile pipettes (Sterilin Limited, Feltham, England).

2.2. Buffers and reagents

2.2.1. General cell culture reagents

Stock 10x Versene solution

20 PBS tablets

0.742 g Na_2EDTA

0.1 g phenol red

to 200 ml with d H_2O

pH was adjusted to 7.4 using 1 M sodium hydroxide, the solution filtered and stored at room temperature after autoclaving.

Trypsin-Versene solution

10 ml stock 10x Versene solution

10 ml 10x trypsin solution

to 100 ml with d H_2O

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

1% (w/v) crystal violet

1 g crystal violet

to 100 ml with d H_2O

0.1 M citric acid with 0.1% (w/v) crystal violet

1.92 g citric acid

0.1 g crystal violet

to 100 ml with d H_2O

PBS, pH 7.4

5 PBS tablets

to 500 ml with d H_2O

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

10 µg/ml EGF

0.1 mg EGF (tissue culture grade) was dissolved in 10 ml sterile PBS, pH 7.4, and 500 µl aliquots stored frozen at -20°C.

25 mM H-7

5 mg H-7

to 6.86 ml with DMSO

Aliquots (100 µl) were stored dessicated at -20°C.

20 mM R59022

50 mg R59022

to 5.44 ml with DMSO

Aliquots (100 µl) were stored dessicated at -20°C.

2.2.3. Electron microscopy solutions

0.18 M sodium cacodylate

19.26 g sodium cacodylate

to 500 ml with d H_2O

The solution was stored at 4°C.

0.05 M sodium cacodylate with 0.2% glutaraldehyde

5.35 g sodium cacodylate

1 g glutaraldehyde

to 500 ml with d H_2O

The solution was stored at 4°C.

2.2.4. LDH cytotoxicity assay solutions

3.5 mM NADH

9.92 mg NADH

to 4 ml with d H_2O

The solution was prepared only on the day of use and kept on ice until used.

32 mM sodium pyruvate

14.08 mg sodium pyruvate

to 4 ml with d H_2O

The solution was prepared only on the day of use and kept on ice until used.

2.2.5. Stock reagents for quantitative assay of PKC by PAGE

20% (w/v) Triton X-100

20 g Triton X-100

to 100 ml with d H_2O

The solution was dissolved with heat and stirring and stored at room temperature. All other solutions of Triton X-100 were prepared by the appropriate dilution of this stock.

Anode buffer stock

75.6 g Tris base

to 1000 ml with d H_2O

pH was adjusted to 7.51 using concentrated hydrochloric acid and the buffer stock stored at 4°C.

The stock was diluted 1:10 with d H_2O before use to give 0.0625 M Tris-HCl, pH 7.51.

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Cathode buffer stock

28.5 g Tris base

17.3 g glycine

to 500 ml with d H_2O

pH was adjusted to 8.90 using concentrated hydrochloric acid and the buffer stock stored at 4°C.

For 1 litre of cathode buffer, stock was diluted as follows:

100 ml cathode buffer stock

10 ml 20% Triton X-100

890 ml d H_2O

Diluted cathode buffer: 0.0426 M Tris-HCl, 0.0461 M glycine, 0.2% Triton X-100, pH 8.90.

Stacking gel buffer stock

2.96 g Tris base

to 100 ml with d H_2O

pH was adjusted to 6.67 using concentrated phosphoric acid and the stock stored at 4°C.

Resolving gel buffer stock

18.16 g Tris base

to 100 ml with d H_2O

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

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

19.6 g electrophoresis pure acrylamide

0.4 g N,N' electrophoresis pure methylenebisacrylamide

to 50 ml with d H_2O

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

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

3.2 g electrophoresis pure acrylamide

0.8 g N,N' electrophoresis pure methylenebisacrylamide

to 50 ml with d H_2O

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

1:10 TEMED

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

0.01% riboflavin solution

10 mg riboflavin

to 100 ml with d H_2O

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

Potassium persulphate/riboflavin solution (KP/RF)

30 mg potassium persulphate

10 ml 0.01% riboflavin

to 50 ml with d H_2O

The solution was prepared only on the day of use.

Amaranth dye solution

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

1.0 M Tris-HCl, pH 7.4

12.114 g Tris base

to 100 ml with d H_2O

pH was adjusted to 7.4 using concentrated hydrochloric acid and the solution stored at 4°C. 20 mM Tris-HCl, pH 7.4, was prepared by 1:50 dilution of this solution with d H_2O and stored at 4°C.

0.5 M magnesium nitrate

1.282 g magnesium nitrate

to 10 ml with d H_2O

The solution was stored at 4°C.

0.1 M calcium chloride

Analar 1 M calcium chloride (1 ml) was diluted to 10 ml with d H_2O and stored at 4°C. All further dilutions of calcium chloride were made using this stock solution.

100 mM Na_2EDTA, pH 7.4

0.744 g Na_2EDTA

to 20 ml with d H_2O

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

100 mM EGTA, pH 7.4

0.761 g EGTA

to 20 ml with d H_2O

pH was adjusted to 7.4 using 8 M sodium hydroxide and the solution stored at 4°C. Stock 10 mM EGTA, pH 7.4, was prepared by 1:10 dilution of this solution with d H_2O.

Leupeptin stock (10 mg/ml)

Leupeptin (100 mg) was dissolved in 10 ml d H_2O and 200 µl aliquots frozen at -20°C.

Aprotinin stock 10 mg/ml)

Aprotinin (100 mg) was dissolved in 2.5 ml d H_2O and 100 µl aliquots frozen at -20°C.

Homogenization buffer H-8

Used in the preparation of cell extracts for loading onto gel.

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

to 100 ml with d H_2O

The buffer was prepared on the day of use.

Extraction buffer

Extraction buffer (H-8 + 1% NP-40) was prepared as described for homogenization buffer H-8, with the addition of 1 g NP-40 per 100 ml buffer.

Elution buffer

Used for eluting protein from gel slices overnight.

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

to 100 ml with d H_2O

The buffer was prepared on the day of use.

Wash buffer W-3 stock

Used for washing cell monolayers before scraping cells.

12.114 g Tris base

43.830 g sodium chloride

4.505 g glucose

to 500 ml with d H_2O

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

The stock was diluted 1:10 on the day of use using d H_2O and the proteolysis inhibitors leupeptin and aprotinin added.

When diluted, W-3:

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

10% TCA + phosphate

1 kg TCA
100-200 g sodium dihydrogen orthophosphate
to 10 litres with d H_2O

The solution was cooled to 4°C before use.

300 mM ATP

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

Aliquots (50 µl) were frozen at -20°C. When required, an aliquot was thawed rapidly and diluted to 60 mM (1:5 dilution) with d H_2O before addition to the assay mix.

Assay mix-protamine

For one assay the following components were mixed:

4 µl 1 M Tris-HCl, pH 7.4
4 µl 0.5 M magnesium nitrate
0.08 µl 60 mM ATP
100 µg protamine
0.5 µCi [γ-^{32}P]-ATP

to 50 µl with d H_2O

The assay mix was prepared on the day of use. Usually, assay mix sufficient for 200-300 assays was required.

Assay mix-histone

For one assay the following components were mixed:

4 µl 1 M Tris-HCl, pH 7.4

4 µl 0.5 M magnesium nitrate

0.08 µl 60 mM ATP

50 µg histone H1

20 µl 3.75 mM calcium chloride

0.5 µCi [γ-^{32}P]-ATP

The assay mix was prepared on the day of use. Usually, assay mix for sufficient for 200-300 assays was required.

Phospholipid mix

Phospholipid mix was required for the assay of PKC using histone as substrate.

For one assay, 100 µg PS (10 mg/ml stock solution in 95% chloroform /5% methanol) and 10 µg diolein (1 µl, 10 mg/ml stock solution in 95% chloroform/5% methanol) were aliquoted into a plastic mini-scintillation vial on ice and protected from light. After complete evaporation of the solvent using nitrogen, 50 µl 20 mM Tris-HCl, pH 7.4, was added. Micelles of phospholipid were formed by sonicating for 30 s with 30 s cooling periods for a total time of 5 min (MSE sonicator, setting 26). After sonication, the phospholipid mix was overlaid with nitrogen, tightly capped and maintained at 4°C until required for the assay.

2.2.6. Stock solutions for measurement of $[Ca^{2+}]_i$ using cells cultured on microcarrier beads

Cytodex 1 microcarrier beads

5 g Cytodex 1

to 250 ml with PBS, pH 7.4

Beads were allowed to swell in a silanised glass bottle and autoclaved before use.

40 mM quin-2/AM

10 mg quin-2/AM

to 300 µl with DMSO

Aliquots (20 µl) were stored at -20°C in a dessicator.

1 mM fura 2/AM

1 mg fura 2/AM

to 998 µl with DMSO

Aliquots (50 µl) were stored at -20°C in a dessicator.

10 mM SRI 62-834

56.7 mg SRI 62-834

to 10 ml with d H_2O

The solution was stored at -20°C.

Neutrophil buffer

8.18 g sodium chloride

0.38 g potassium chloride

0.24 g sodium hydrogencarbonate

1.5 ml 1 M calcium chloride solution

0.2 g magnesium chloride

0.014 g magnesium sulphate

1 g glucose

2.38 g Hepes (free acid)

to 1 litre with d H_2O

pH was adjusted to 7.2 using 1 M sodium hydroxide and the buffer stored at -20°C.

250 mM EGTA, pH 7.2

1.903 g EGTA

to 20 ml with d H_2O

pH was adjusted to 7.2 using 8 M sodium hydroxide and the solution stored at 4°C.

2.2.7. Mixed micellar assay stock solutions

200 mM Tris-HCl, pH 7.5

2.423 g Tris base

to 100 ml with d H_2O

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

20 mM Tris-HCl, pH 7.5, 200 µM $CaCl_2$ and 0.015% (w/v) Triton X-100

10 ml 200 mM Tris-HCl, pH 7.5

20 µl 1 M stock calcium chloride solution

75 µl 20% (w/v) Triton X-100

to 100 ml with d H_2O

The buffer was stored at 4°C.

6 mM calcium chloride

Stock 0.1 M calcium chloride solution (100 µl) was diluted to 1.666 ml with d H_2O and stored at 4°C.

3% (w/v) Triton X-100

Stock 20% (w/v) Triton X-100 (150 µl) was diluted to 1 ml with d H_2O and stored at room temperature.

2.2.8. Miscellaneous reagents

All phorbol esters, mezerein and bryostatins were stored as 1 mM stock solutions with the exception of PDBu which was 2 mM. Stocks were stored frozen at -20°C. When required, further dilutions were made in DMSO as appropriate such that the concentration of DMSO never exceeded 0.5%. Diluted stocks which were frozen and thawed frequently were discarded after 3-5 weeks.

Stock solutions of DAGs and all synthesized compounds were dissolved in DMSO immediately prior to use and were not stored.

SECTION 3: METHODS

SECTION 3: METHODS

3.1. Cell culture

3.1.1. Maintenance of cell cultures

A549 cells were obtained from the American Type Culture Collection, Rockville, Maryland, USA. Cultures were maintained in Nutrient Hams F-12 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (10 pg/ml) and 20 mM glutamine. 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 trypsinisation. Typically, cells in logarithmic growth exhibited a doubling time of 23-24 h.

3.1.2. Detachment of cells by trypsinization

Medium was aspirated from cultures and cells were washed briefly with Versene solution to remove debris. After the removal of Versene by aspiration, trypsin-Versene solution was added and the cells incubated at 37°C for 5-10 min until detached. An equal volume of medium was then added to inactivate the trypsin.

In the case of routine subculturing, approximately 1/30 of the original culture was reseeded into a fresh tissue culture flask.

3.1.3. Storage of cultures in liquid nitrogen.

Cells in logarithmic growth were trypsinized as described and pelleted using a Heraeus Christ labofuge 6000 centrifuge at a speed of 1000 rpm for 5 min. The supernatant was removed carefully by aspiration and the pellet gently resuspended at a density of 1 x 10^6 cells/ml in Nutrient

Hams F-12 medium supplemented with 12.5% FCS, 10% DMSO and antibiotics. Aliquots of the cell suspension were transferred to cryogenic vials (1 ml/vial) and cooled to -80°C for 3 h before immersion in liquid nitrogen.

When cultures were removed from the cell bank, vials were rapidly thawed and the contents seeded into culture flasks. To enhance the plating efficiency of the culture, cells were grown in medium supplemented with 20% FCS for the first two days before reverting to medium supplemented with 10% FCS.

3.1.4. Counting of cells

A model ZM coulter counter (Coulter Electronics Ltd., Luton, Bedfordshire, England) was routinely used to count cells. A 200 µl aliquot of single cell suspension was diluted to 10 ml with isoton (Coulter Electronics Ltd.) and counted at the predetermined settings of current : 130, attenuation : 16, lower threshold (T_L) : 12.0, upper threshold (T_U) : 99.9. Occasionally cell suspensions were counted using a haemocytometer (Weber Scientific International Ltd.). In order to facilitate accurate counts when cell numbers were low ($<5 \times 10^4$), cells were pelleted by centrifugation at 1000 rpm for 5 min and resuspended in an appropriate volume of PBS, pH 7.4, before counting.

3.1.5. Growth curves

Between 2-5 x 10^4 cells were seeded into 35 mm-diameter six-well multidishes in 3 ml medium. Compounds dissolved in DMSO were added 4 h after seeding, when the cells had attached to the plastic. An equivalent volume of DMSO was added to control cultures (final concentration in the medium <0.5%). Cell numbers were counted daily, usually over a 6

day period, after removing cells from the wells by trypsinisation.

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

Cells (2 x 10^5 per well) were seeded into 35 mm-diameter six-well multidishes and compounds added after cells had attached to the plastic surface. Cultures were routinely incubated for 24 h or for shorter time periods in the case of the experiments in which the time course of incorporation of [^{3}H]TdR was studied. At the end of the incubation time cells were briefly washed with medium before incubating for 1 h in 1 ml medium containing 1 µCi [^{3}H]TdR. Cultures were then washed six times with ice-cold PBS, pH 7.4, before fixing with 50% (v/v) methanol/10% (v/v) acetic acid and storing dishes at 4°C for > 30 min. Wells were washed once more with PBS and the remaining insoluble fraction solubilized in two 0.5 ml aliquots of 1% SDS and added to 10 ml Optiphase MP scintillation fluid. Radioactivity was counted in a Packard Tricarb CA2000 scintillation counter.

Cell counts were also performed in conjunction with the assay and results expressed as % control incorporation/10^5 cells.

3.1.7. Clonogenic analysis

Between 1 x 10^2 and 1 x 10^3 (usually 5 x 10^2) cells from a single cell suspension were seeded into 35 mm-diameter six-well multidishes (3 ml medium) or 55 mm-diameter Petri dishes (5 ml medium) and allowed to attach for 24 h at 37°C in a gassing incubator in an atmosphere of 95% air/5% CO_2. Test compounds were then added to the medium in DMSO carrier. Controls received DMSO alone (final concentration

< 0.5%).

Dishes were further incubated for 6-10 days, medium and compound being routinely replaced every 3 days, until colonies in control dishes were of sufficient size (> 50 cells/colony) to be visible to the naked eye when stained. Medium was removed, dishes washed briefly in PBS, pH 7.4, and cells fixed with 10% formalin. Colonies were stained using 1% (w/v) aqueous crystal violet for 5 min, after which excess stain was washed off using PBS, pH 7.4.

The colony-forming efficiency (CFE) of cultures was determined by counting the number of discrete colonies visible using a low power microscope (4 x magnification). CFE was calculated as follows:

$$CFE = \frac{\text{no. of colonies counted/dish}}{\text{initial no. of cells seeded/dish}} \times 100$$

3.1.8. Isolation of TPA-resistant clones

Cells (4×10^3) were seeded into 55 mm-diameter Petri dishes and allowed to attach for 24 h at 37°C in a gassing incubator in an atmosphere of 95% air/5% CO_2. TPA was then added to the medium to give a final concentration of 10 nM, controls receiving carrier DMSO only (< 0.5%).

Dishes were incubated for 14-21 days, medium and phorbol ester being routinely replaced every 3 days. During this period all dishes were examined regularly and colonies which continued to grow in the presence of TPA noted. When these resistant colonies were deemed to be of sufficient size (200+ cells) they were isolated.

After removal of medium by aspiration under aseptic conditions, sterile silicone grease was placed around the

base of a sterile cloning ring and the ring firmly affixed to the base of the Petri dish around a colony whose position had been previously marked. An aliquot (200 µl) of trypsin-Versene solution was pipetted into the ring and the dish incubated at 37°C for 10-20 min. Detached cells were then seeded into a tissue culture flask with 5 ml medium supplemented with 20% FCS. Cultures were then incubated in the absence of TPA for 7 days. Cultures which continued to grow were cultured until sufficient cells were available for experiments.

The above procedure was performed 20 times, of which 3 led to clones, designated as clones 1, 2 and 3.

3.1.9. Electron microscopy

Cells (5 x 10^3) were seeded into 50 mm PetriPerm dishes and incubated at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. After a 4 h attachment period, cultures were treated with the test compound and further incubated for 24 h. After aspiration of the culture medium, cells were briefly washed with 5 ml 0.18 M sodium cacodylate before fixing for 1 h in 5 ml 0.05 M sodium cacodylate with 0.2% (w/v) glutaraldehyde.

The samples were then processed by Dr. Stuart Townsend at the MRC Radiobiology Unit, Harwell, Didcot, Oxon, England. 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.

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

LDH catalyses the reaction :

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

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

Cells (1×10^5) were seeded into 35 mm-diameter six-well multidishes and allowed to attach for 4 h. Medium was then replaced by 1.5 ml medium supplemented with 1% FCS, antibiotics and glutamine. The reduction in FCS levels was found to be necessary as endogenous NADH oxidising enzymes present in serum interfered significantly with the assay at higher concentrations. The test compound was added (carrier DMSO, < 0.3%) to give final concentrations ranging from 10^{-4} to 10^{-9} M, controls received DMSO alone. Dishes were incubated overnight before the medium was transferred to polystyrene centrifuge tubes and stored at 4°C. Wells were then further treated with the compound as described above (to mimic multiple additions) before incubation for a further 5 h. The two aliquots of medium were then combined and centrifuged at a speed of 1000 rpm for 5 min to remove cell debris, before assaying for LDH activity.

To a quartz cuvette, 1 cm path length, were added 2.4 ml PBS, pH 7.4, 100 µl stock NADH solution (final assay concentration: 0.12mM) and 400 µl sample. The assay cocktail was allowed to equilibrate at 37°C for 5 min before

before initiating the reaction by the addition of 100 µl stock sodium pyruvate solution (final assay concentration: 1.0 mM). The rate of change of absorbance over 5 min 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 , 160 µl of 20% Triton X-100 was added to previously untreated cells in 3 ml medium supplemented with 1% FCS. After 5 min incubation, 400 µl of supernatant was assayed for LDH activity and the rate obtained used to represent 100% cell death, all other rates being expressed as a percentage of this value. In order to correct for cell growth at non-toxic or marginally toxic concentrations, cell counts were performed in conjunction with the assay at all concentrations tested, and results expressed as; % Triton-releasable LDH activity/10^5 cells.

3.1.11. Estimation of cell viability by trypan blue exclusion

Approximately 100 µl of cell suspension was mixed with 50 µl of filtered 0.5% trypan blue in 0.9% sodium chloride solution. After 1 min the suspension was introduced into a haemocytometer and examined under a light microscope. The number of cells which excluded trypan blue were counted and values expressed as a percentage of the total number of cells.

3.2. Quantitative assay of PKC by non-denaturing PAGE

3.2.1. Procedure

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 then frozen and sliced into 1 mm sections before eluting protein from the slices overnight. The eluates were assayed for PKC activity by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into lysine-rich histone or protamine sulphate.

3.2.2. Preparation of polyacrylamide gels

Gels were prepared only one day before sample application, and a maximum of twelve gels could be run in an experiment. All gels were cast in borosilicate glass rods of length 14-15 cm, outer diameter 0.7 cm and inner diameter 0.5 cm. All gel casting was performed at 4°C. Unwashed tubes were sealed with parafilm at one end before the addition of gel mixes.

In all experiments resolving (lower) gels were prepared at a total monomer concentration of 10% acrylamide with 2% bisacrylamide crosslinks. For twelve 10% polyacrylamide gels, 6 ml resolving gel acrylamide stock, 6 ml resolving gel buffer stock, 6 ml potassium persulphate/riboflavin solution and 6 ml d H_2O were mixed in a brown bottle on ice. The gel mix was deaerated by stirring on ice for 5 min while passing nitrogen through the bottle. Triton X-100 (20%, 240 µl) and TEMED solution (240 µl) were added in order to initiate polymerisation. Gel mix (1.6 ml) was added to the glass tubes to form gels of approximately 6 cm length. The top of the gels were overlaid with d H_2O and photopolymerised by illuminating with fluorescent light at 4°C for 30 min. After polymerisation was complete, the gel

surface was rinsed 2-3 times with d H_2O, residual H_2O removed by aspiration and the tubes dried carefully.

Stacking (upper) gels were prepared at a total monomer concentration of 3.5% acrylamide with 20% bisacrylamide crosslinks. It was essential that the volume of the stacking gel (usually 700 µl) was at least twice that of the sample volume loaded onto the gel (usually 200 µl). For twelve gels, 6 ml stacking gel acrylamide stock, 3 ml stacking gel buffer stock and 3 ml potassium persulphate/riboflavin solution were mixed in a brown bottle on ice. The gel mix was deaerated as described for the resolving gel before adding Triton X-100 (20%, 120 µl) and TEMED solution (120 µl) to initiate polymerisation. The gel mix (700 µl) was poured into the tubes, overlaid with d H_2O and photopolymerised as described for the resolving gel.

3.2.3. Preparation of subcellular fractions

Cells were cultured in 140 mm diameter Petri dishes. The cultures were maintained at 37°C in a humidified atmosphere of 95% air with 5% CO_2. Cells were used in experiments when 70-100% confluent. A maximum of six dishes (twelve gel samples) were used per experiment. Samples could only be prepared one day before partial purification by PAGE.

When required, compounds were dissolved in DMSO and added to cultures (final DMSO concentration < 0.5%).

After the growth medium had been discarded the cell monolayer was rapidly washed three times with 10 ml ice-cold wash buffer W-3. The wash buffer was carefully removed by aspiration and 0.5 ml homogenization buffer H-8 added. The cells were carefully scraped off the dish and transferred to

a 10 ml thick-walled centrifuge tube on ice. Cells remaining on the dish were rinsed and scraped with a further 0.5 ml H-8 buffer and combined in the centrifuge tube. If necessary, the volume was adjusted to 1.5 ml using H-8 buffer.

Cells were then disrupted by sonication (3 x 5 s, setting 24, MSE sonicator). The cell preparation was centrifuged at 100000g at 4°C for 30 min, yielding a membrane pellet and the supernatant cytosol. The latter was supplemented with 15% (w/v) glycerol and immediately frozen at -20°C in two aliquots, one of which was used for determination of protein.

The membrane pellet was resuspended in 1.5 ml extraction buffer (H-8 + 1% (w/v) NP-40) before sonication and centrifugation as described for the cytosolic extract. The resulting supernatant, designated as the membrane fraction, was supplemented with 15% glycerol and frozen at -20°C overnight in two aliquots as described above for the cytosol sample.

3.2.4. Sample application to gels

Cytosol and membrane fractions were rapidly thawed and 270 µl pipetted into a 1.5 ml Eppendorf tube. Cytosol samples were mixed with 30 µl cathode buffer containing 2% Triton X-100 (final concentration 0.2%) and Amaranth front dye (sufficient for the dye to be visible when loading onto the gel), whilst membrane samples received 30 µl cathode buffer containing Amaranth front dye only.

After removal of Parafilm from the ends of the glass tubes and rinsing of the gel surface 2-3 times with d H_2O, 200 µl

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

3.2.5. Electrophoresis running conditions

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 and the temperature of the system was maintained at approximately 4°C by circulating ethylene glycol antifreeze (precooled to -20°C using a Haake DK12 refrigeration unit) through the central cooling core of the gel apparatus.

Running conditions for the gels were as follows: voltage fixed at 100 V whilst the dye boundary moved through the stacking gel (running time approximately 4 h), and 160-180 V while the boundary moved through the resolving gel (total running time 7-9 h). Throughout electrophoresis, a current in excess of 0.8 mA per gel was avoided if possible.

3.2.6. Gel slicing and elution of enzyme activity from gel slices

Electrophoresis was complete when the dye boundary reached the end of the resolving gel. Gels were extruded from tubes by using a needle and syringe to force d H_2O up the inner rim of the tube until the gel was free. After discarding the stacking gel, the resolving gel was carefully laid in a polystyrene centrifuge tube and frozen by placing on a block of dry ice. Gels were sliced into 1 mm sections using a Mickle gel slicer (Mickle Laboratory Engineering Co. Ltd., UK) and the gel discs transferred to 3.5 ml polystyrene centrifuge tubes containing 300 µl elution buffer. Enzyme activity was eluted from slices overnight by gentle shaking

at 4°C.

3.2.7. Protein kinase assay using protamine sulphate as substrate

PKC activity was assayed in gel eluates using a microtitre assay technique adapted from the method of Kikkawa et al (1983a) which is similar to that described by Granet and Mastro (1987).

96-well U-form microtitre plates were taped securely to the top of a perspex box designed such that water heated and circulated by a thermostirrer maintained solutions in the microtitre wells at 32°C (see Fig. 3). Gel eluate (150 µl) was pipetted into wells and allowed to equilibrate to 32°C. The enzyme reaction was initiated by the addition of 50 µl protamine-assay mix (0.5 µCi [γ-^{32}P]ATP/assay) to the wells using a multichannel pipette (Flow Laboratories Ltd.). Final concentrations of assay components in the incubate were: 20 mM Tris-HCl, pH 7.4, 10 mM $Mg(NO_3)_2$, 24 µM ATP and 100 µg protamine. After 10 min incubation 150 µl of the reaction mix was removed using the multichannel pipette and immediately spotted onto Whatman 17Chr filter paper (approximate dimensions 20 mm x 6 mm) which had previously been 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. All papers were washed by shaking gently in 500 ml of 10% (w/v) TCA for 1 h, the washing solution being changed every 15 min. They were then briefly soaked in methanol (to aid drying) and placed in a hot oven until dry. Dried papers were transferred to 10 ml scintillation vials and 5 ml

Optiphase MP scintillation fluid added. Radioactivity was counted using a Packard Tricarb CA2000 scintillation counter.

Unless otherwise indicated in this thesis, one unit of PKC activity is defined as the amount of enzyme which incorporates 1 pmol of ^{32}P into protamine sulphate substrate per minute at 32°C.

3.2.8. Protein kinase assay using histone H1 as substrate

Phosphorylation of histone H1 by PKC is dependent on the presence of calcium and phospholipid and only activity of the holoenzyme is detected (protamine sulphate is a substrate for both the holoenzyme and PS/Ca^{2+}/DAG-independent proteolytic fragments).

Assay of PKC by this method is similar to that described above for protamine with the following exception: gel eluate (100 µl) and phospholipid mix (50 µl) were pipetted into wells and allowed to equilibrate at 32°C. The reaction was then initiated by the addition of 50 µl histone assay mix. Final concentrations of assay components in the mixture were: 20 mM Tris-HCl, pH 7.4, 10 mM $Mg(NO_3)_2$, 300 µM $CaCl_2$, 24 µM ATP, 100 µg PS, 10 µg diolein and 50 µg histone H1. Basal activity was measured by incubating samples in the absence of Ca^{2+}, PS and diolein, but in the presence of 0.5 mM EGTA.

3.2.9. Determination of protein

Protein was determined by the method of Bradford (1976) using Bio-Rad dye reagent. Dilutions of protein sample were made (1:100 and 1:200) and 0.8 ml mixed with 0.2 ml dye reagent. Samples were vortexed and allowed to stand for 5

min before reading absorbance at 595 nm using a Beckman DU-7 spectrophotometer. Blank readings were obtained using d H_2O in place of protein sample. A standard curve was constructed using bovine serum albumin and used to estimate protein concentrations.

3.3. Culture of cells on Cytodex 1 microcarrier beads

3.3.1. Initiation and maintenance of cultures

Two 10 ml aliquots (400 mg total) of Cytodex 1 microcarrier beads were transferred to separate 30 ml sterile Universal tubes and the beads sedimented by allowing them to stand for 2-3 min. The supernatant was carefully removed using a sterile pasteur pipette and the beads washed with 20 ml medium. This wash procedure was repeated once before aliquoting the washed microcarriers into a silanised flask specially designed for the maintenance of microcarrier cultures (see Figure 4).

Cells (1.5-3.0 x 10^7) were trypsinised from flasks and pelleted by centrifugation in sterile 30 ml Universal tubes. After aspiration of the supernatant, the cell pellet was gently resuspended in 6 ml medium before mixing with the washed microcarrier beads in the culture flask. The volume of medium was increased to 80 ml and the culture briefly gassed with 95% air/5% CO_2. The culture was maintained at 37°C by means of a water jacket supplied by a thermostirrer incorporating a water pump. Cells were allowed to attach to the beads for 4 h, the flask being gently shaken for 2 min every 30 min in order to ensure even distribution of cells between beads and to minimise clumping.

After the attachment period, the medium volume was increased to 400 ml (final bead density 1 mg/ ml), the flask regassed and the culture stirred at 30-50 rpm overnight using a low speed stirrer. The speed of stirring was adjusted to the minimum at which the beads remained in suspension.

3.3.2. Counting of cells cultured on microcarriers

A simple method of monitoring cell growth on microcarrier cultures is described by Van Wezel (1973). Cells growing on microcarriers are incubated in a hypotonic solution and nuclei released by lysis stained by a dye in this solution.

A 1 ml sample of evenly suspended culture was aliquoted into a polystyrene centrifuge tube. After the microcarriers had settled (1-3 min) the supernatant was discarded and the microcarriers resuspended in 1 ml 0.1 M citric acid containing 0.1% (w/v) crystal violet. The contents of the tube were vortexed briefly, capped securely to prevent evaporation and incubated at 37°C for 1 h. After incubation the tube was briefly vortexed again and the released stained nuclei counted with a haemocytometer.

3.3.3. Determination of $[Ca^{2+}]_i$ in microcarrier cultures

In all cases, microcarrier cultures which had been stirring overnight were used in experiments.

Stirring of the culture was stopped and the microcarrier beads allowed to settle for 1-2 min before reducing the volume of the culture to 200 ml. An aliquot (40 µl) of a stock solution of fura-2/AM was added to give a final concentration of 2 µM. Stirring was then resumed and the culture allowed to load with fura-2 for 1 h. (Note: in

initial experiments which utilised quin-2, cells were loaded in the presence of 20 µM quin-2 for 1 h).

After loading with fura-2, the medium supernatant was aspirated using a pipette and the microcarrier beads washed with 300 ml fresh medium. After removal of the wash supernatant the culture was finally resuspended in 200 ml medium and the flask gassed with 95% air/5% CO_2. When required, 9.5 ml of bead suspension (19 mg beads, approximately 2.4 x 10^6 cells) were harvested, washed and resuspended in 2 ml of neutrophil buffer. They were then transferred to a 1 cm^2 quartz cuvette and the fluorescence was monitored at 37°C in a Perkin-Elmer LS-5 spectrometer equipped with a thermally controlled cuvette holder and magnetic stirrer. Fluorimeter settings were: excitation wavelength 340 nM, emission wavelength 510 nM, excitation slit setting 5 nM and emission slit setting 20 nM.

After cuvettes had been allowed to equilibrate at 37°C, test compounds were added to the cuvette and the incubation continued for a further 10-15 min maximum. All test compounds were added dissolved in DMSO (final concentration < 0.5%) except the ether lipid SRI 62-834 which was an aqueous solution. Controls received carrier alone and did not themselves alter intracellular calcium levels over the course of incubations.

$[Ca^{2+}]_i$ was calibrated as described by Tsien et al (1982). Intracellular calcium was first equilibrated with external calcium (maximal fluorescence, F_{max}) by lysing cells in 1% Triton X-100 (100 µl, 20% Triton X-100). Minimal fluorescence (F_{min}) was then achieved by the addition of 200

µl 250 mM EGTA, pH 7.2 (final concentration, 20 mM EGTA) to chelate all Ca^{2+}. $[Ca^{2+}]_i$ could then be calculated from the ratio of the two values:

$$[Ca^{2+}]_i = 218 \times \frac{F_{obs} - F_{min}}{F_{max} - F_{obs}}$$

Units = nM

where F_{obs} is the observed fluorescence, F_{max} and F_{min} are as described and 218 is the dissociation constant (K_d), units nM, of Ca^{2+} for fura-2.

3.4. Mixed micellar assay for phorbol ester binding

3.4.1. Procedure

The assay measures phorbol ester binding to PKC by separating [^{3}H]PDBu bound to PKC in the presence of Triton X-100/phospholipid mixed micelles from free [^{3}H] PDBu by molecular sieve chromatography (Hannun and Bell, 1987).

3.4.2. Preparation of columns

Silanized glass Pasteur pipettes were packed with equal volumes of Ultrogel AcA 202 (approximately 2 ml) and washed with 20 mM Tris-HCl, pH 7.5. Columns were stored at 4°C in buffer containing 0.02% sodium azide when not in use.

3.4.3. Preparation of mixed micelles

An appropriate amount of PS in 95% chloroform/5% methanol was transferred to a glass centrifuge tube (calculated to give a final micellar concentration of 20 mol% as a mole fraction of Triton X-100, where 1 mol% PS = 43 µM) and the solvent evaporated under a stream of nitrogen. The PS was then solubilised in 3% (w/v) Triton X-100 by vortexing vigorously for 1-5 min before incubating at 27°C for 10 min.

then solubilised in 3% (w/v) Triton X-100 by vortexing vigorously for 1-5 min before incubating at 27°C for 10 min.

3.4.4. Assay method

For one assay, the following reagents were mixed in a 3 ml polystyrene tube: 10 µl 200 mM Tris-HCl, pH 7.5, 10 µl 6 mM $CaCl_2$, 10 µl mixed micelles (3% (w/v) Triton X-100 + 20 mol% phosphatidylserine) and 50 µl [^{3}H] PDBu (diluted with d H_2O to give the appropriate final concentration). Where a saturating concentration of [^{3}H] PDBu was required, a final assay concentration of 75 nM was used.

The assay reagents were then split equally into two tubes and 10 µM unlabelled PDBu (5 µl 2 mM stock PDBu/1 ml assay mix, final DMSO concentration 0.5%) added to one of them to determine nonspecific binding. Aliquots of both fractions were then transferred to 3 ml polystyrene tubes and the binding reaction initiated by the addition of 20 µl of protein sample. In these preliminary experiments, crude cytosolic fractions prepared as described in Section 3.2.3 were employed. The final concentrations of assay components (volume 100 µl) were: 20 µM Tris-HCl, pH 7.5, 200 µM $CaCl_2$ (concentration of free Ca^{2+} in the assay determined by the amount of EGTA in the protein sample), 0.3% Triton X-100 and 1-100 nM [^{3}H]PDBu (usually 75 nM). After 10 min, when binding equilibrium had been reached, the tubes were transferred to ice.

An aliquot of the reaction mixture (50 µl) was then added to a Pasteur containing 2 ml AcA 202 gel previously equilibrated with a buffer containing 20 mM Tris-HCl, pH 7.5, 200 µM $CaCl_2$ and 0.015% (w/v) Triton X-100. The column

This fraction contained PKC, mixed micelles and bound [^{3}H]PDBu and was designated as the total binding fraction. The gel was then washed with a further 2 ml of buffer and the eluate collected in different scintillation vials. This fraction was designated as the free [^{3}H]PDBu fraction.

Scintillation fluid (20 ml) was added to the vials and radioactivity counted using a Packard Tricarb CA2000 scintillation counter.

Non-specific binding was determined by collecting the first 1 ml when the binding reaction was carried out in the presence of 10 µM unlabelled PDBu. This was then subtracted from the total binding to give the specific binding.

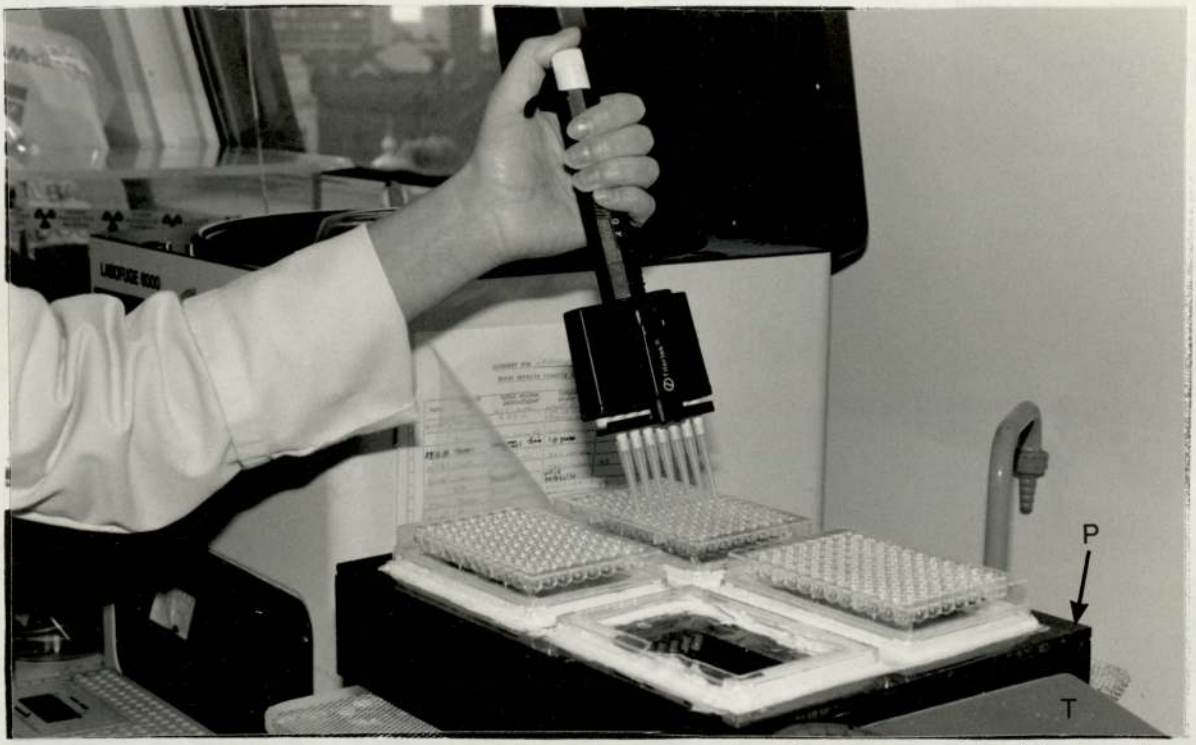


Figure 3. Apparatus for the assay of PKC activity using microtitre plates. A thermostirrer (T) was used to heat and circulate water through the base of 96 well U-form microtitre plates taped securely to the top of a perspex box (P), such that solutions in the microtitre wells were maintained at 32°C.

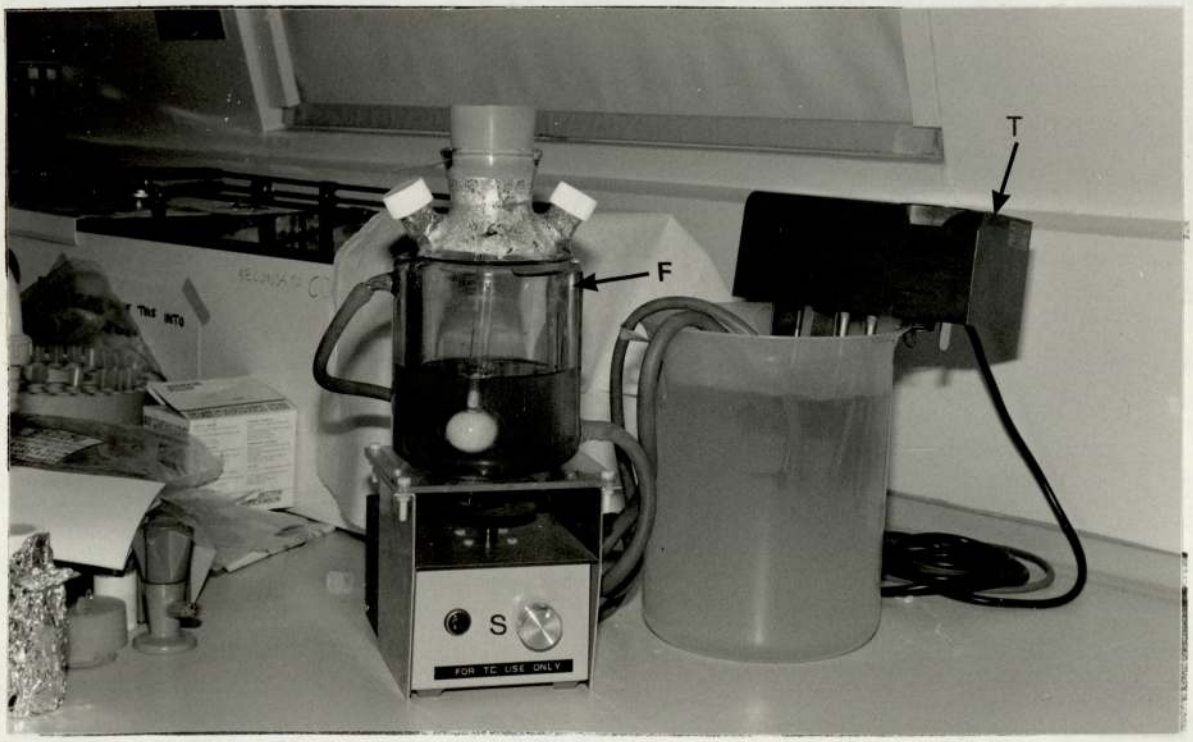


Figure 4. Apparatus for the culture of cells on Cytodex 1 microcarrier beads. Cells were cultured in a silanized culture flask (F) and stirred using a slow speed stirrer (S). Cultures were maintained at 37°C by the circulation of water through a water jacket surrounding the flask using a thermostirrer (T).

SECTION 4: RESULTS AND DISCUSSION

SECTION 4: RESULTS AND DISCUSSION

Section 4.1. Studies of the antiproliferative properties of compounds on A549 cell growth

Section 4.1.1. Introduction

TPA potently inhibits the growth of the A549 human lung carcinoma cell line (Gescher and Reed, 1985). Incubation of cultures with 10 nM TPA caused complete inhibition of cell growth for 5-6 days, after which cells began to grow at a rate similar to that of control cells. When treated cells were trypsinised and reseeded sensitivity to TPA was regained. However, cells subcultured in the continued presence of 10 nM TPA for several weeks gradually became completely refractory to the growth-inhibitory properties of the phorbol ester, such that after 9 weeks continued exposure TPA was unable to slow cell growth.

In this section, the ability of phorbol esters and related compounds to influence A549 cell growth was investigated in more detail in order to elucidate mechanism(s) of growth inhibition. In particular, the growth-inhibitory potential of mezerein, DAGs and bryostatins have been evaluated. Like the phorbol esters, these compounds are known activators of the enzyme PKC. It is believed that the ability to activate PKC is at least partly responsible for the many diverse cellular responses elicited by phorbol esters (see Introduction).

Section 4.1.2. Dependence of growth of A549 cells on concentration of phorbol esters and related compounds

4.1.2.1. Phorbol esters

A549 cells were incubated with various concentrations of phorbol ester for 5 days before growth was assessed by counting cells. Alternatively, cells were treated for 24 h before the incorporation of [^{3}H]TdR into cells was measured as a quantitative index of cell growth. Both methods yielded almost identical IC_{50} values.

TPA potently inhibited cell growth as assessed by cell count with an IC_{50} value of 0.2 nM (Fig. 5 and Table 1). Similarly, incorporation of [^{3}H]TdR was inhibited by TPA with an IC_{50} value of 0.2 nM (Fig. 6). In both cases, the extent of growth inhibition at the maximal inhibitory concentration (> 5 nM) was 90-95%. Assay of LDH release from TPA-treated cells indicated that cytotoxicity was only evident at micromolar concentrations, three orders of magnitude greater than the observed IC_{50} value for TPA (Table 1).

PDBu inhibited the incorporation of [^{3}H]TdR into cells with an IC_{50} of 16 nM (Fig. 6). PDA was only weakly growth-inhibitory at concentrations much higher than those observed for TPA or PDBu with an IC_{50} value for the inhibition of [^{3}H]TdR incorporation of 0.8 µM (Fig. 6). In the presence of PDA at 1 µM, [^{3}H]TdR incorporation was reduced to 41 $\pm$ 7% of controls. 4-α-PDD, which is not a tumour promoter, lacked growth-inhibitory properties at concentrations of up to 1 µM (Fig. 6).

Treatment of cells for 24 h with 10 nM TPA in the presence of the PKC inhibitor H-7 at concentrations of up to 100 µM did not alter the ability of the phorbol ester to inhibit [^{3}H]TdR incorporation into cells (Fig. 7), and did not block the typical morphology change associated with growth inhibition (see Section 4.1.7).

4.1.2.2. Mezerein

Like phorbol esters, mezerein was found to be a potent inhibitor of the growth of A549 cells. Inhibition of growth determined by cell counting after incubation of cells in the presence of various concentrations of mezerein indicated an IC_{50} of 3.9 nM (Fig. 5A and Table 1). DNA synthesis, as measured by incorporation of [^{3}H]TdR into cells, was similarly inhibited with an IC_{50} of 5 nM (Figure 5B). Therefore the potency of mezerein as an inhibitor of the growth of A549 cells is approximately a twentieth of that of TPA. Maximal inhibition of growth was 90-95% and it was evident at concentrations of 50 nM and higher. Like TPA, a cytotoxic influence of mezerein was only observed at concentrations much higher than the determined IC_{50} value (Table 1).

4.1.2.3. DAGs

Cells were treated with the synthetic cell-permeable PKC-activating DAGs 1-oleoyl-2-acetylglycerol (OAG) or 1,2-dioctanoylglycerol (1,2-diC_8) 9 times during a period of 72 h. Multiple additions were performed to maintain the DAGs at sufficiently high concentrations, as it has been

shown that inactivation by metabolism is rapid in biological systems (see Welsh and Cabot, 1987).

Figure 9A illustrates that treatment of low density cultures of A549 cells with 1,2-diC$_8$ 9 times over a 3 day period resulted in a concentration-dependent decrease in cell numbers when counted after 5 days. Cell growth was inhibited with an IC_{50} value of 44 µM (Table 1). Visual examination of cultures treated with 1,2-diC$_8$ revealed that the concentrations used were rapidly cytotoxic to cells. At a concentration of 100 µM, 1,2-diC$_8$ caused 100% cell death, with many detached cells seen earlier than 15 min after the first addition, while only 30% of those still attached to the plastic excluded trypan blue. Assessment of cytotoxicity by assay of LDH release from treated cells (Fig. 9B and Table 1) supported the observation that 1,2-diC$_8$ was merely exerting a cytotoxic influence, as the LC_{50} value of the DAG, 56 µM, was not significantly different from the IC_{50} value. At non-cytotoxic concentrations, a morphology change such as that observed with TPA was not seen.

The isomer 1,3-diC$_8$, which unlike 1,2-diC$_8$ is not a PKC activator (Boni and Rando, 1985), was also tested for its growth inhibitory potential and it displayed growth-inhibitory (IC_{50} 50 µM) and cytotoxic (LC_{50} 43 µM) properties similar to that of 1,2-diC$_8$ (Table 1).

OAG inhibited cell growth in a manner similar to that of diC$_8$, with an IC_{50} value of 70 µM (Table 1). Visual indications of cytotoxicity were evident within 15 min at concentrations above 10 µM. After treatment for 15 min with

100 µM OAG, many detached cells were seen and fewer than 50% of those still attached were able to exclude trypan blue. Cytotoxicity as assessed by the release of LDH from cells indicated an LC_{50} value of 75 µM (Table 1), supporting the conclusion that toxicity was high at growth-inhibitory concentrations of DAGs.

Interestingly, when the growth-inhibitory properties of OAG and 1,2-diC_8 were assessed by [^{3}H]TdR incorporation after treatment with the DAG every 8 h for 24 h (Fig. 10), the growth-inhibitory influence of the DAG was not as pronounced as in experiments in which growth inhibition was determined by counting cell numbers after 5 days (Table 1). To investigate this discrepancy, the cytotoxic influence of 100 µM 1,2-diC_8 at various cell densities was investigated following treatment every 8 h for 24 h by assay of LDH leakage from treated cells (Fig. 11). At low cell densities corresponding to those used in the cell counting experiments (2.1 x 10^3 cells/cm^2 or 5.2 x 10^3 cells/cm^2), treatment with 1,2-diC_8 for 24 h resulted in 100% cytotoxicity. However, at higher cell densities cytotoxicity was markedly reduced, such that at cell densities corresponding to those used in [^{3}H]TdR incorporation assays (2.1 x 10^4 cells/cm^2), cytotoxicity was reduced to 47 $\pm$ 10% (SD; n=4) (Fig. 11).

In order to reduce potential cellular metabolism of DAGs, cells were treated every 8 h for 24 h with 1,2-diC_8 at weakly or non-toxic concentrations (< 25 µM) in the presence of R59022, a reported inhibitor of DAG kinase (de Chaffoy de Courcelles *et al*, 1985), before measurement of

[^{3}H]TdR incorporation (Fig. 12). Treatment of cultures with concentrations of R59022 > 10 µM was observed to result in inhibition of [^{3}H]TdR into cells, but no difference was observed between cultures treated with diC_8 and concentrations of up to 50 µM R59022 and those treated with diC_8 alone (Fig. 12).

4.1.2.4. Bryostatins

Inhibition of DNA synthesis by both bryostatins 1 and 2 exhibited biphasic concentration-response curves (Fig. 13). Bryostatin 1 inhibited the incorporation of [^{3}H]TdR incorporation maximally at a concentration of 10 nM. The extent of inhibition was between 60 and 80%. However arrest of DNA synthesis was diminished or abolished at higher concentrations (Fig. 13A). Similarly, bryostatin 2 decreased incorporation of [^{3}H]TdR into cells maximally at 100 nM, whereas higher concentrations were less inhibitory (Fig. 13A).

Inhibition of growth by bryostatin 1 was also assessed by cell counting after 5 days incubation (Fig. 13B). The characteristic biphasic concentration-response curve was seen, with cell number reduced by 64 $\pm$ 4% compared to controls at a concentration of 5 nM.

4.1.2.5. EGF

In order to determine if the polypeptide growth factor EGF was capable of modulating A549 cell proliferation, cultures were treated with EGF at concentrations of 100 and 10 ng/ml for 5 days before counting cells. No influence of EGF on

growth or cellular morphology was observed when cells were cultured in either 10% or 1% FCS (Fig. 14), nor was any modulation of the growth-inhibitory influence of 10 nM TPA by 100 and 10 ng/ml EGF noted.

4.1.2.6. Discussion

In many cultured cell lines, TPA has been shown to be mitogenic; there are rather less examples in the literature in which the growth of cells was inhibited (for a review see Gescher, 1985).

In agreement with the findings of Gescher and Reed (1985) biologically active phorbol esters were found to be potent inhibitors of the growth of A549 cells. After treatment for 5 days with various concentrations of TPA, cell numbers were reduced with an IC_{50} value of 0.2 nM. Similarly, inhibition of DNA synthesis as assessed by [^{3}H]TdR incorporation after 24 h treatment with the phorbol ester yielded an identical IC_{50} value. It is important to note that, in certain instances, incorporation of [^{3}H]TdR is not necessarily a good indicator of cell proliferation, as the amount of thymidine incorporated into a cell depends not only on overall DNA synthetic activity but also on factors such as intracellular pools of nucleotides, activity of thymidine transport mechanisms and the activities of a number of enzymes including thymidine kinase (Pardee et al, 1986). However, in A549 cells results obtained by measurement of [^{3}H]TdR incorporation always closely mirrored those of cell counts with the exception of DAGs (for reasons discussed below) and the bryostatins (for reasons discussed in Section

4.1.3).

The phorbol esters PDBu (IC_{50} 16 nM) and PDA (IC_{50} 0.8 μM) also inhibited cell growth, although less potently than did TPA. The relative potency of phorbol ester analogues in inhibiting proliferation of A549 cells therefore coincides with their efficiency in promoting tumours (Blumberg, 1980). Growth inhibition was not due to cytotoxic effects as (i) cell numbers were never lower than those seeded, and (ii) LDH cytotoxicity data did not indicate significant cell death , even at micromolar concentrations. 4-α-PDD, which is inactive as a tumour promoter, did not affect cell growth.

The PKC inhibitor H-7, first described by Hidaka et al (1984), has been utilized in many studies in support of a role for PKC in a variety of physiological responses (reviewed by Hidaka and Hagiwara, 1987). For example, phorbol ester-induced phenotypic differentiation of HL-60 cells was reported to be inhibited by H-7 in a concentration-dependent manner, such that 7.5 μM H-7 restored the growth rate of cells treated with 0.5 nM TPA to the level of cells treated with inhibitor alone (Matsui et al, 1986). In A549 cells, the inability of H-7 to block growth inhibition by 10 nM TPA at concentrations as high as 100 μM seems to indicate that PKC does not play an important role in phorbol ester-induced growth inhibition. However, one must draw this conclusion with caution as (i) the specificity and efficacy of H-7 in inhibiting PKC activity is questionable (Garland et al, 1987; Watson et al, 1988), and (ii) although reported to be cell-permeable (Hidaka et

al, 1984), it is possible that concentrations of H-7 present in A549 cells were insufficient for inhibition of PKC activity.

The response of A549 cells to phorbol esters can be compared with that of other cell lines where growth is inhibited by TPA. Osborne and co-workers demonstrated that in MCF-7 human mammary carcinoma cells TPA-induced blockage of cell replication was accompanied by stimulation of protein synthesis resulting in hypertrophied cells. After 10-12 days of exposure to TPA cells became detached from the culture dish (Osborne et al, 1981). Although cells were unable to proliferate in the continued presence of TPA, removal of the phorbol ester resulted in the resumption of growth. Eppenberger and co-workers have studied the influence of phorbol esters on the growth of a panel of human mammary carcinoma cells, the MCF-7, T-47-D, ZR-75-1, MDA-MB-231, HBL-100 and BT-20 cell lines (Roos et al, 1986). Only the T-47-D line did not show significant growth inhibition by TPA, whilst in the other cell lines half-maximal inhibition was observed at TPA concentrations ranging from 0.5 nM to 30 nM, while maximal growth inhibition was obtained with 300 nM TPA. The highest sensitivity towards potent tumour promoters with respect to growth arrest was exhibited by MCF-7 cells. As is the case in A549 cells, the potency of the phorbol ester analogues paralleled their activity as tumour promoters. Teleocidin and aplysiatoxin, which exert TPA-like tumour promoting activity but are structurally unrelated to phorbol esters (Sugimura, 1982), inhibited the proliferation of MCF-7 cells

with a concentration-dependency similar to that seen with TPA.

The A431 human epidermoid carcinoma cell line is unusual in that it displays an extremely high number of EGF receptors (Haigler et al, 1978) yet its growth is inhibited by concentrations of EGF that are mitogenic with other cell lines (Gill and Lazar, 1981; Barnes, 1982). Smith and co-workers reported that addition of TPA and EGF to A431 cells produced an almost immediate cessation of cell growth in a concentration-dependent manner within 24 h in both low and high density cultures. TPA enhanced the growth-inhibitory effect of EGF in high density cultures (Smith et al, 1983). TPA was found to inhibit the high affinity binding of ^{125}I-EGF to A431 cells (Davis and Czech, 1984) in a way similar to that reported for several other cell lines in culture (Brown et al, 1979; Shoyab et al, 1979) and to mediate the phosphorylation of specific EGF receptor peptides via activation of PKC (Cochet et al, 1984; Davis and Czech, 1984; Iwashita and Fox, 1984). On the basis of these findings it was hypothesized that phosphorylation of the EGF receptor by TPA might play a role in the ability of tumour promoters to potentiate the mitogenic effect of EGF. In this study it has been demonstrated that at concentrations reported to be growth-inhibitory in A431 cells (Smith et al, 1983), EGF did not influence the growth of A549 cells and did not modulate growth inhibition by TPA. Therefore, it would appear that EGF or its receptor does not play a role in the growth inhibition of A549 cells caused by phorbol esters. A549

cells, which display normal numbers of EGF receptors (Haeder et al, 1988), are therefore distinct from a number of malignant cell lines growth inhibited by EGF which express high levels of EGFR (Lifshitz et al, 1983; Filmus et al, 1985; Kamata et al, 1986).

Mezerein induces many of the same biological responses observed when cells in culture are treated with TPA. Therefore, it is perhaps not surprising that mezerein was found to inhibit the growth of A549 cells in a fashion similar to TPA, although with a potency only a twentieth of that of the phorbol ester. However, it should be noted that although mezerein, like TPA, was growth-inhibitory in the HL-60 promyelocytic cell line, the induction of monocytic maturation observed was qualitatively different (Morin et al, 1987). Mezerein is a potent activator of PKC (Miyake et al, 1984). Therefore the observation that two structurally different PKC activators inhibit cell proliferation apparently in the same manner argues for the involvement of PKC in mediating growth inhibition.

DAGs are the presumed physiological ligands of PKC, generated by the receptor-mediated hydrolysis of phosphoinositides by PI-PLC (Nishizuka, 1984; Bell, 1986). It now appears that the biological activity of phorbol esters is due, at least in part, to the ability of these agents to readily enter the cell and to imitate the action of DAGs via activation of PKC. A priori, one might therefore expect exogenously added synthetic cell-permeable DAGs such as OAG (Mori et al, 1982) and 1,2-diC_8 (Davis et al, 1985) to induce the same biological responses observed with

phorbol esters. Indeed, certain studies support this hypothesis. Both DAGs and phorbol esters cause a reduction of the high affinity binding of EGF to its receptor (Davis et al, 1985), induce mitogenesis of 3T3 fibroblasts (Rozengurt et al, 1984) and DAGs mimic some of the biochemical changes associated with tumour promotion by TPA in mouse epidermis (Smart et al, 1986). Recently, Verma (1988) has reported that 1,2-diC_8 is active as a second stage tumour promoter. Equally however, in other model systems DAGs are unable to mimic phorbol ester action, such as in the induction of differentiation of the U937 monoblastoid cell line (Ways et al, 1987). As previously discussed in Section 1.5.2, the possibility that DAGs parallel TPA in inducing the monocytic differentiation of HL-60 cells has been discussed in several recent papers (Ebeling et al, 1985; Kreutter et al, 1985; Morin et al, 1987; Yamamoto et al, 1985), although to date no conclusive proof that DAGs are able to induce HL-60 cell differentiation has been reported.

In A549 cells, neither OAG or diC_8 were observed to mimic the non-cytotoxic growth inhibitory effect of phorbol esters. Rather, a cytotoxic effect was noted in cell count experiments at concentrations of greater than 10 μM and 100% cytotoxicity was evident at 100 μM. This effect was certainly non-specific and unlikely to be mediated via PKC as the 1,3-diC_8 isomer, which is not a PKC activator (Boni and Rando, 1985), was as potently cytotoxic as the PKC activator 1,2-diC_8. A reduced influence of DAGs was observed in experiments in which their growth-inhibitory

potential was assessed by [^{3}H]TdR incorporation rather than by cell counting, presumably due to (i) reduced cytotoxicity of DAGs at high cell densities, and (ii) normalization of data by expression of results as "[^{3}H]TdR incorporation/10^5 cells", thereby correcting for any decrease in cell numbers caused by toxicity.

It seems reasonable to assume that the hydrophobic DAGs exert their toxicity by disruption of cellular membranes. It is interesting to note that the toxic concentrations of DAGs in A549 cells were of an order of magnitude lower than those reported in HL-60 cells, 50 µM versus > 300 µM (Ebeling et al, 1985; Morin et al, 1987). Presumably, this phenomenon reflects the fact that cell densities achieved in suspension cultures of HL-60 cells far exceed those possible in monolayer cultures of A549 cells. This argument is supported by the observation that the cytotoxicity of 1,2-diC$_8$ in A549 cells was diminished at high cell densities.

Treatment of A549 cells with non-cytotoxic concentrations of DAGs (< 10 µM) appeared to have no effect on cell growth. Furthermore, the DAG kinase inhibitor R59022, which should potentiate any biological influence of DAGs in cells by preventing the metabolism of free DAG to phosphatidic acid (de Chaffoy de Courcelles et al, 1985), did not alter the cellular response to 1,2-diC$_8$ at the concentrations tested. Inhibition of DAG kinase by R59022 has been reported to potentiate short-term responses mediated by PKC in intact platelets (de Chaffoy de Corcelles et al, 1985; Joseph et al, 1988) and neutrophils (Muid et al, 1987). The compound

has also been reported to modulate long-term mitogenesis in Swiss 3T3 cells by potentiating PKC activation and DNA synthesis by bombesin (Morris et al, 1988). Interestingly, an inhibition of [^{3}H]TdR incorporation at R59022 concentrations > 6 µM was observed in 3T3 cells (Morris et al, 1988), a phenomenon also noted in A549 cells at concentrations > 5 µM. Presumably this inhibition of growth by R59022 represents non-specific effects of higher (> 10 µM) concentrations, as has been reported in a number of cell lines (Mahadevappa, 1988; Nunn et al, 1987).

The inability of OAG and 1,2-diC$_8$ to mimic phorbol ester-induced growth inhibition in A549 cells is in direct contrast to recent reports which investigated the antiproliferative influence of 1,2-diC$_8$ in the MCF-7 breast cancer cell line using an experimental protocol very similar to that employed for A549 cells (Issandou and Darbon, 1988; Issandou et al, 1988). Treatment of MCF-7 cells with 1,2-diC$_8$ 3 times a day for 5 days was observed to result in a concentration-dependent inhibition of proliferation with an IC$_{50}$ value of 32 µM, compared with an IC$_{50}$ value of 44 µM in 1,2-diC$_8$-treated A549 cultures (Section 4.1.2). Although cell densities used were very similar to those used in experiments which investigated the antiproliferative influence of 1,2-diC$_8$ in A549 cells, no cytotoxic influence of the DAG in MCF-7 cells was discussed even though concentrations as high as 248 µM were employed. It is therefore possible that 1,2-diC$_8$ was exerting a non-specific cytotoxic effect in MCF-7 cells rather than a PKC-mediated influence as suggested by the authors (Issandou and Darbon,

1988).

From the finding that DAGs, activators of PKC, are unable to mimic TPA-induced growth arrest in A549 cells, one might conclude that the activation of PKC alone is not sufficient to trigger inhibition of the growth of A549 cells, and that phorbol esters exert their influence via a cellular receptor other than PKC. However, this conclusion cannot be drawn with any certainty as a number of other factors have to be considered. It is well documented that phorbol esters, unlike DAGs, are poorly metabolized in biological systems (Cabot, 1984). The stability and metabolic fate of DAGs is poorly understood, although it has been assumed that the much greater biological potency of phorbol esters compared to DAGs is due to the high metabolic stability of the former. Welsh and Cabot compared the stability of TPA and acylacetylglycerol DAGs such as OAG in HL-60 cells (Welsh and Cabot, 1987). Their results showed that DAGs were rapidly metabolized both by cells and serum lipases. It is therefore conceivable that the inability of DAGs to cause non-toxic growth inhibition in A549 cells was due to metabolic inactivation by non-specific esterases. The role of DAG kinase in the metabolism of DAGs would appear to be small as the enzyme inhibitor R59022 was without effect. It is doubtful that the cells possess the ability to completely metabolize the high concentrations of DAGs administered, but the possibility that DAG was inactivated by serum cannot be ruled out. However, this possibility seems unlikely as DAG concentrations were maintained by regular replenishment. The best way to ascertain that the DAGs were penetrating the

cells at effective concentrations and that PKC activation was occurring would be to study the phosphorylation patterns of intracellular proteins. In view of the recent identification and characterisation of several PKC isozymes with varying biological properties (reviewed by Nishizuka, 1988), an alternative reason for the inability of DAG to mimic the action of phorbol esters in A549 cells could be that the phorbol esters modulate the activity of PKC isozyme(s) which are not activated by DAG.

Study of the effects of treatment of A549 cells with bryostatins 1 and 2 revealed the dual nature of the efficacy of these compounds previously reported in other cell lines. Both bryostatins 1 and 2 inhibited DNA replication and growth in a similar manner to TPA, with maximal inhibition occurring at 10 and 100 nM, respectively, although the degree of inhibition by bryostatin 2 was slightly greater than that of bryostatin 1. However, unlike TPA, at higher concentrations they blocked both their own inhibitory action and the antireplicative action of TPA and mezerein (see also section 4.1.8).

Bryostatins have previously been shown to mimic TPA in that they, for example, inhibit phorbol ester binding and caused mitogenesis in Swiss 3T3 cells (Smith et al, 1985), stimulate polymorphonuclear leukocytes (Berkow and Kraft, 1985) and induce ornithine decarboxylase and inhibit cell-cell communication in keratinocytes (Sako et al, 1987; Pasti et al, 1988). In contrast, bryostatins block the induction of differentiation of HL-60 cells caused by phorbol esters (Kraft et al, 1986), human colon cancer cells

(McBain et al, 1988) and primary mouse epidermal cells (Sako et al, 1987). They also restore the differentiation response in Friend erythroleukaemia cells in which drug-induced differentiation has been suppressed by PDBu (Dell'Aquila et al, 1987).

Here it has been shown that bryostatins mimic phorbol esters in the growth inhibition of A549 cells at nanomolar concentrations but at higher concentrations, unlike TPA, growth arrest is reduced or overcome completely. Other ways in which the bryostatins were observed to differ from TPA in A549 cells are discussed in Sections 4.1.3, 4.1.6 and 4.1.8.

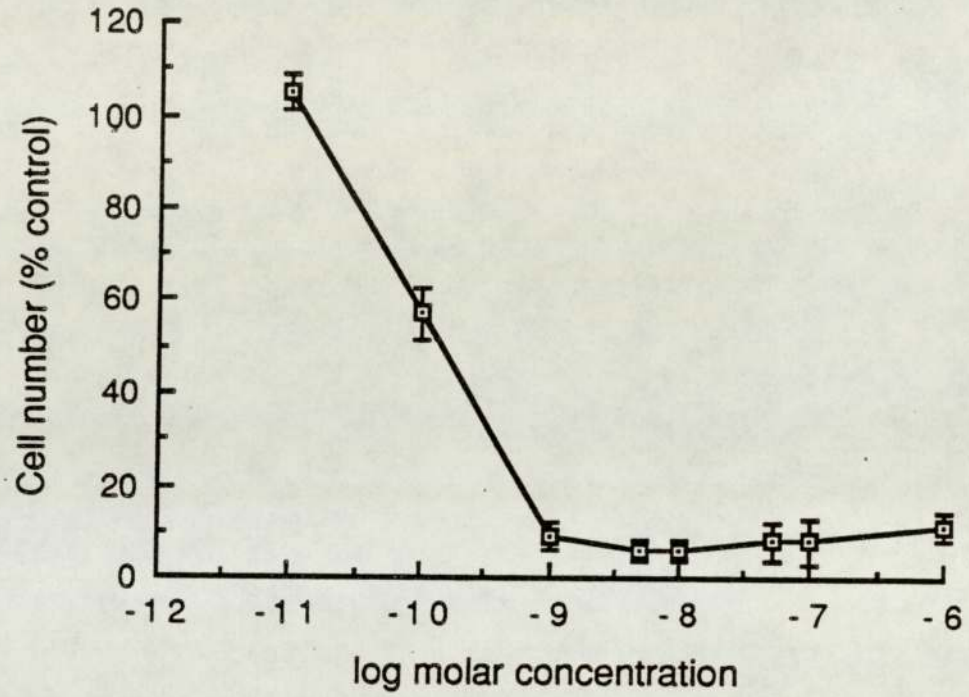


Figure 5. The effect of different concentrations of TPA on A549 cell growth assessed by cell counting. Cells (2×10^4) were treated for 5 days before counting cell numbers (mean $\pm$ SD (n=4) in one experiment representative of 3).

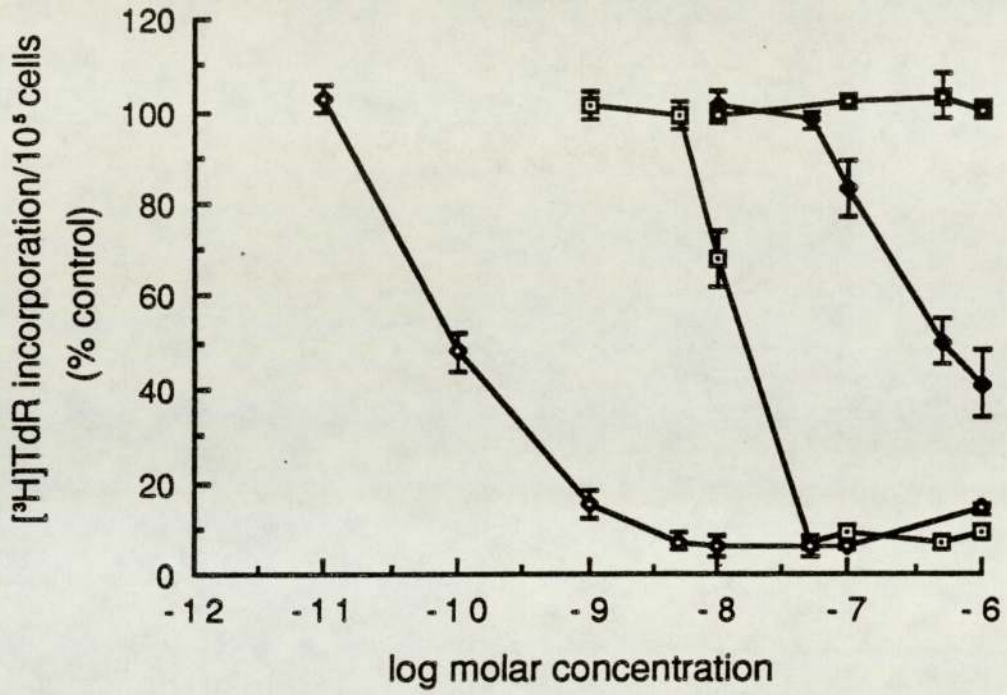


Figure 6. The effect of different concentrations of phorbol esters on A549 cell growth assessed by [³H]TdR incorporation. Cells (2 x 10^5) were treated with phorbol ester for 24 h before measurement of [³H]TdR incorporation (mean ± SD (n=4-6) in experiments representative of 3-4). ◇; TPA, ◻; PDBu, ◆; PDA, ■; 4-α-PDD.

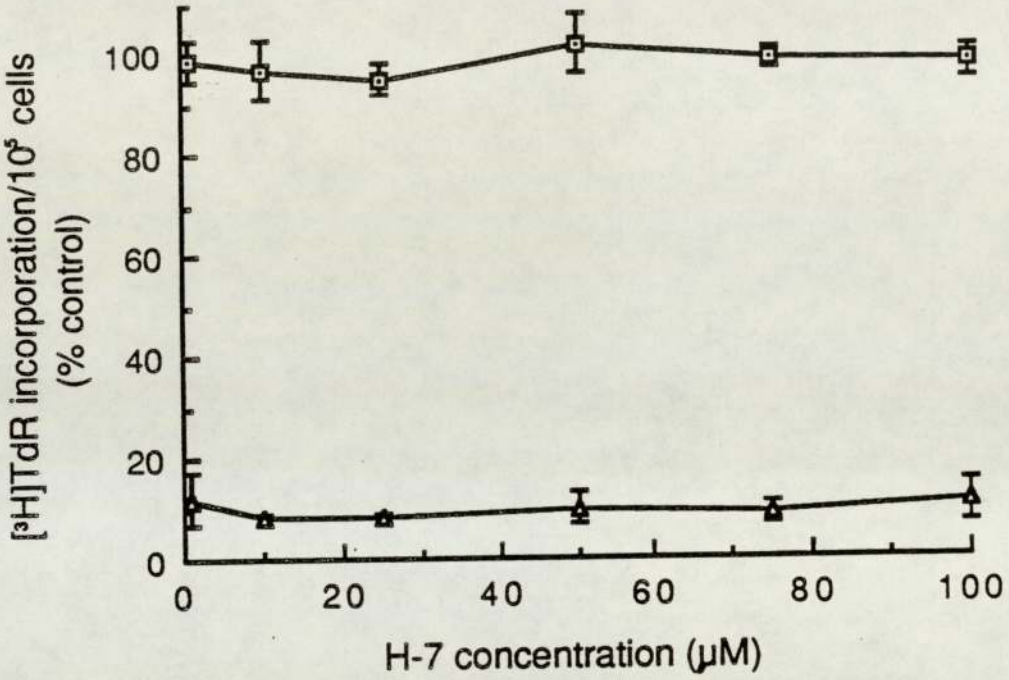


Figure 7. The effect of the PKC inhibitor H-7 on the growth-inhibitory influence of 10 nM TPA in A549 cells. Cells (2 x 10^5) were treated with H-7 in the presence (▲) and absence (□) of 10 nM TPA for 24 h before measurement of [³H]TdR incorporation (mean ± SD (n=4) in one experiment representative of 2).

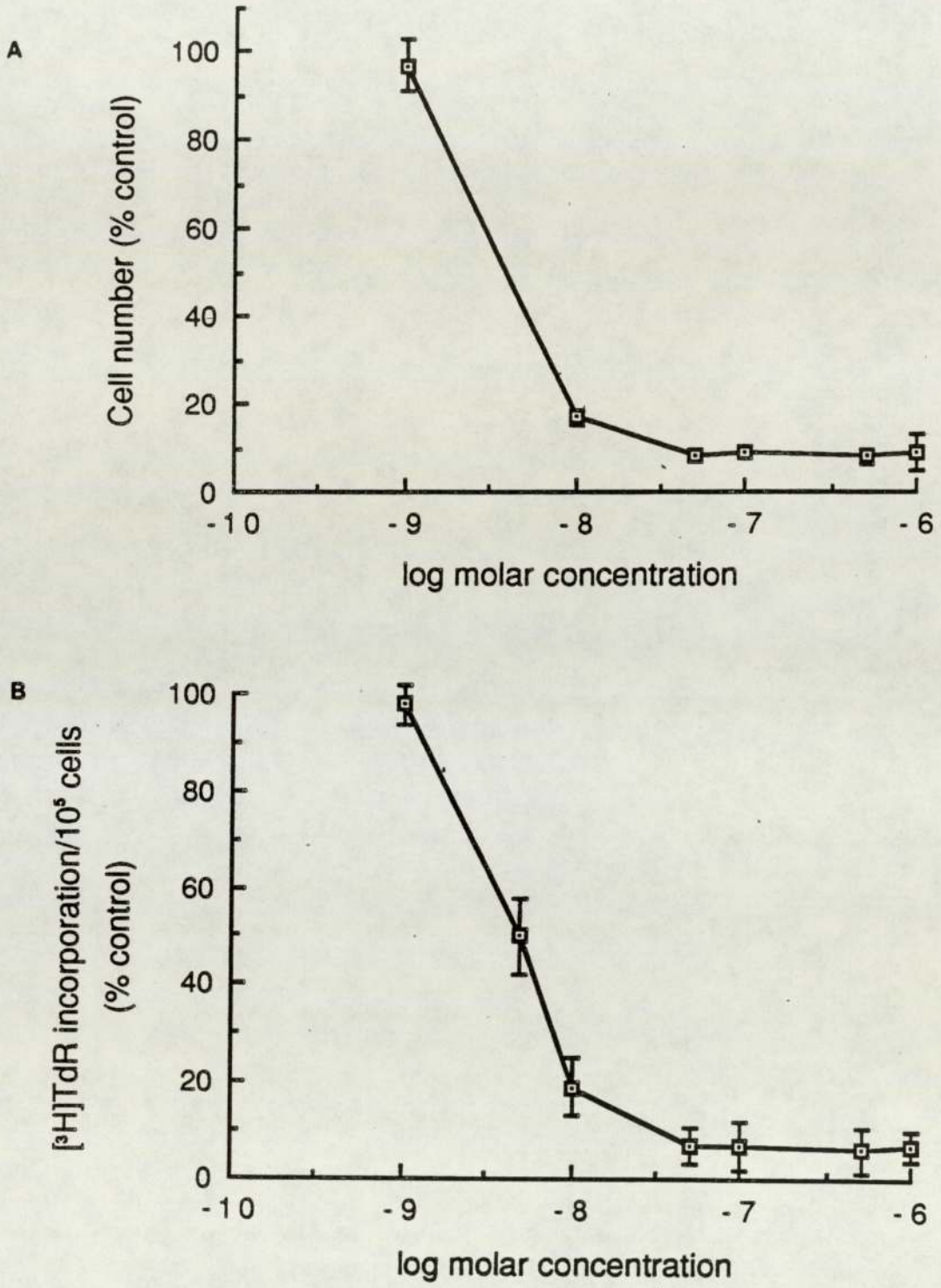


Figure 8. The effect of different concentrations of mezerein on A549 cell growth assessed by (A) cell counting and (B) [^{3}H]TdR incorporation.

(A) Cells (2 x 10^4) were treated for 5 days before counting cell numbers (mean ± SD (n=4) in one experiment representative of 3).

(B) Cells (2 x 10^5) were treated for 24 h before measurement of [^{3}H]TdR incorporation (mean ± SD (n=4) in one experiment representative of 2).

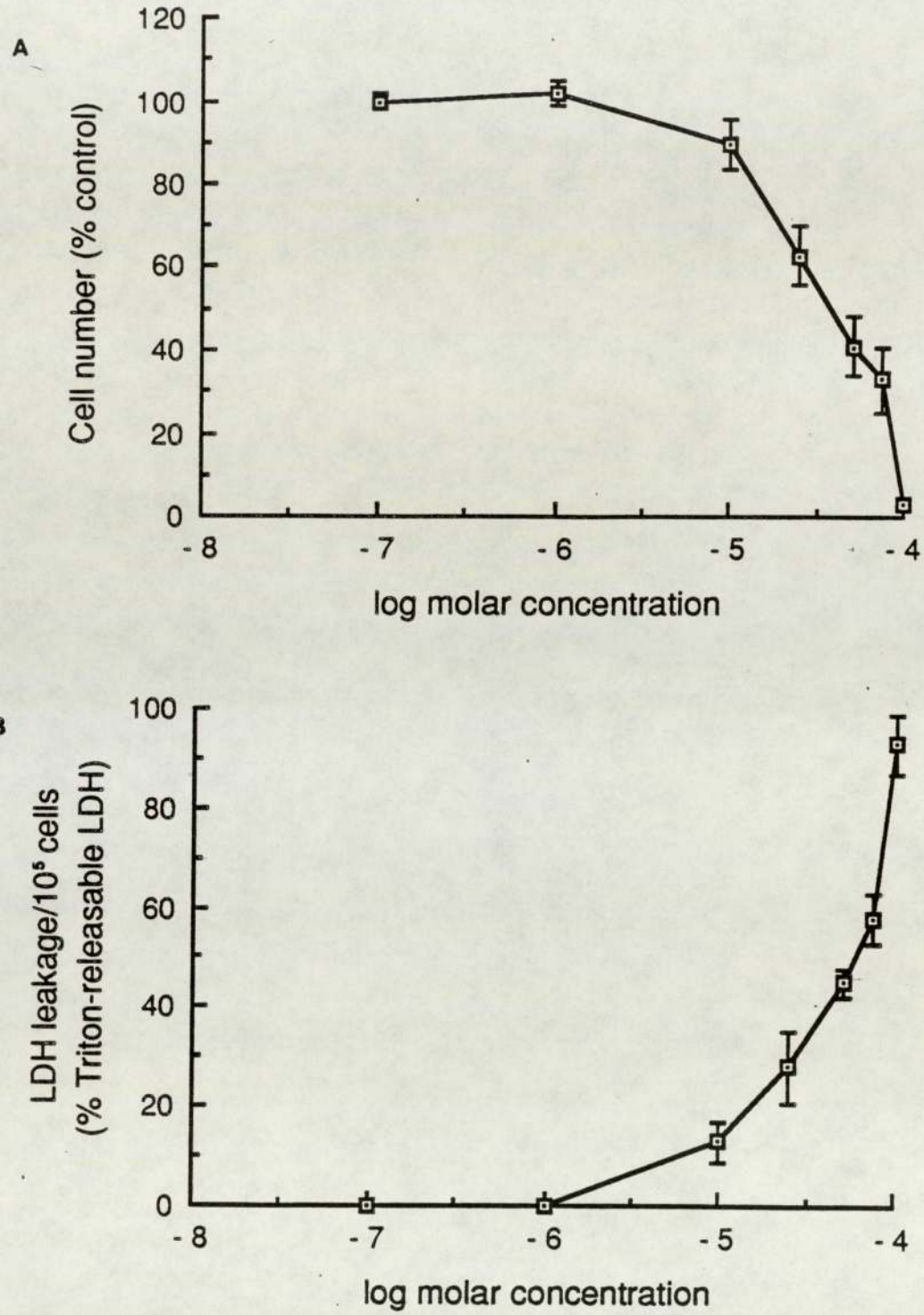


Figure 9. The (A) growth-inhibitory and (B) cytotoxic influence of different concentrations of 1,2-diC_8 on A549 cells.

(A) Cells (5 x 10^4) were treated 3 times a day for 3 days and cell numbers counted after 5 days (mean ± SD (n=4) in one experiment representative of 5).

(B) Cells (1 x 10^5) were treated twice over a period of 24 h before measurement of LDH release from cells (mean ± SD (n=3)).

Compound	Growth inhibition $IC_{50}(\mu M)$[a]	Cytotoxicity $LC_{50}(\mu M)$[b]
TPA	0.0002 (0.0001, 0.0004)[c]	> 1
Mezerein	0.0039 (0.0027, 0.0055)	> 1
OAG	70 (60, 81)	75 (67, 82)
1,2-diC_8	44 (35, 53)	56 (42, 68)
1,3-diC_8	50 (36, 65)	48 (40, 56)

Table 1. Growth-inhibitory properties and cytotoxicities of TPA, mezerein, OAG, 1,2-diC_8 and 1,3-diC_8 in A549 cells. Cells (2×10^4) were treated with compounds as described in Methods and cell numbers counted after 5 days incubation.

[a] Concentration which caused half-maximal inhibition of growth determined by cell counting.

[b] Concentration which caused half-maximal LDH release from cells.

[c] Values were obtained by linear regression analysis of the linear portion of the concentration-response curve constructed with values measured at at least 5 concentrations in each of 4 separate experiments. Values in parenthesis are the 90% confidence limits.

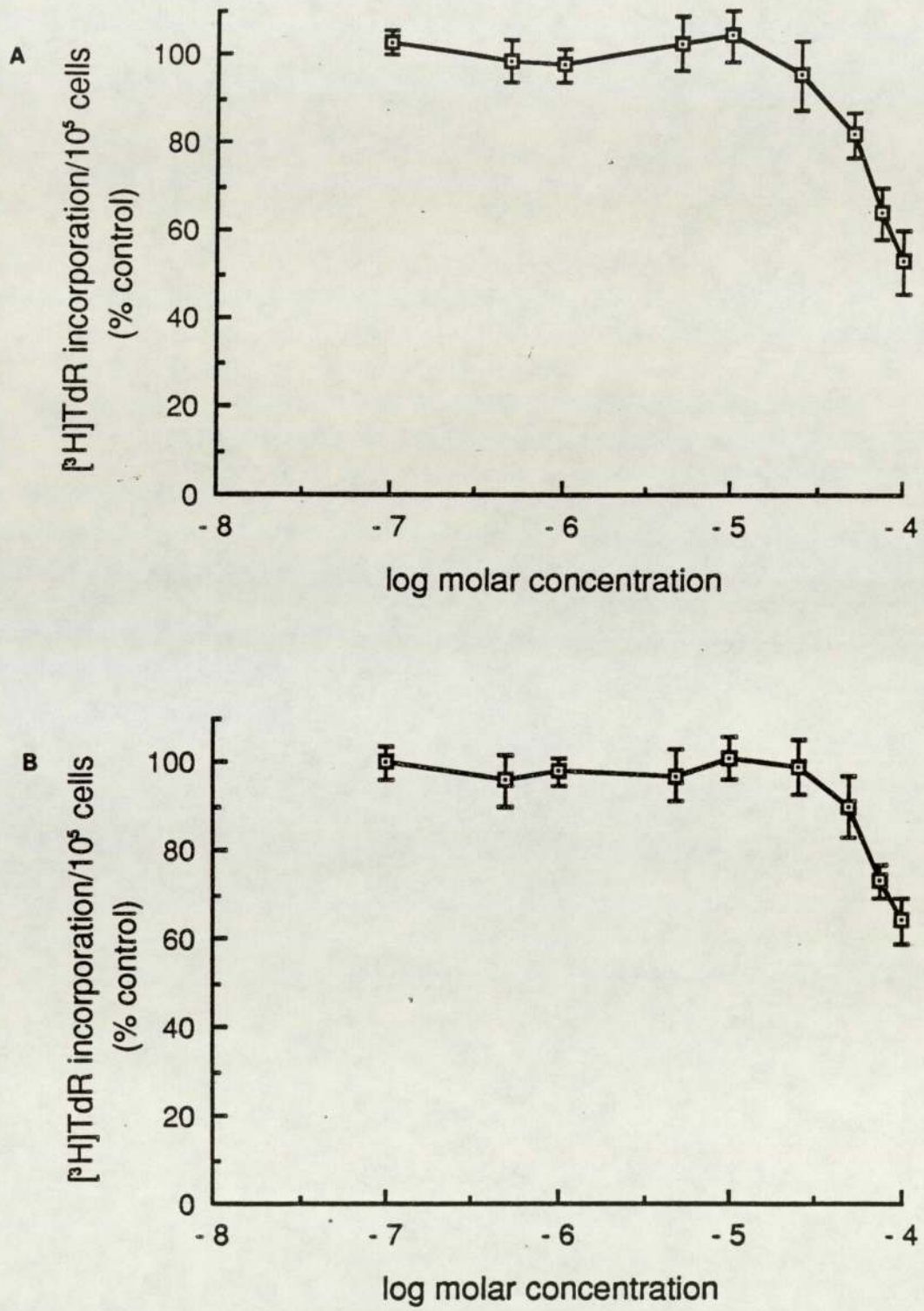


Figure 10. The effect of different concentrations of (A) OAG and (B) 1,2-diC_8 on A549 cell growth assessed by [^{3}H]TdR incorporation. Cells (2 x 10^5) were treated 3 times over a period of 24 h before measurement of [^{3}H]TdR incorporation (mean ± SD (n=6) in one experiment representative of 3).

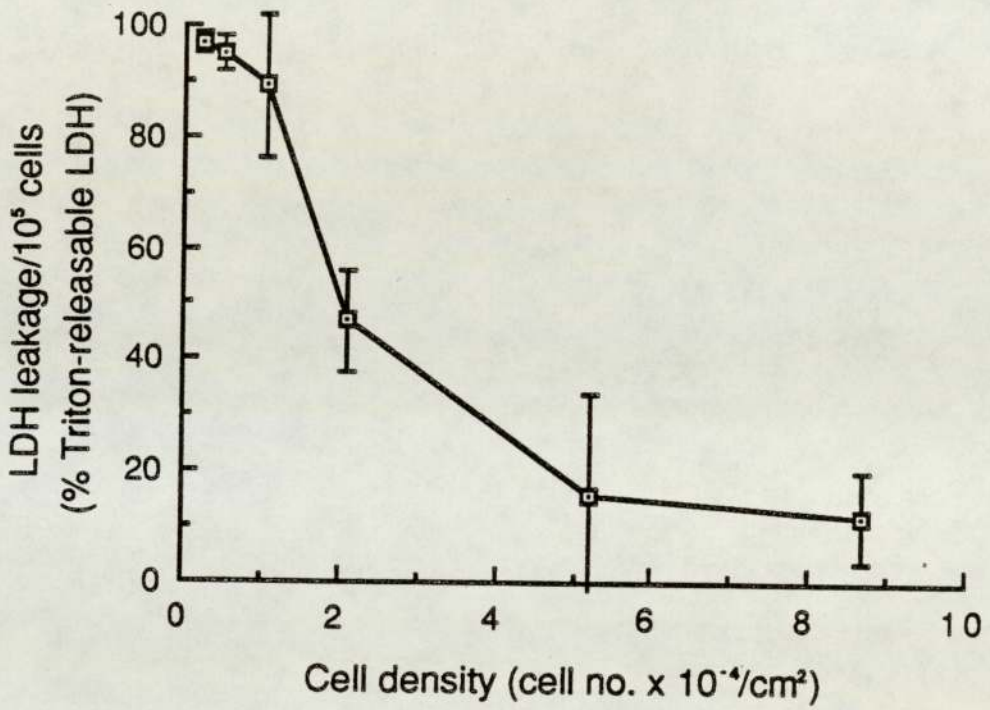


Figure 11. Dependence of the cytotoxic influence of 1,2-diC_8 on cell density. Cells were seeded at the densities indicated and treated twice with 1,2-diC_8 over a period of 24 h before assay of LDH release from cells (mean ± SD (n=4)).

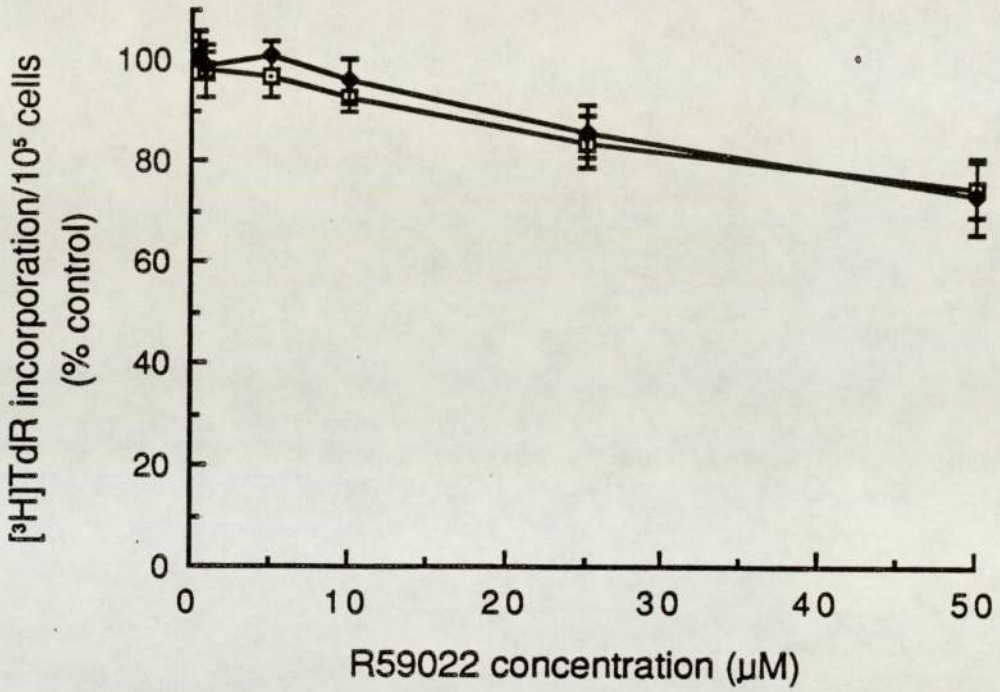


Figure 12. The effect of the DAG kinase inhibitor R59022 on the growth-inhibitory influence of 25 µM 1,2-diC$_8$ assessed by [^{3}H]TdR incorporation. Cells (2 x 10^5) were incubated with various concentrations of R59022 in the presence (⧈) and absence (◆) of 25 µM 1,2-diC$_8$, which was replenished 3 times over a period of 24 h, before measurement of [^{3}H]TdR incorporation (mean ± SD (n=4) in one experiment representative of 2).

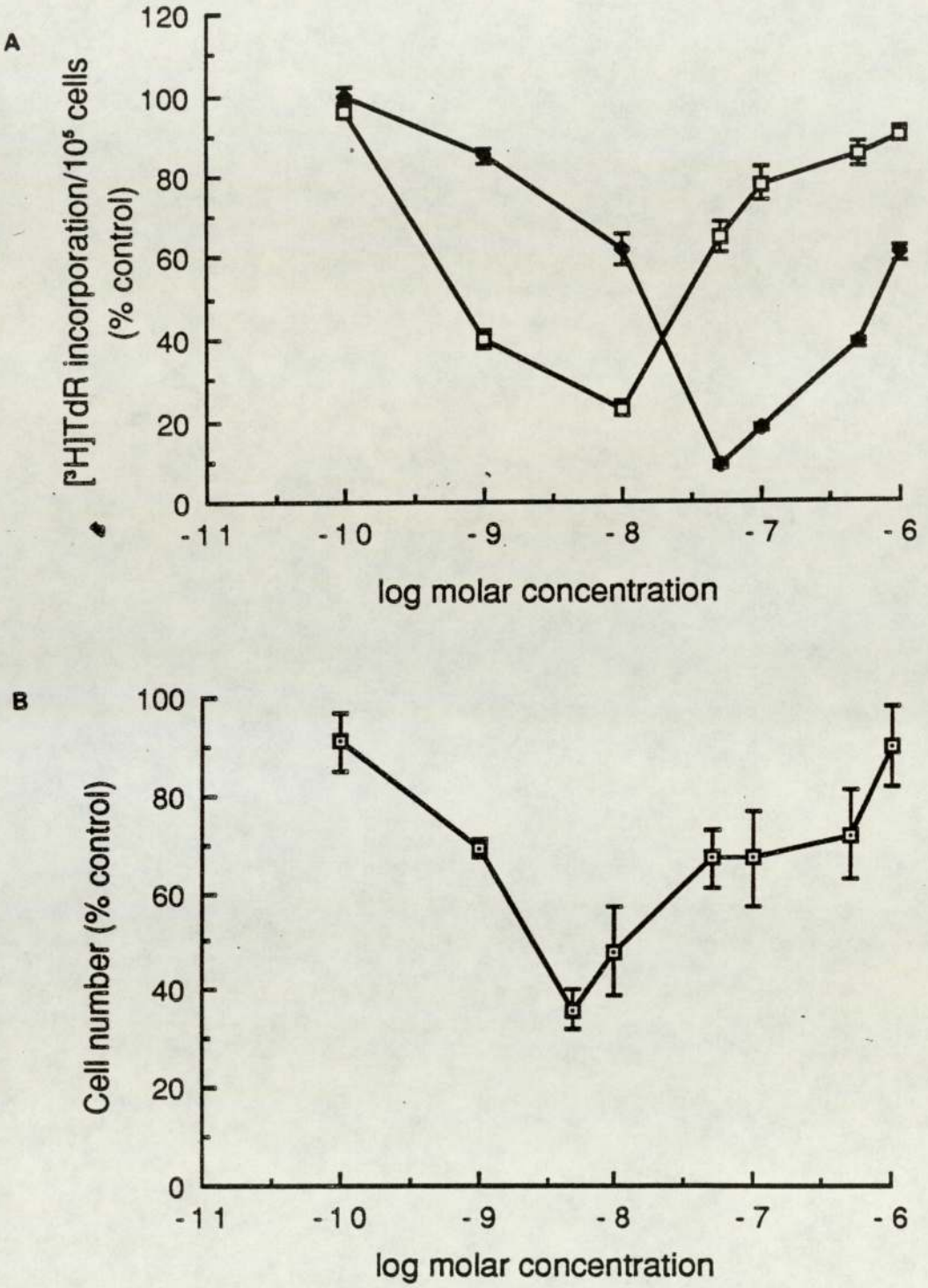


Figure 13. The effect of different concentrations of bryostatins 1 and 2 on A549 cell growth assessed by (A) [³H]TdR incorporation and (B) cell counting.

(A) Cells (2 x 10^5) were treated with bryostatin 1 (□) or bryostatin 2 (■) for 24 h before measurement of [³H]TdR incorporation (mean ± SD (n=4) in one experiment representative of 5).

(B) Cells (2 x 10^4) were treated with bryostatin 1 for 5 days before counting cell numbers (mean ± SD (n=4)).

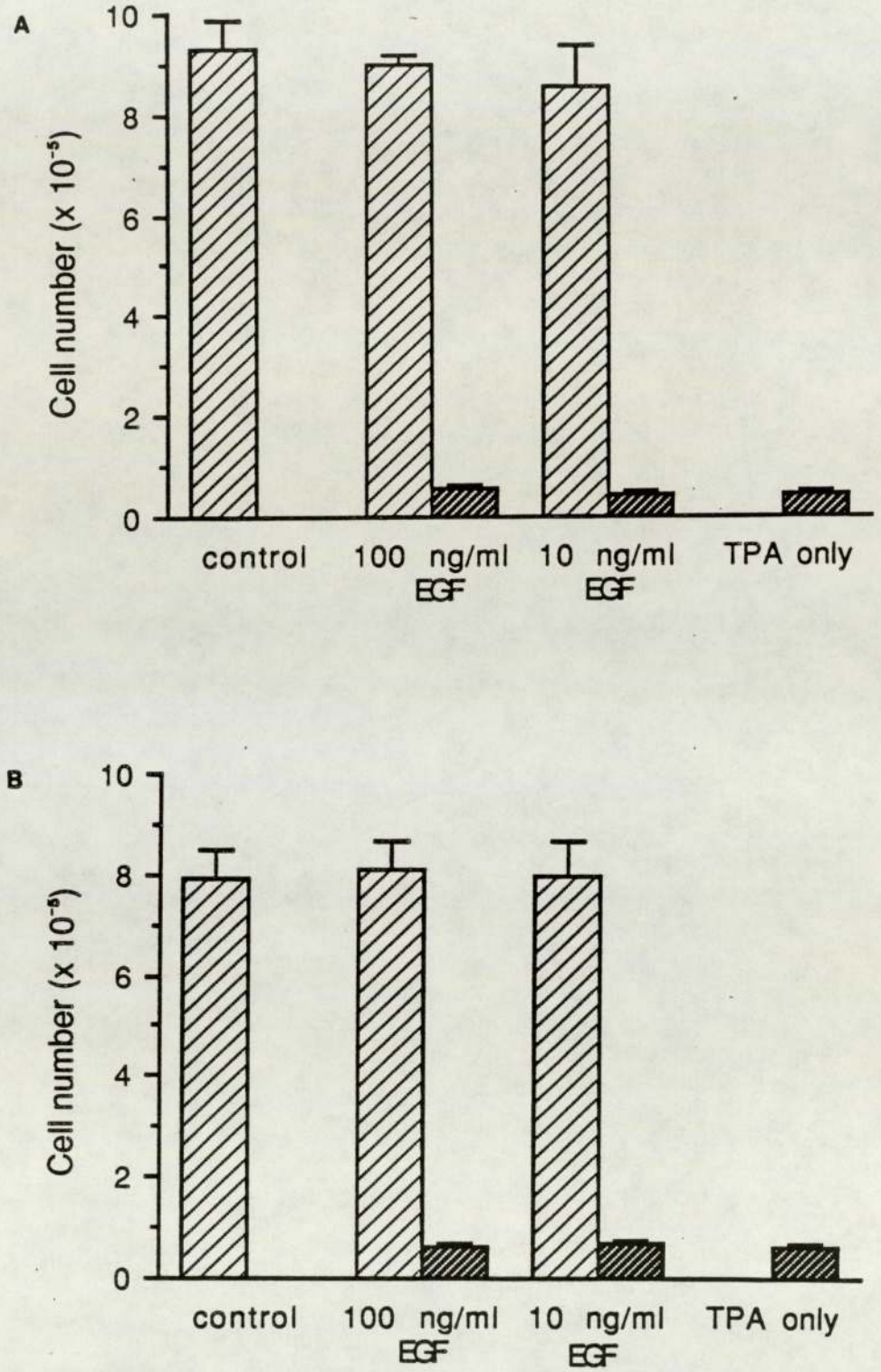


Figure 14. The effect of EGF on A549 cell growth in medium supplemented with (A) 10% FCS and (B) 1% FCS. Cells (2×10^4) were treated with EGF as indicated in the absence (▨) and presence (▩) of 10 nM TPA for 5 days before counting cell numbers (mean ± SD (n=6) in one experiment representative of 2).

Section 4.1.3. Desensitisation of A549 cells to the growth-inhibitory influence of phorbol esters, mezerein and bryostatins

4.1.3.1. Phorbol esters and mezerein

As shown in Figure 15, the growth of cells cultured in the presence of 10 nM TPA (Fig. 15A) or 100 nM mezerein (Fig. 15B) was arrested completely for 4-6 days, after which cells began to proliferate at a rate similar to that of control cells. Resistance was lost on trypsinisation and reseeding but cells with an altered phenotype which retained their resistance to growth inhibition even after trypsinisation, designated A549-TPA and A549-MEZ, were obtained on culturing in the presence of either TPA or mezerein for 60-70 days (see Section 4.1.4).

Treatment of A549 cells with 100 nM PDBu also resulted in a transient inhibition of proliferation similar to that observed with TPA (Fig. 16A). The ability of PDBu to induce a A549-PDBu resistant phenotype was not investigated.

The removal of 100 nM PDBu from the medium after treatment of cells for 24 h resulted in a rapid reversal of the growth-inhibitory influence of the phorbol ester such that cells were proliferating at a rate similar to controls after only 3 days (Fig. 16B). PDBu was used in preference to TPA in this investigation as the high lipophilicity of the latter makes removal by washing uncertain.

4.1.3.2. Bryostatins

Figure 17A illustrates the growth of cells cultured in the

presence of various concentrations of bryostatin 1 for up to 6 days. Maximal inhibition of growth was observed on treatment with 10 nM bryostatin 1, with cell numbers reduced by approximately 50% after 6 days when compared to controls. Inhibition of growth at a concentration of 100 nM was only slight, whilst no growth inhibition was observed when cells were cultured in the presence of 1 μM bryostatin 1 (Fig. 17A). This trend is consistent with the concentration-response curve observed for the inhibition of incorporation of [^{3}H]TdR into cells by bryostatin 1 (Section 4.1.2).

As expected, inhibition of A549 cell growth by bryostatin 2 reflected that of bryostatin 1 rather than that of the phorbol esters. Incubation in the presence of 100 nM bryostatin 2, the concentration at which maximal inhibition of incorporation of [^{3}H]TdR into cells was observed in the bryostatin 2 concentration-response curve (Section 4.1.2), resulted in the inhibition of A549 cell growth (Fig. 17B). In contrast, growth inhibition was not observed when cells were cultured in the presence of 1 μM bryostatin 2 (Fig. 17A). Like bryostatin 1, cell growth was only retarded at concentrations in the region of 100 nM and after 4 days treatment cells appeared to be proliferating at a rate similar to that of controls.

4.1.3.3. Discussion

The A549 cell line is unusual in that growth inhibition by TPA is only transient in nature, as cells regain their proliferative potential after 5-6 days in the continued

presence of the phorbol ester. This is unlike the majority of cultured cell lines whose proliferative capacity is decreased by treatment with phorbol esters. For example, the MCF-7 breast carcinoma cell line, the growth of which is potently inhibited by TPA, did not grow even in the prolonged presence of the phorbol ester. However, recovery of proliferation was observed if TPA was removed from the cells (Osborne et al, 1981). The HL-60 human promyelocytic leukaemia cell line responds to treatment with TPA by terminally differentiating into macrophage-like cells which have permanently lost their proliferative potential (Rovera et al, 1979).

However, the nature of the growth inhibition induced by TPA in A549 cells is not unique. A phorbol ester-tolerant variant of HL-60 promyelocytes has recently been described in which growth was arrested on exposure to TPA for approximately 48 h, after which time they resumed their original rate of replication even in the continued presence of the phorbol ester (MacFarlane et al, 1988). The resistant cells appeared to be as sensitive as the parent cell line to other agents which differentiate HL-60 cells such as retinoic acid, DMSO and 1,25-dihydroxyvitamin D_3, and phosphorylation patterns both before and after exposure to TPA were similar to the parent line (MacFarlane et al, 1988). Transient inhibition of proliferation by TPA has also been reported in the SVK 14 cell line (McKay et al, 1983), which was derived by SV40 transformation of human foreskin keratinocytes. In the presence of 100 nM TPA, the growth of SVK 14 cells was inhibited to 20% of control

growth . Treatment with 100 nM PDBu had a similar effect, although it only achieved inhibition to 40% of controls. However, after 2 days treatment cells began to recover from the growth-inhibitory effects of TPA and PDBu (McKay et al, 1983). In order to explain the kinetics of growth of SVK 14 cells treated with phorbol esters it was suggested that mitogenic growth factors produced by the cells could accumulate and reverse the inhibitory effects of the phorbol esters. It is possible that this is also the case for A549 cells. An alternative explanation is the induction after 4-6 days of enzymes capable of inactivating the growth-inhibitory compounds, such as non-specific esterases. This latter explanation is unlikely to be applicable to the cells studied here, as A549 cells which had acquired resistance towards the growth inhibition by TPA were observed to be completely resensitised after trypsinisation and reseeding.

Prolonged treatment of A549 cells with 100 nM PDBu or 100 nM mezerein resulted in the same transient growth inhibition seen in the case of 10 nM TPA. Higher concentrations of these compounds were employed in this study as their potency as inhibitors of cell growth were lower than that of TPA (Section 4.1.2). Recovery from the antiproliferative influence of PDBu following the removal of the phorbol ester after 24 h incubation was complete within 2-3 days. This result demonstrates that the presence of the phorbol ester is required for the continued blockage of growth during the first 5-6 days of treatment, although cells begin to proliferate even in the presence of PDBu after this time.

In addition to their growth-inhibitory influence, both PDBu and mezerein induced the characteristic morphological change associated with growth inhibition of A549 cells by TPA (Section 4.1.7).

Both bryostatins 1 and 2 inhibited A549 cell growth and induced, to some degree, the morphological change seen on treatment with TPA. However, growth inhibition by both bryostatins was not prolonged for several days. It can be argued that bryostatin 1 induces a potent but short-lived inhibition of growth, probably of less than 24 h duration. Several observations support this conclusion; (i) scrutiny of the concentration-response curve for bryostatin 1 as assessed by cell counts after 5 days treatment indicates that the compound is less potent as a growth inhibitor than suggested by its ability to reduce the incorporation of [^{3}H]TdR into cells after 24 h treatment (Section 4.1.2). This discrepancy could be explained by the transient nature of the effect of the bryostatin, as after 5 days cell growth could have reduced the apparent potency of the bryostatin, (ii) the rate of proliferation of cells after 2-3 days treatment appeared similar to that of controls, suggesting that the cells had completely overcome the growth-inhibitory influence of bryostatin 1 and, (iii) the time course of the inhibition of [^{3}H]TdR indicated that cells were beginning to recover from the growth-inhibitory influence of bryostatin 1 (Section 4.1.6). The transient nature of growth inhibition was also observed on treatment with bryostatin 2, although in this case the time span of the inhibition of growth was possibly longer (see Fig. 17A and Section 4.1.6). However,

this conclusion can only be drawn tentatively on the basis of the data described in this thesis, and further work is required to confirm this hypothesis.

The transient action of bryostatins when compared to phorbol esters could be explained by a greater susceptibility to inactivation by metabolism. However, the observation that bryostatin continued to block TPA-mediated inhibition of A549 cell growth even after 5 days coincubation (see Section 4.1.7) suggests that this is not the case. Other workers have previously demonstrated the metabolic stability of the bryostatins in cell culture (Smith et al, 1985; Kraft et al, 1986).

The short-lived nature of growth inhibition by bryostatin 1 has been previously described in other cell systems. Pasti et al (1988) examined the ability of PDBu and bryostatin 1 to inhibit cell-cell communication in keratinocytes as quantitated by microinjection of Lucifer yellow dye and measurement of the number of cells into which it transferred. PDBu was shown to rapidly suppress dye transfer and this suppression was maintained for 8-24 h. Similarly, treatment with bryostatin 1 caused the rapid suppression of dye transfer in these cells but, in contrast to the phorbol ester, the action of bryostatin 1 was transient, with activity returning to control levels between 2 and 4 h. It has been suggested that the shorter duration of action for bryostatin 1 may account for some of the differences between the activities of bryostatin 1 and the phorbol esters, though it cannot account for all the differences (for a review see Blumberg, 1988).

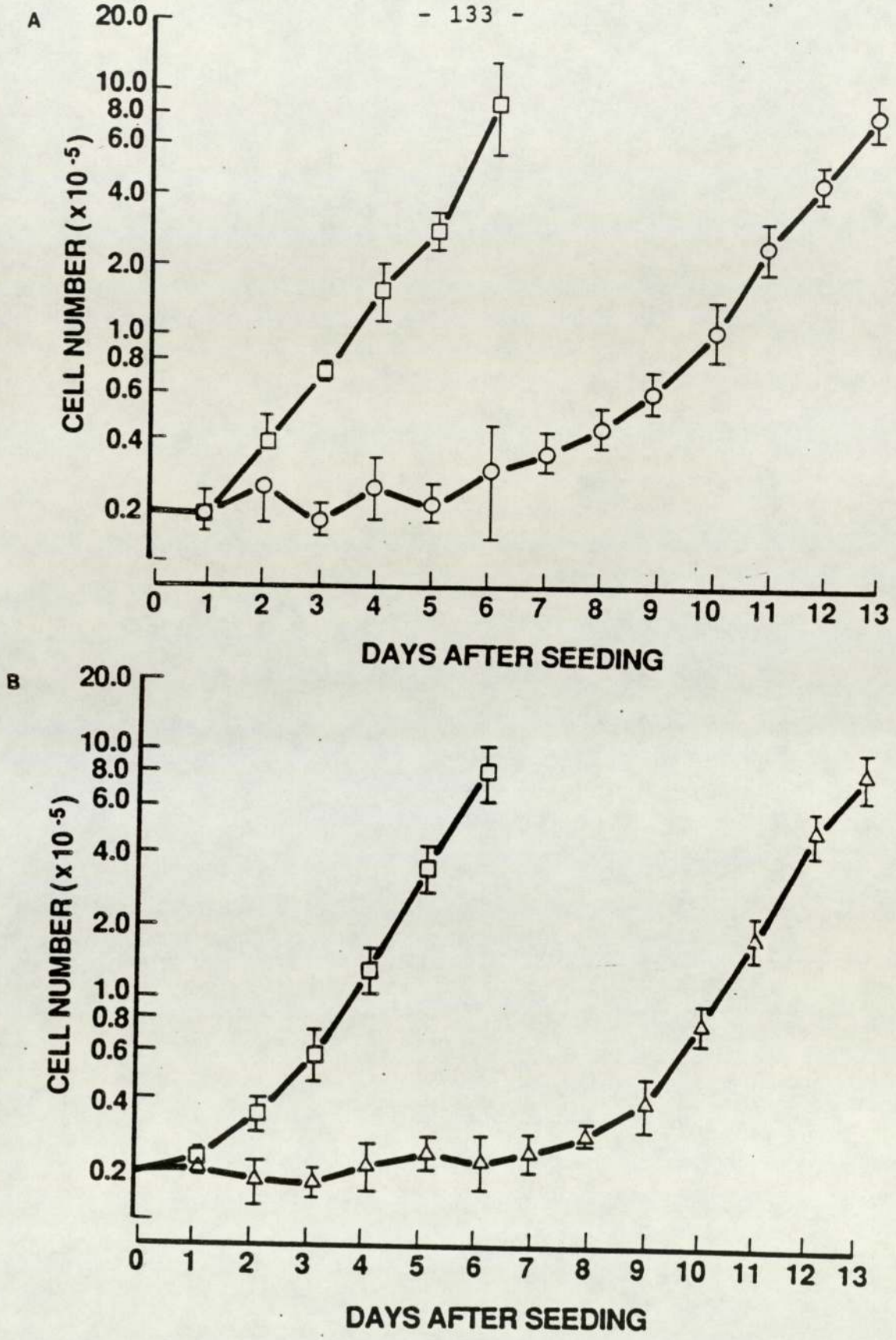


Figure 15. Desensitisation of A549 cells to the growth-inhibitory influence of TPA and mezerein. Cells (2 x 10^4) were treated with (A) 10 nM TPA and (B) 100 nM mezerein and cell numbers counted at the time intervals indicated. The growth of control cultures is indicated in each case (□). Medium and compound were replenished every 2 days (mean ± SD (n=4-6)).

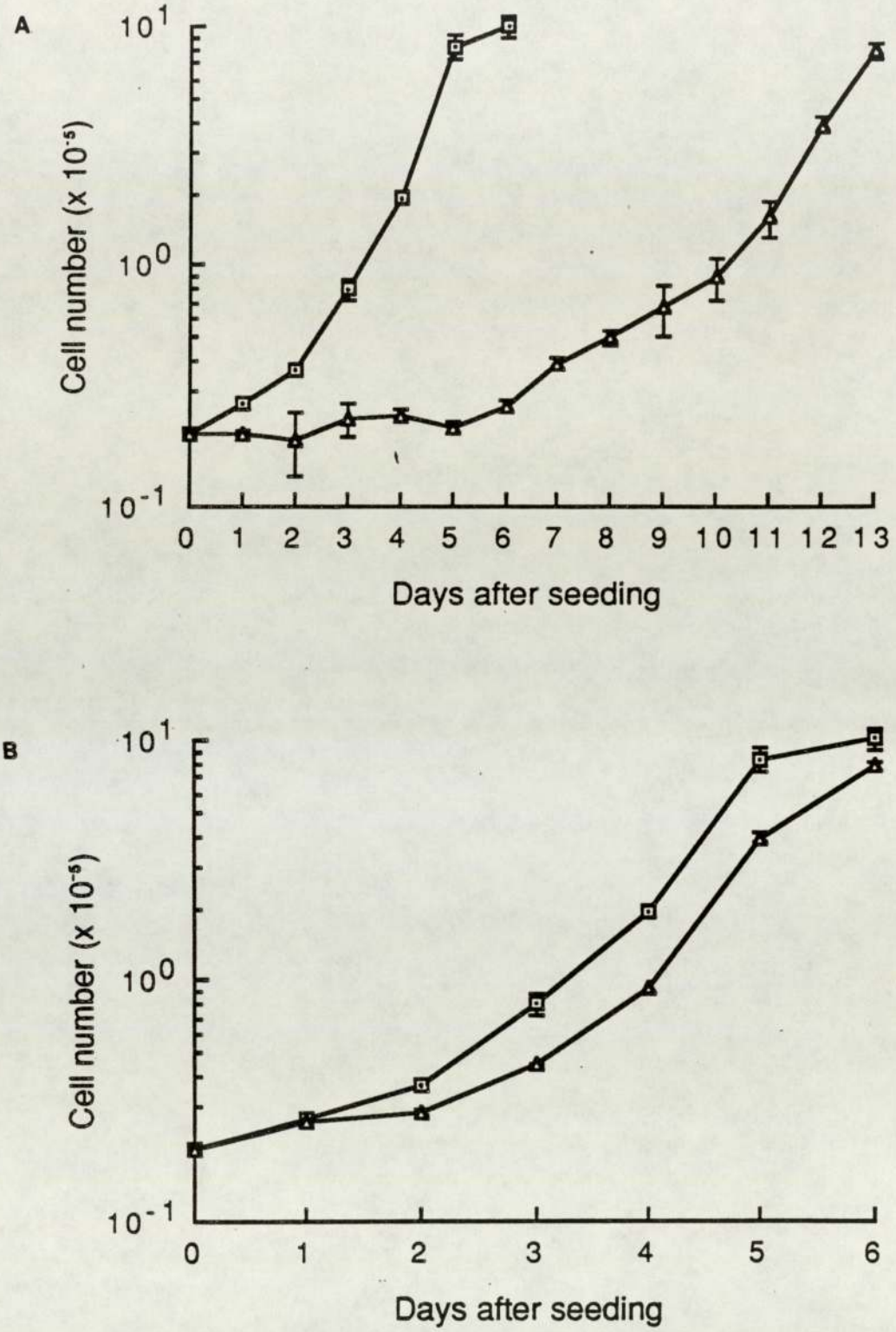


Figure 16. The growth-inhibitory influence of 100 nM PDBu on the growth of A549 cells. Cells (2 x 10^4) were treated with 100 nM PDBu either (A) for the duration of the investigation or (B) for 24 h before washing the phorbol ester from cells and subsequently incubating in medium only. Cell numbers were counted at the time intervals indicated (mean ± SD (n=4) in one experiment representative of 2). ▫; 100 nM PDBu, ▲; control

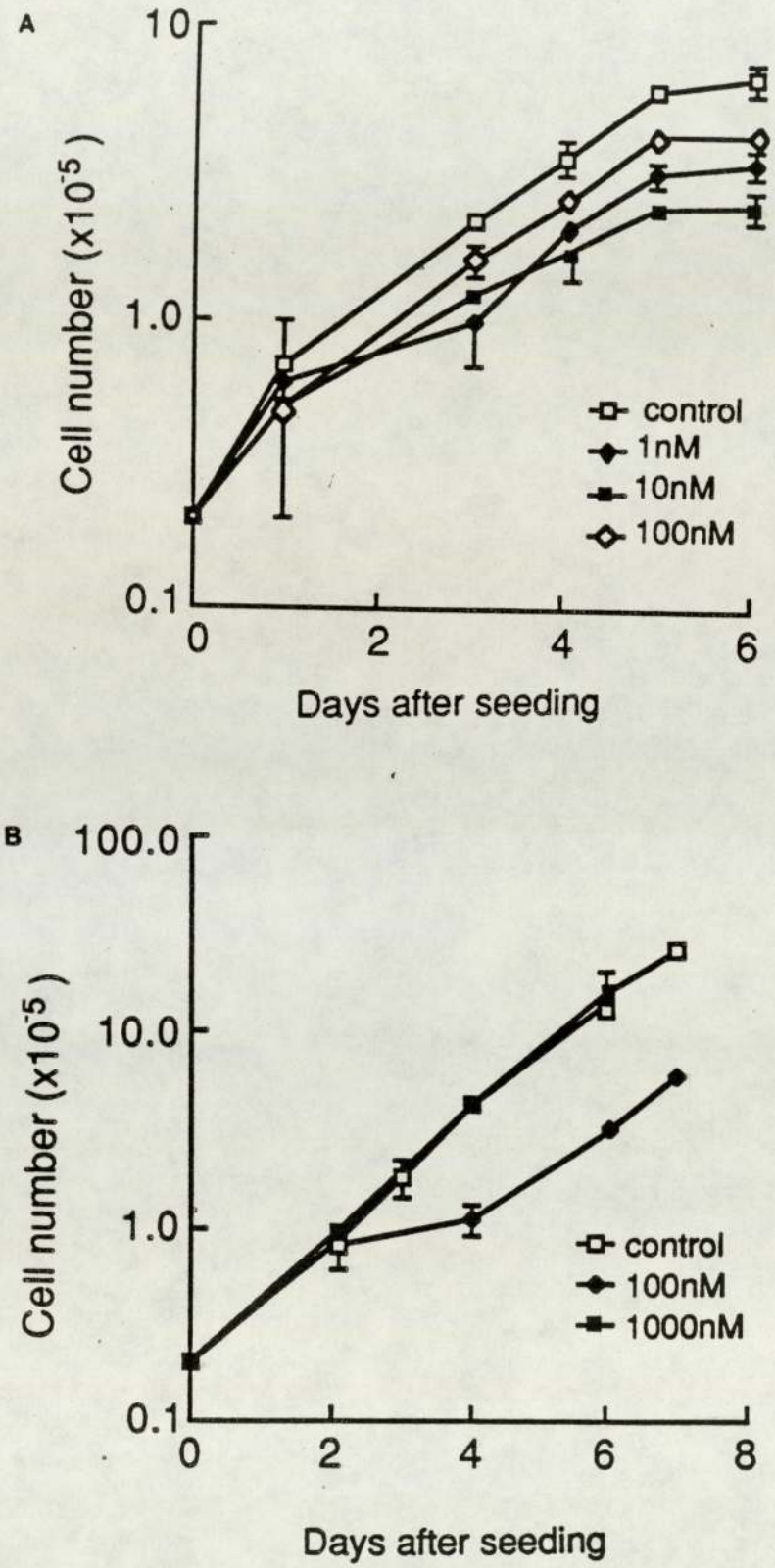


Figure 17. The effect of bryostatins 1 and 2 on A549 cell growth. Cells (2 x 10^4) were treated with (A) bryostatin 1 and (B) bryostatin 2 and cell numbers counted at the time intervals indicated. Medium and bryostatin were replenished every 2 days (mean $\pm$ SD (n=3) in one experiment representative of 3).

Section 4.1.4. Resistant cell sublines A549-TPA and A549-MEZ

4.1.4.1. Results

When subcultured in the presence of 10 nM TPA or 100 nM mezerein for several weeks, cultures which were resistant to the growth-inhibitory effects of the tumour promoters even after trypsinisation were established. Cultures which possessed this more resistant phenotype were designated A549-TPA and A549-MEZ, respectively. Figure 18 illustrates that both A549-TPA and A549-MEZ cells grew at a rate similar to that of "naive" A549 cells (cells unexposed to tumour promoter) whether cultured in the absence or presence of tumour promoter.

The stability of the resistance of A549-TPA and A549-MEZ cells to the growth-inhibitory influence of TPA or mezerein was investigated by culturing cells in untreated medium. Cells were subcultured when confluent, after 7 and 14 days, at which time the sensitivity of cells to growth inhibition was assessed by retreating A549-TPA cells with 10 nM TPA or A549-MEZ cells with 100 nM mezerein for 24 h before measuring [^{3}H]TdR incorporation (Fig. 18). The growth characteristics of cells cultured in the absence of TPA or mezerein for 14 days were further investigated by the means of growth curves (Fig. 19). In both cases, full sensitivity to the growth inhibitory influence of the compounds was observed to have been regained, a conclusion also supported by clonogenic assay data (Section 4.1.5). Resensitized cells also exhibited the cell morphology

typical of naive A549 cells (not shown).

A549-TPA cells were treated with concentrations up to 1 µM of TPA, mezerein or bryostatin 1 for 24 h before the measurement of [^{3}H]TdR incorporation. Figure 20 illustrates that none of the compounds were growth-inhibitory at any concentration tested. Additionally, cells did not display "rounded cell" morphology to the same degree as treated naive cells (Section 4.1.7).

4.1.4.2. Discussion

When cultured in the presence of 10 nM TPA for several weeks, A549 cells were observed gradually to become resistant to the growth-inhibitory influence of the phorbol ester even after trypsinisation (Gescher and Reed, 1985). The present study has demonstrated that exposure of A549 cells to the tumour promoter mezerein, in addition to inhibiting the proliferation of A549 cells in a manner similar to TPA (Sections 4.1.2 and 4.1.3), also results in the establishment of a resistant cell population A549-MEZ with characteristics apparently the same as A549-TPA cells.

In an effort to understand the mechanism(s) of action of phorbol esters, many laboratories have isolated cell line variants which are resistant to the influence of phorbol esters observed in the parental cell line, such as the Friend erythroleukaemia cell line (Giroldi et al, 1986), the KG-1 acute myeloid leukaemia cell line (Shoji et al, 1987) and T-lymphocytes (Mills et al, 1988). A number of investigations have reported the isolation of HL-60 human promyelocytic leukaemia cell line variants which are

resistant to the differentiation-inducing activity of phorbol esters and maintain their proliferative capacity in the presence of high concentrations of the promoters (Anderson et al, 1985; Diamond et al, 1985; Homma et al, 1986; Perrella et al, 1986; Solanki et al, 1981).

In many cases, the isolated variants of these cell types exhibited stable phenotypes for many subcultures even when cultured in the absence of phorbol esters. This is unlike the A549-TPA and A549-MEZ variants, which were observed to lose their resistance to the tumour promoters within 14 days, in which they were subcultured only twice. It is therefore clear that the phenotypic change which renders A549-TPA and A549-MEZ cells resistant to growth inhibition is unstable, and that they rapidly revert to express the phenotype typical of sensitive cells. In this regard the A549 variants are similar to a variant HL-60 cell line (Diamond et al, 1985; Perrella et al, 1986) and a Friend cell variant (Yamasaki et al, 1984), which rapidly lose their resistance to phorbol esters within 3-4 days. The HL-60 variant displayed only 40% and the Friend cell variant only 20% of phorbol ester receptor binding sites when compared to the parental cell line, in comparison to the A549-TPA cell variant which was reported to possess a concentration of receptor binding sites 77% of that of control cells following a 24 h incubation period with 100 nM PDBu (Gescher and Reed, 1985).

In A549-TPA cells, the recovery of receptors following the removal of the phorbol ester was very rapid, with a significant up-regulation of receptor binding noted after

only 3 h. It would therefore seem that no link exists between the up-regulation of binding sites and the return of cells to the sensitive phenotype, as the former occurred within hours whereas the latter was observed only after culture in phorbol ester-free medium for a number of days. It is interesting to note that although the down-regulation of receptor binding of A549-TPA cells was never greater than 23%, the loss of functional PKC activity was total (Section 4.3). Possible reasons for this discrepancy are discussed in Section 4.3.5.

The lack of correlation in A549-TPA cells between up-regulation of phorbol ester receptors and the return of biological responsiveness to phorbol esters is in contrast to findings using the reversibly resistant HL-60 cell variant (Perrella et al, 1986), which underwent a rapid up-regulation of phorbol ester receptors and PKC functional activity which occurred in parallel with the return of biological sensitivity to TPA.

The A549-TPA cell variant was completely resistant to the growth-inhibitory effects of TPA at concentrations as high as 1 μM. Furthermore, cells were also refractory to the influence of mezerein and bryostatin 1. One may therefore suggest that these compounds share a common mechanism(s) by which they cause growth arrest in A549 cells, which is lost or overridden in resistant cells. The loss of cellular responsiveness to the influence of phorbol esters following chronic exposure has also been reported in a number of other cell lines and has led to the suggestion that the down-regulation of PKC activity is responsible for this

effect (Gainer and Murray, 1985; Glynn et al, 1986; Katakami et al, 1986; Rodriguez-Pena and Rozengurt, 1984). It is tempting to speculate that the lack of detectable functional PKC activity in A549-TPA cells (Section 4.3) is responsible for their resistance to TPA, mezerein and bryostatin 1. However, one must be aware that down-regulation occurs only after activation of the enzyme and phorbol esters have been reported to have effects independent of the activation of PKC (Kikkawa and Nishizuka, 1986).

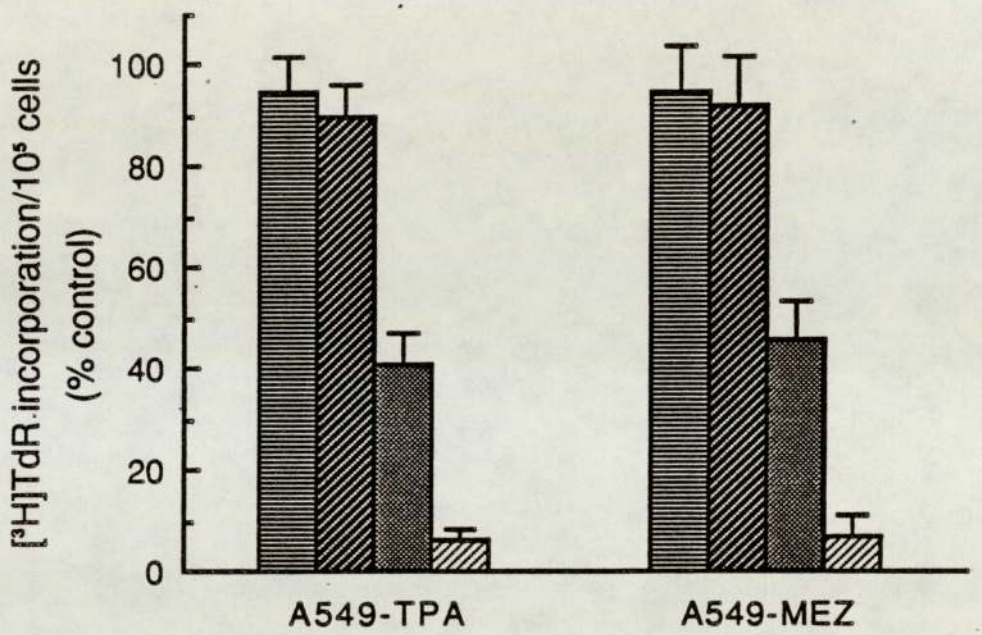


Figure 18. Growth characteristics of resistant cell sublines A549-TPA and A549-MEZ. Cells (2 x 10^4) were treated with carrier only ▤ or with tumour promoter ▨ (A549-TPA, 10 nM TPA; A549-MEZ, 100 nM mezerein) following previous culture in medium containing tumour promoter. Resistance was also determined after culture in the absence of tumour promoter for 7 days ▩ and 14 days ▧. Values are expressed as a percentage of the incorporation of [³H]TdR into untreated "naive" cells (mean ± SD (n=4)) in one experiment representative of 2).

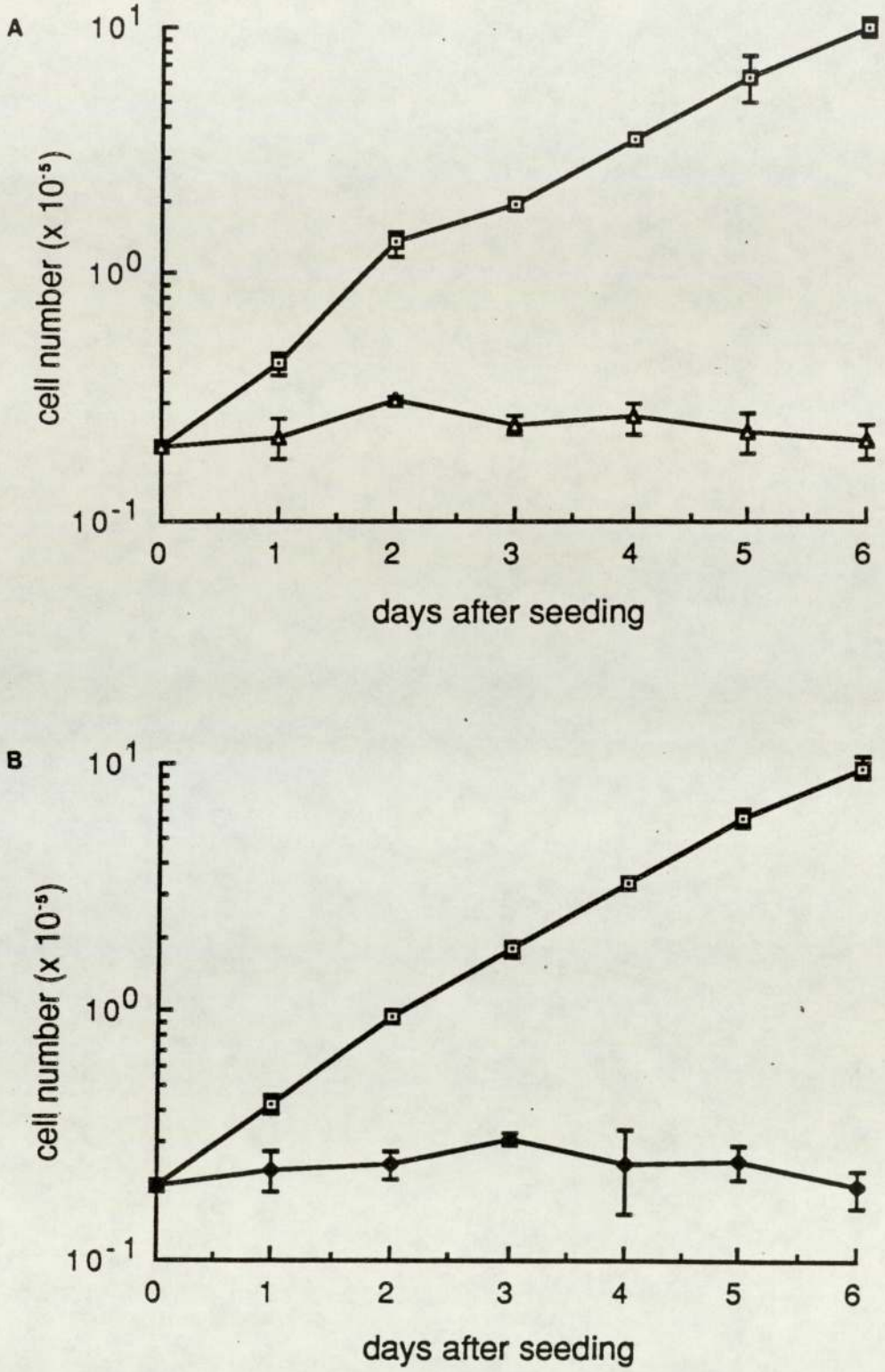


Figure 19. The effect of (A) 10 nM TPA and (B) 100 nM mezerein on the growth of A549-TPA and A549-MEZ cells previously cultured in the absence of tumour promoter for 14 days. Cells (2 x 10^4) were treated with (A) 10 nM TPA (▲), (B) 100 nM mezerein (◆) or carrier only (□). Cell numbers were counted at the time intervals indicated (mean ± SD (n=4) in one experiment representative of 2).

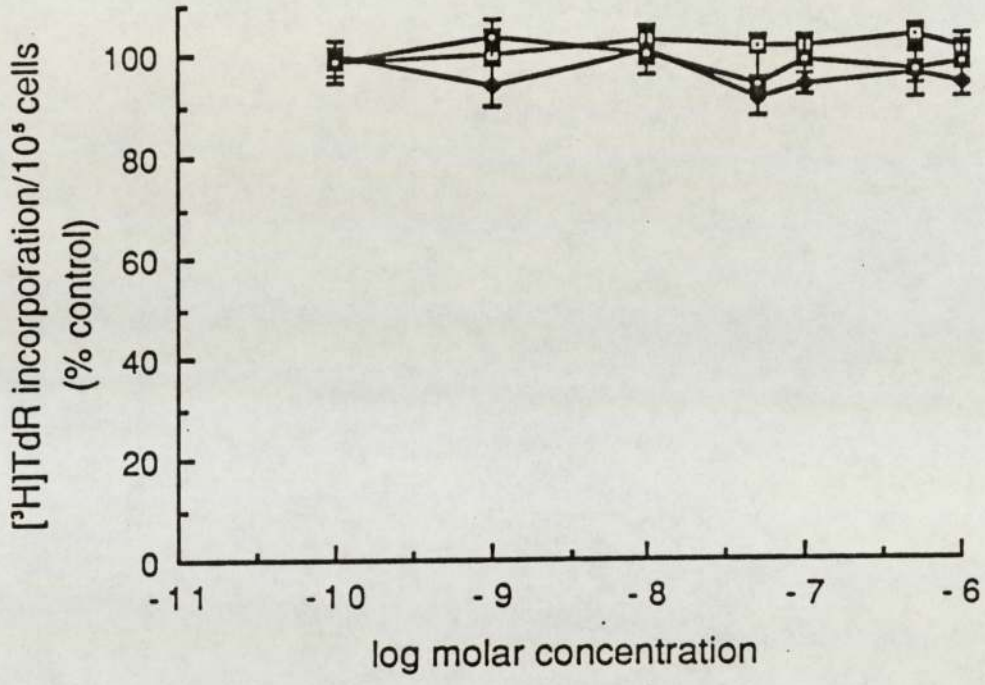


Figure 20. The effect of different concentrations of TPA, mezerein and bryostatin 1 on A549-TPA cell growth assessed by [³H]TdR incorporation. A549-TPA cells (2 x 10^5) were treated with compound for 24 h before measurement of [³H]TdR incorporation (mean ± SD (n=4) in one experiment representative of 2).

▫; TPA, ◆; mezerein, ▪; bryostatin 1

Section 4.1.5. Clonogenicity of cells

4.1.5.1. Clonogenicity of A549, A549-TPA and A549-MEZ cells

A549 cells cultured under clonogenic assay conditions grew well without the requirement for any modification of culture conditions. Untreated cultures exhibited a CFE of 36 ± 7% (SD; n=24) (Table 2), with dense, packed colonies easy to count after staining. As expected, cultures of A549 cells exposed to 10 nM TPA showed no colonies if counted after 7 days exposure to the phorbol ester. However, when counted after 21 days exposure to 10 nM TPA, many colonies were visible and the CFE was determined to be 34 ± 6% (SD; n=6) (Table 2).

After 7 days incubation, A549-TPA cells displayed a CFE of 34 ± 6% (SD; n=14) when cultured in the absence of 10 nM TPA or 32 ± 4% (SD; n=5) in the presence of the phorbol ester (Table 2), as expected considering their observed resistance to growth inhibition. A549-TPA cells resensitised to the growth-inhibitory influence of 10 nM TPA by culture in phorbol ester-free medium for 14+ days (see also Section 4.1.4) were found to be sensitive to growth inhibition by the phorbol ester in clonogenic assays and displayed the phenotype typical of naive cells. Colonies were not observed after incubation for 7 days with 10 nM TPA, but after 21 days a CFE of 32 ± 6% (SD; n=4) was noted (Table 2).

Similar to A549-TPA cells, A549-MEZ cells exhibited resistance to the growth-inhibitory influence of 100 nM

mezerein, with respective CFE's of 36 ± 6% (SD; n=5) and 34 ± 5% (SD; n=5) when cultured in the absence and presence of 100 nM mezerein (Table 3). Resensitisation of A549-MEZ cells cultured in the absence of mezerein for 14+ days was noted, as no colonies were visible when resensitised cells were treated with 100 nM mezerein under clonogenic assay conditions for 7 days, but a CFE of 33 ± 6% (SD; n=5) was determined when colonies were counted after 21 days (Table 3).

4.1.5.2. Isolation and characterisation of TPA-resistant clones of A549 cells

In order to investigate further the phenotypic characteristics of TPA-resistant A549 cells, 3 clones were isolated following the exposure of naive cells to 10 nM TPA for 2-3 weeks and designated as clones 1-3. The isolated clones were grown to confluency in the absence of TPA after a period of 4-7 weeks before trypsinisation and assessment of their resistance to TPA.

The growth of clones and their relative resistances to TPA immediately following isolation are illustrated in Figure 21A. The ability of cells to incorporate [^{3}H]TdR was assessed following incubation in the presence of medium only or with 10 nM TPA for 24 h. When cultured in the absence of the phorbol ester, clones 1 and 2 proliferated only slowly when compared to the parental cell line but clone 3 grew at a rate similar to that of controls (Fig. 21A). All three clones exhibited the morphology typical of A549 cells. When cultured in the presence of 10 nM TPA, clones 1 and 2

exhibited a partial resistance (Fig. 21A) and only a certain degree of rounded cell morphology typical of TPA-treated parental cells was observed (not shown). In contrast, the growth of clone 3 was potently inhibited by 10 nM TPA in a manner similar to that of naive A549 cells (Fig. 21A) and cell morphology became rounded (not shown).

Clonogenic analysis immediately after isolation indicated that clones 1 and 2 were resistant to growth inhibition by 10 nM TPA, whereas clone 3 was sensitive (Table 4). In the absence of 10 nM TPA, all clones displayed CFE's similar to those of A549 and A549-TPA cells after 6 days growth (clone 1, 42 $\pm$ 8%; clone 2, 40 $\pm$ 7%; clone 3, 37 $\pm$ 4% (SD; n=5)). However, when incubated in the presence of 10 nM TPA, the resistance of clones 1 and 2 and sensitivity of clone 3 was noted (clone 1, 35 $\pm$ 7%; clone 2, 35 $\pm$ 7%; clone 3, no colonies visible (SD; n=6)).

In order to investigate the permanence of resistance of clones 1 and 2 to phorbol ester-induced growth arrest, they were further cultured in the absence of TPA for 14 days before reassessment of resistance by clonogenic assay (Table 4) and measurement of [^{3}H]TdR incorporation (Fig. 21B). It was clear that both clones had completely regained their sensitivity and exhibited the phenotype of parental cells.

4.1.5.3. Discussion

The ability of A549 cells to overcome the growth-inhibitory influence of phorbol esters after 5-6 days could be due to either the outgrowth of a resistant subpopulation, or alternatively the restoration of proliferative capacity in

the whole cell population. The results presented here show that after 21 days the clonogenicity of TPA-treated A549 cells was similar to that of untreated cultures. This clearly indicates that a phenotypic change in the entire A549 cell population had occurred which allowed cells to override the block induced by TPA. If a resistant subpopulation had been selected one would have expected a reduction in the CFE. Roos et al (1986) have similarly reported that the ability of the MCF-7 breast cancer cell line to overcome the growth-inhibitory effects of TPA following trypsinisation and replating was not a result of the selection of resistant variants. Cells were kept for 3 months in 100 nM TPA before cloning. Evaluation of a number of clones indicated that all of them were still sensitive to TPA (Roos et al, 1986).

The resistant A549-TPA and A549-MEZ cells displayed CFE's comparable to that of naive A549 cells whether cultured in the presence or absence of 10 nM TPA. This suggests that the resistance of A549-TPA and A549-MEZ populations to growth inhibition even after trypsinisation is also a property of the whole A549 cell population. The reversion of resistant A549-TPA and A549-MEZ to a sensitive phenotype after culture in the absence of TPA for 14 days (see also Section 4.1.4) is another indication that this temporary resistance is a property of the whole A549 cell population.

The cloning of cells which proliferated in the presence of TPA also suggested that all A549 cells can overcome growth inhibition. In experiments performed as soon as sufficient cells were available, clones were observed to display

varying degrees of resistance to TPA. In fact, clone 3 was found to possess a TPA-sensitive phenotype identical to that of "naive" A549 cells when sufficient cells were available for experiment. One can explain this apparent anomaly when one considers that after isolation clones were cultured in the absence of TPA. It seems clear that during the time taken for clone 3 cells to grow to confluency (48 days), sufficient time had elapsed for cells to revert to the sensitive phenotype. On the other hand, clones 1 and 2 were observed to display some resistance to growth inhibition by TPA. However, subsequent subculture of both these clones in TPA-free medium for 14 days resulted in the return of the sensitive phenotype, indicating that these clones were only temporarily resistant to growth inhibition by TPA.

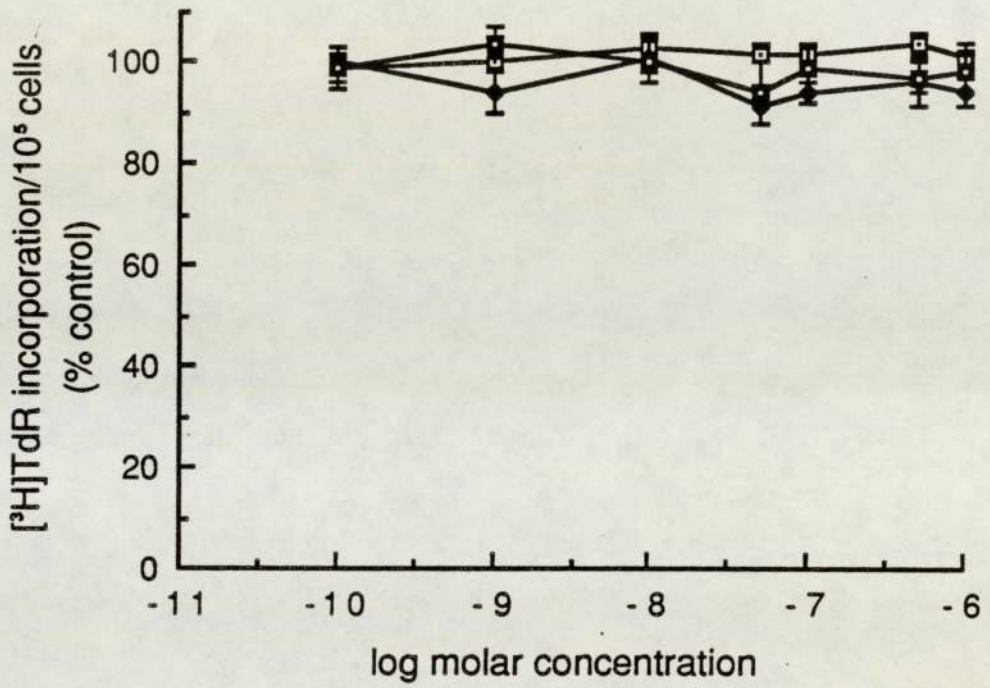


Figure 20. The effect of different concentrations of TPA, mezerein and bryostatin 1 on A549-TPA cell growth assessed by [³H]TdR incorporation. A549-TPA cells (2 x 10^5) were treated with compound for 24 h before measurement of [³H]TdR incorporation (mean ± SD (n=4) in one experiment representative of 2).

▫; TPA, ◆; mezerein, ▪; bryostatin 1

Cell population	Colony forming efficiency (%)		
	- 10 nM TPA	+ 10 nM TPA	
	7 days	7 days	21 days
A549	36 ± 7 (n=24)	0 (n=15)	34 ± 6 (n=6)
A549-TPA	34 ± 6 (n=14)	32 ± 4 (n=5)	ND
A549-TPA (resensitised)	35 ± 4 (n=4)	0 (n=4)	32 ± 6 (n=4)

Table 2. The effect of 10 nM TPA on the clonogenicity of A549 and A549-TPA cells. Cells (5×10^2) were treated with TPA as described in Methods and the number of colonies/dish counted after 7 or 21 days indicated. A549-TPA (resensitized) cells had been incubated in the absence of 10 nM TPA for 14 days prior to clonogenic analysis. Values in parenthesis indicate number of experiments.

Cell population	Colony forming efficiency (%)		
	- 100 nM MEZ	+ 100 nM MEZ	
	7 days	7 days	21 days
A549	36 ± 7 (n=24)	0 (n=8)	38 ± 3 (n=8)
A549-MEZ	34 ± 5 (n=5)	30 ± 5 (n=4)	ND
A549-MEZ (resensitised)	36 ± 4 (n=5)	0 (n=5)	33 ± 6 (n=5)

Table 3. The effect of 100 nM mezerein on the clonogenicity of A549 and A549-MEZ cells. Cells (5 x 10^2) were treated with mezerein as described in Methods and the number of colonies/dish counted after 7 or 21 days as indicated. A549-MEZ (resensitized) cells had been incubated in the absence of 10 nM TPA for 14 days prior to clonogenic analysis. Values in parenthesis indicate number of experiments.

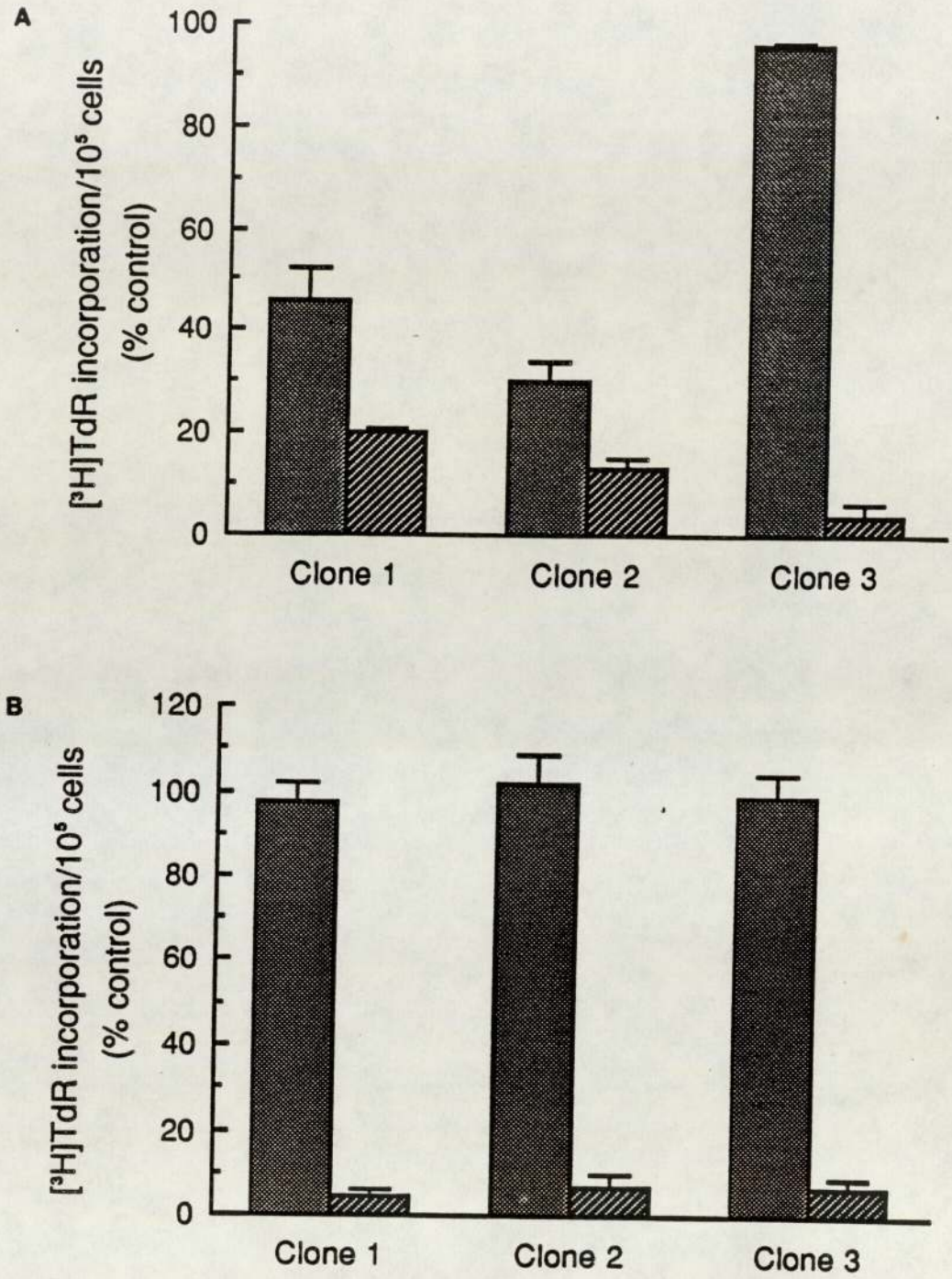


Figure 21. Growth characteristics of TPA-resistant A549 clones. Growth characteristics of clones were determined in the presence ▨ and absence ▩ of 10 nM TPA; (A) immediately following isolation and (B) following culture of isolated cells for a further 14 days in TPA-free medium. At these times, cells (2 x 10^5) were treated with 10 nM TPA and incubated for a further 24 h in the presence of the phorbol ester before cell growth was assessed by measurement of [^{3}H]TdR incorporation. Values are expressed as a percentage of [^{3}H]TdR incorporation into "naive" untreated cells (n=4-8, $\pm$ SD).

Cell population	Colony forming efficiency (%)		
	- 10 nM TPA	+ 10 nM TPA	
	7 days	7 days	21 days
Clone 1	42 ± 8 (n=5)	35 ± 7 (n=5)	ND
Clone 1 (resensitised)	37 ± 5 (n=5)	0 (n=5)	35 ± 4 (n=5)
Clone 2	40 ± 7 (n=5)	38 ± 5 (n=5)	ND
Clone 2 (resensitised)	36 ± 6 (n=5)	0 (n=5)	36 ± 4 (n=5
Clone 3	37 ± 4 (n=5)	0 (n=5)	36 ± 2 (n=5)

Table 4. The effect of 10 nM TPA on the clonogenicity of A549 cell clones 1, 2 and 3. Cells (5×10^2) were treated with TPA as described in Methods and the number of colonies/dish counted at the times indicated. Resensitized clones 1 and 2 had been incubated in the absence of 10 nM TPA for 14 days prior to clonogenic analysis. Values in parenthesis indicate number of experiments.

Section 4.1.6. Study of the time course of inhibition of [^{3}H]TdR incorporation by phorbol esters, mezerein and bryostatins

4.1.6.1. Results

In order to study the rate and manner in which growth-inhibitory compounds reduce cell proliferation, their influence on the incorporation of [^{3}H]TdR into cells was measured at different time intervals after commencement of incubation. Figures 22 and 23 illustrate that 10 nM TPA (Fig. 22A), 100 nM mezerein (Fig. 22B), 10 nM bryostatin 1 (Fig. 23A) and 100 nM bryostatin 2 (Fig. 23B) affected DNA synthesis in a nearly identical manner. [^{3}H]TdR incorporation was decreased by 10-25% within 1 h of incubation, but little further reduction was evident during the subsequent 4-5 h. However, upon longer incubation incorporation was increasingly arrested until it reached a minimal value after incubation for 12 h. Inhibition of [^{3}H]TdR incorporation remained maximal after 24 h incubation in all cases except that of bryostatin 1, where incorporation increased from 22 $\pm$ 1% after 12 h to 40 $\pm$ 5% after 24 h.

4.1.6.2. Discussion

Study of the rate and profile of the inhibition of [^{3}H]TdR into cells imparts little information as to the mechanism(s) by which compounds cause inhibition of growth in A549 cells. However, the similarities observed in the rate and extent of inhibition by TPA, mezerein and the bryostatins seems to

indicate that they induce growth arrest by the same, admittedly as yet undefined, mechanism(s).

One might speculate that PKC plays a role in the observed gradual inhibition of [^{3}H]TdR incorporation. The early events might be mediated via enzyme translocation and activation whereas later events might be related to down-regulation of PKC activity (see Section 4.3).

It is interesting to note that after incubation with bryostatin 1 for 24 h it appeared that cells were beginning to regain their proliferative capacity (Fig. 23A) whereas inhibition of [^{3}H]TdR incorporation by the other compounds, including bryostatin 2 (Fig. 23B), remained maximal. This observation supports the conclusion, already discussed in Section 4.1.2, that growth inhibition by the bryostatins is equally as potent as that of the phorbol esters and mezerein, but is more transient in nature.

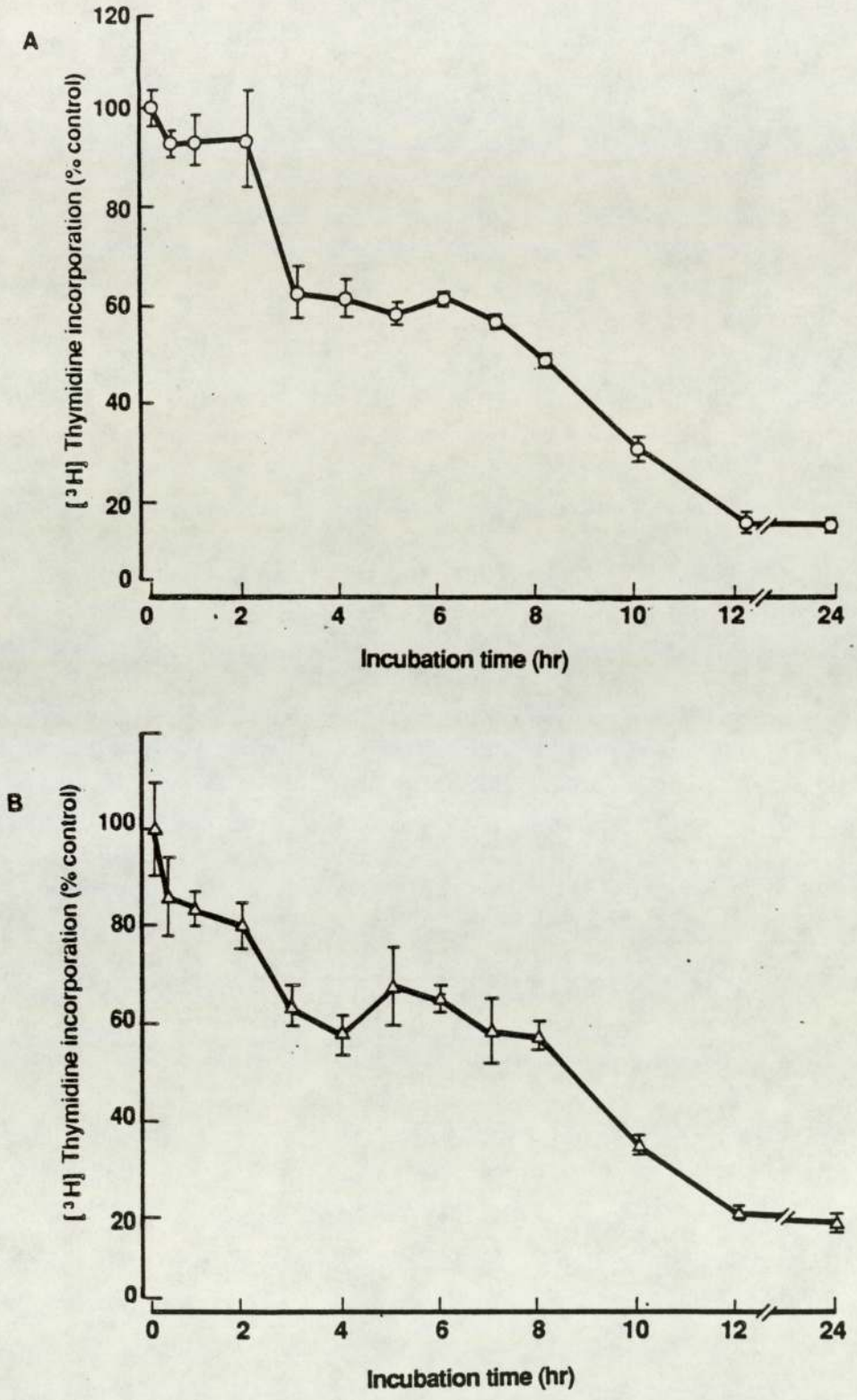


Figure 22. Time-course of the inhibition of [^{3}H]TdR incorporation following treatment of A549 cells with (A) 10 nM TPA and (B) 100 nM mezerein. Cells (2 x 10^5) were treated with compound at time 0 and incubated for various time intervals before measurement of [^{3}]TdR incorporation (mean $\pm$ SD (n=4) in one experiment representative of 3).

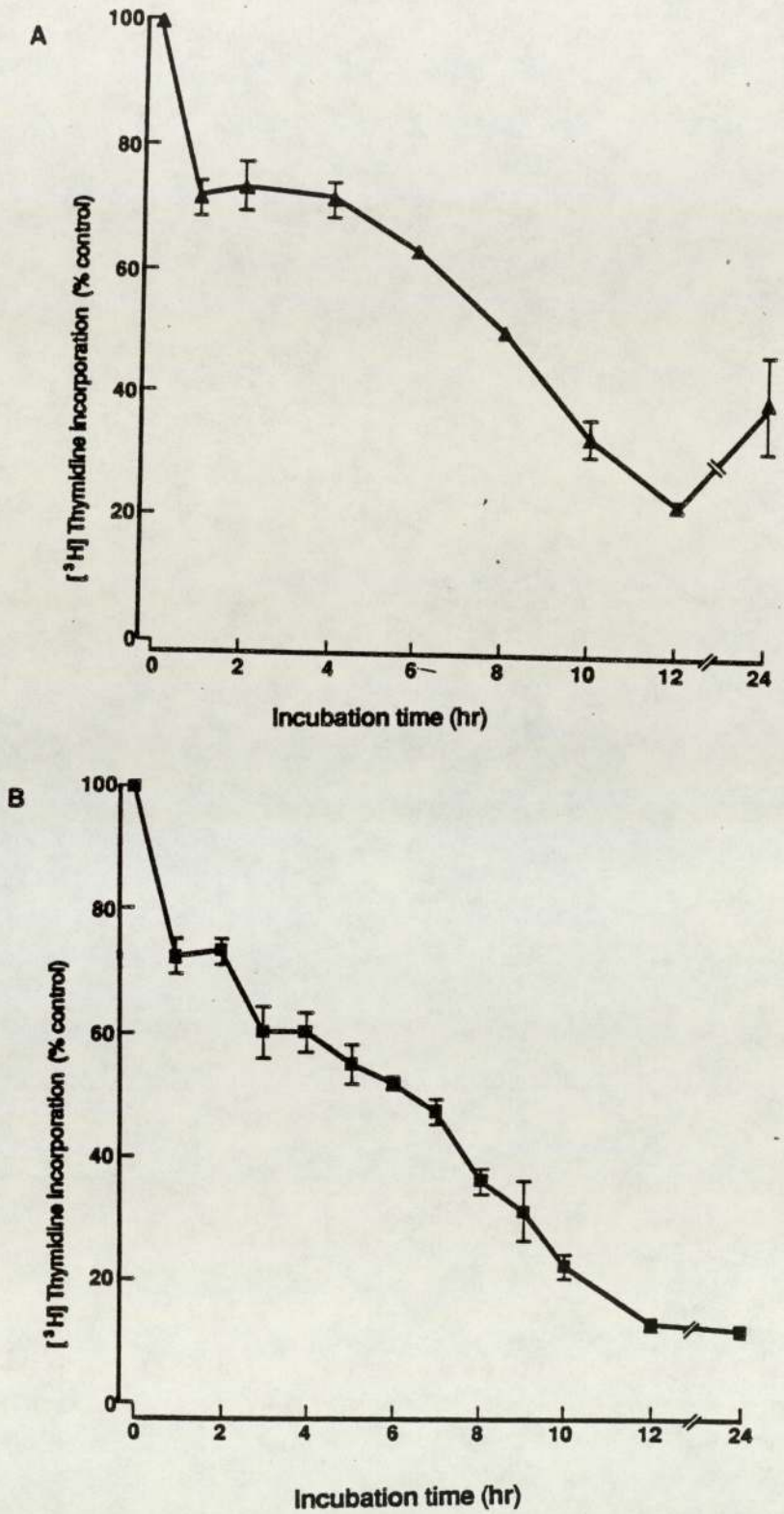


Figure 23. Time-course of the inhibition of [^{3}H]TdR incorporation following treatment of A549 cells with (A) 10 nM bryostatin 1 and (B) 100 nM bryostatin 2. Cells (2 x 10^5) were treated with compound at time 0 and incubated for various time intervals before measurement of [^{3}]TdR incorporation (mean $\pm$ SD (n=4) in one experiment representative of 3).

Section 4.1.7. Changes in cell morphology induced by tumour promoters and bryostatins

4.1.7.1. Light microscopy studies

A549 cells in culture exhibited cellular morphology typical of an epithelial cell line (Fig. 24A). A characteristic rounding up of cells associated with growth inhibition by TPA began to appear after 2-3 h incubation with the phorbol ester and was most marked at those concentrations which also induced maximal growth inhibition (Fig. 24B). A less marked morphological change was visible at 0.1 nM TPA.

The morphological change associated with growth inhibition by TPA was also observed on treatment with PDBu (Fig. 24C), PDA (not shown) and mezerein (Fig. 24D) at those concentrations at which maximal growth inhibition occurred. However, the non-tumour promoting phorbol ester 4-α-PDD, which was not growth-inhibitory in A549 cells (Section 4.1.2), did not cause any change in cell morphology at concentrations as high as 1 µM (not shown). DAGs, which did not mimic phorbol ester-induced growth arrest in A549 cells (Section 4.1.2), did not induce any noticeable change in cell morphology except when at toxic concentrations (not shown).

In the case of either bryostatin, the characteristic morphology change seen with phorbol esters was observed at growth-inhibitory concentrations, although to a smaller degree than in the case of TPA (Figs. 24E, 24F). However, at high non-inhibitory concentrations (> 100 nM) the bryostatins caused little or no change in cell shape.

Cells cultured in the presence of growth-inhibitory concentrations of phorbol esters, mezerein or bryostatins still displayed a rounded cell morphology even when proliferative potential had been regained, although not to the same degree as cells still sensitive to growth inhibition by these compounds (not shown). Similarly, A549-TPA cells more permanently resistant to growth inhibition by phorbol esters (see also Section 4.1.4) did not display the rounded cell morphology to the same extent as sensitive cells (Fig. 24G).

4.1.7.2. Electron microscopy studies

Possible alterations in A549 cell ultrastructure induced by treatment with 10 nM TPA, 100 nM PDBu, 100 nM mezerein, 10 nM bryostatin 1 or 100 nM bryostatin 2 were assessed after treatment for 24 h.

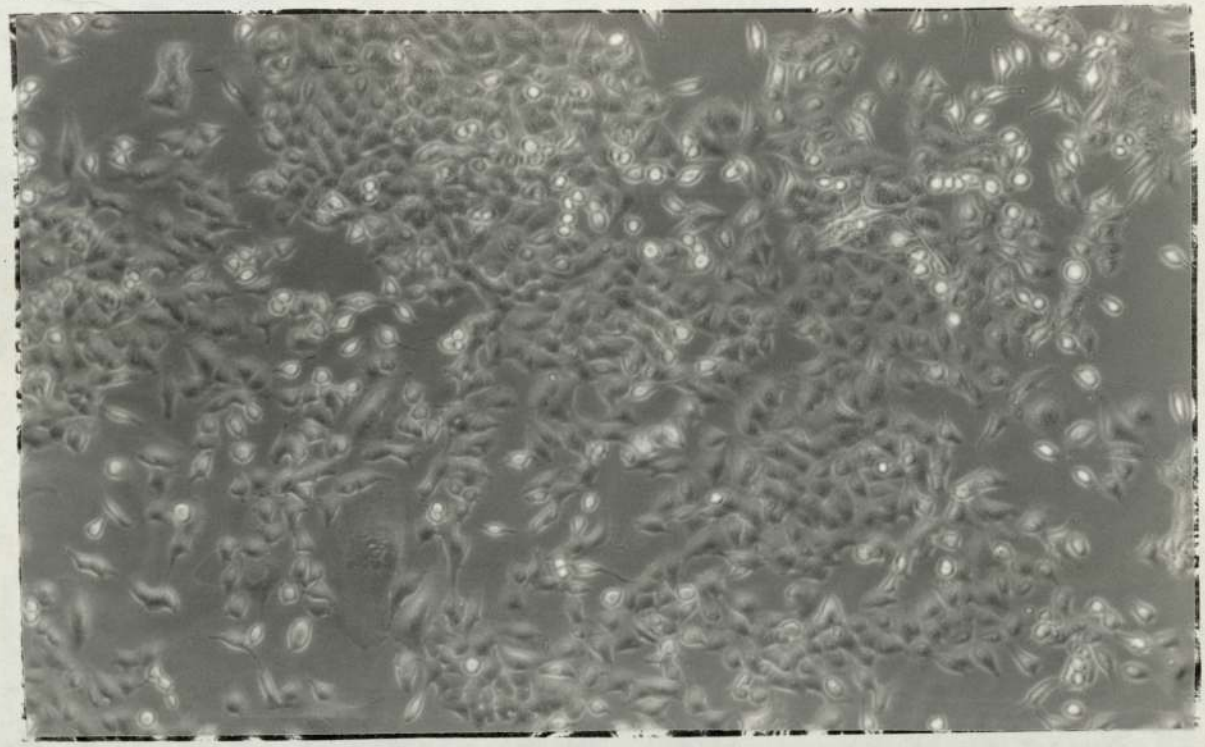
With respect to ultrastructure, no apparent differences between control and treated cells were noted. For brevity, only micrographs of control (Figs. 25A, 25B) and 10 nM TPA-treated cultures (Fig. 25C) are shown. All cells showed varying numbers of multivesicular bodies and myelin figures as has been previously reported (Lieber et al, 1976; Mason and Williams, 1980), the latter being a feature typical of Type II alveolar cells. The Golgi apparatus was dilated and distended in most cases. Junctional complexes between adjacent cells were not observed in either untreated or treated cells.

4.1.7.3. Discussion

Growth-inhibitory phorbol esters, mezerein and the bryostatins have all been shown to be able to induce the characteristic change in A549 cell morphology previously reported following TPA treatment by Gescher and Reed (1985). The finding that no morphological alteration occurs in cells treated with high, non growth-inhibitory concentrations of bryostatins further indicates that this morphology change is intimately associated with growth inhibition. However, the observation that cells which had recovered their proliferative potential in the continued presence of these compounds, including A549-TPA cells, displayed a certain degree of rounded cell morphology suggests that this shape change is not necessarily an indication that cells have completely lost their capacity for growth.

Although light microscopy indicated profound changes in cell morphology, ultrastructural changes as seen by electron microscopy were not apparent. Both treated and untreated cells displayed dilated Golgi apparatus and numerous multivesicular bodies, indicative of a secretory cell type.

(A) A549, control



(B) A549, + 10 nM TPA

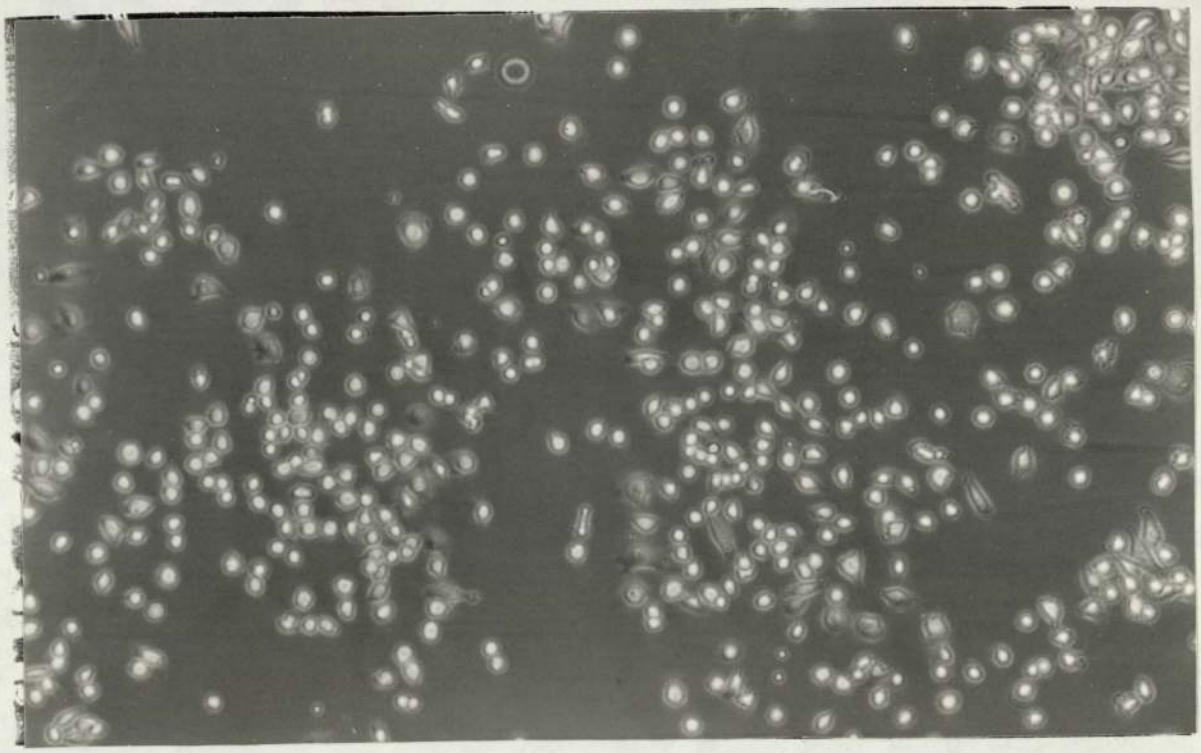
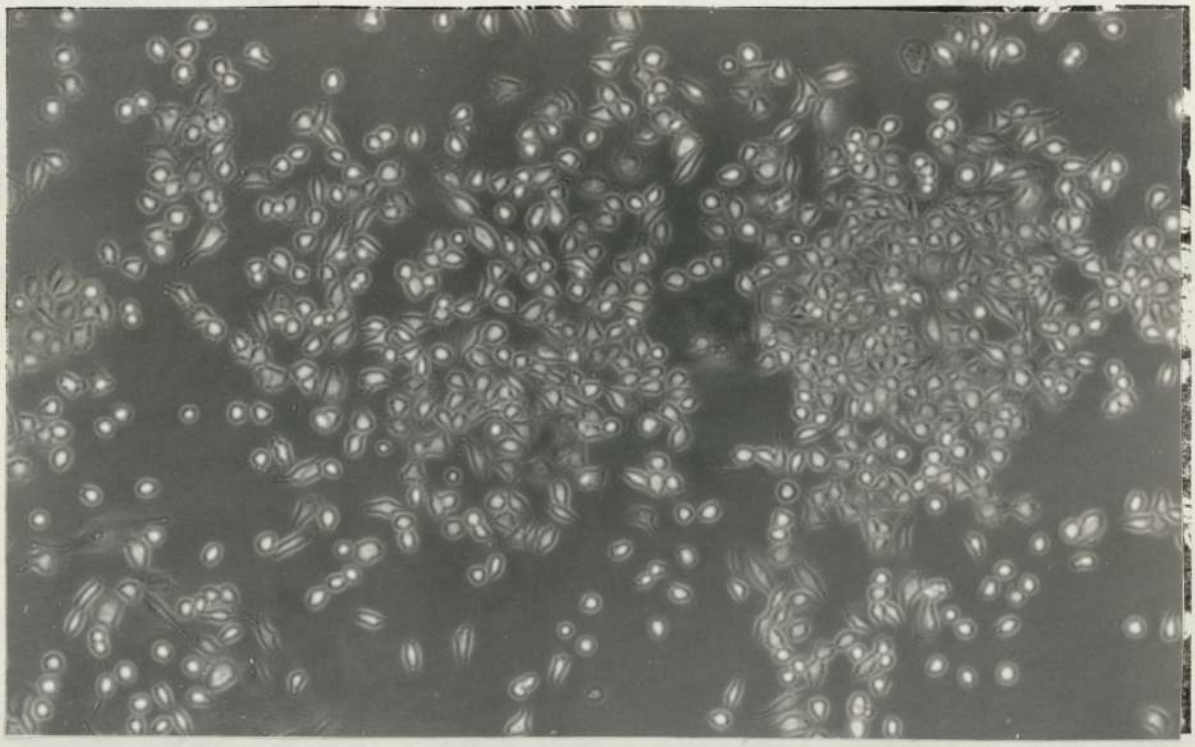


Figure 24. Phase contrast micrographs of cells following incubation with phorbol esters, mezerein or bryostatins. Cells (1 x 10^5) were photographed following treatment with compounds as indicated for 24 h (magnification= x 20).

(C) A549, + 100 nM PDBu



(D) A549, + 100 nM mezerein

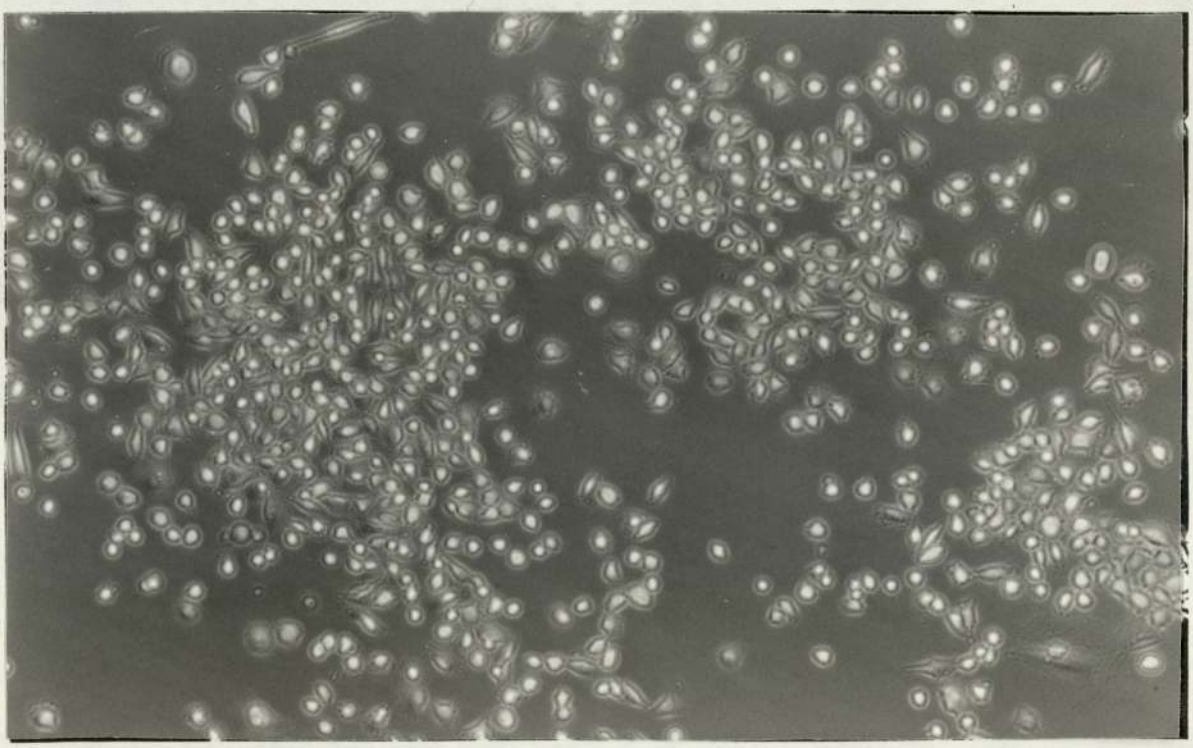
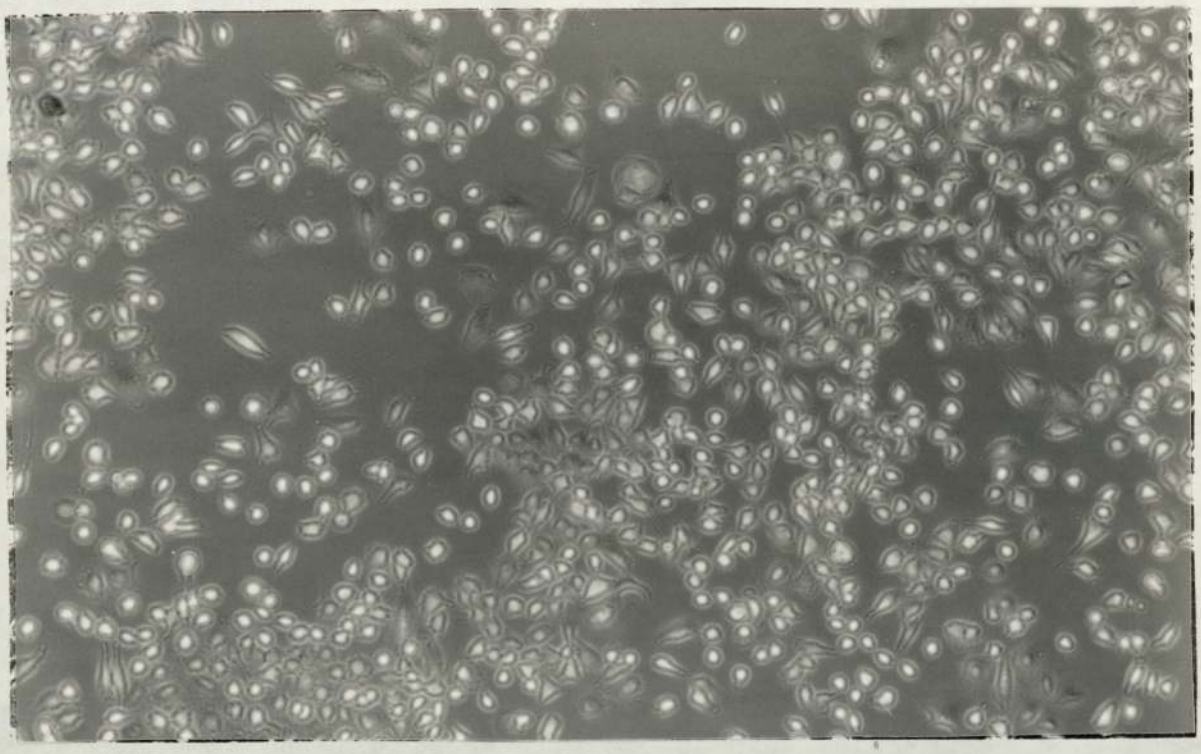


Figure 24, continued

(E) A549, + 10 nM bryostatin 1



(F) A549, + 100 nM bryostatin 2

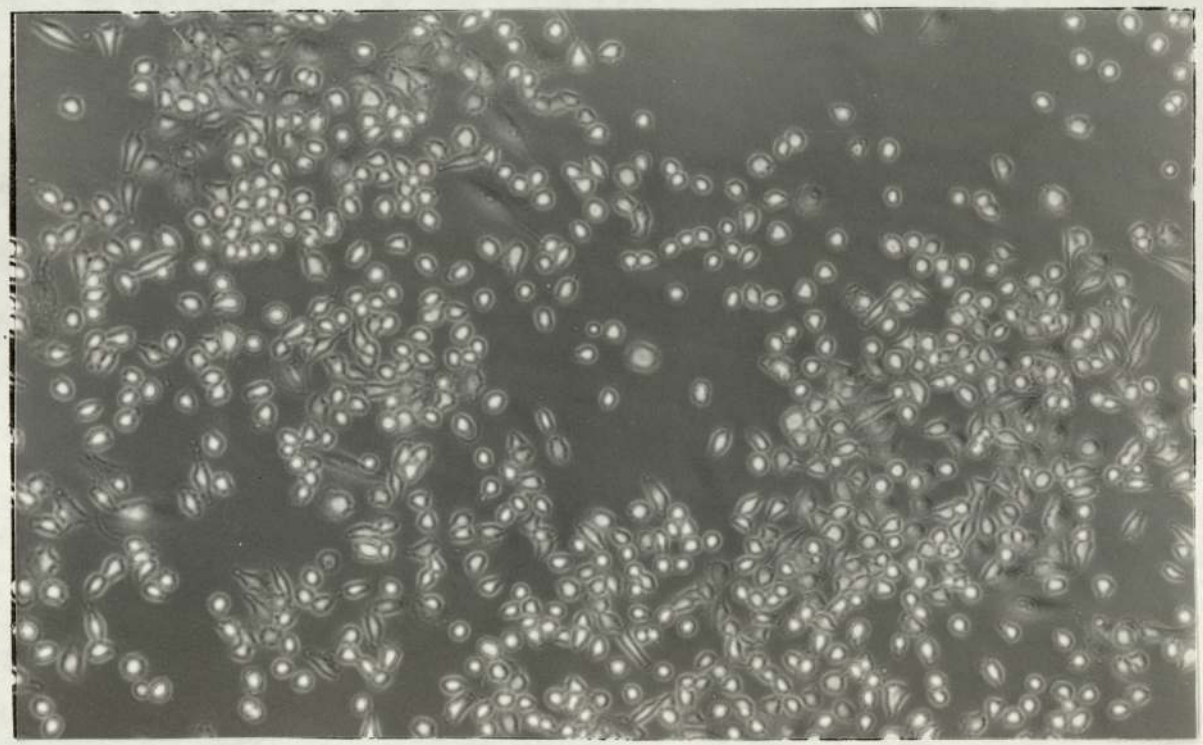


Figure 24, continued

(G) A549-TPA, + 10 nM TPA

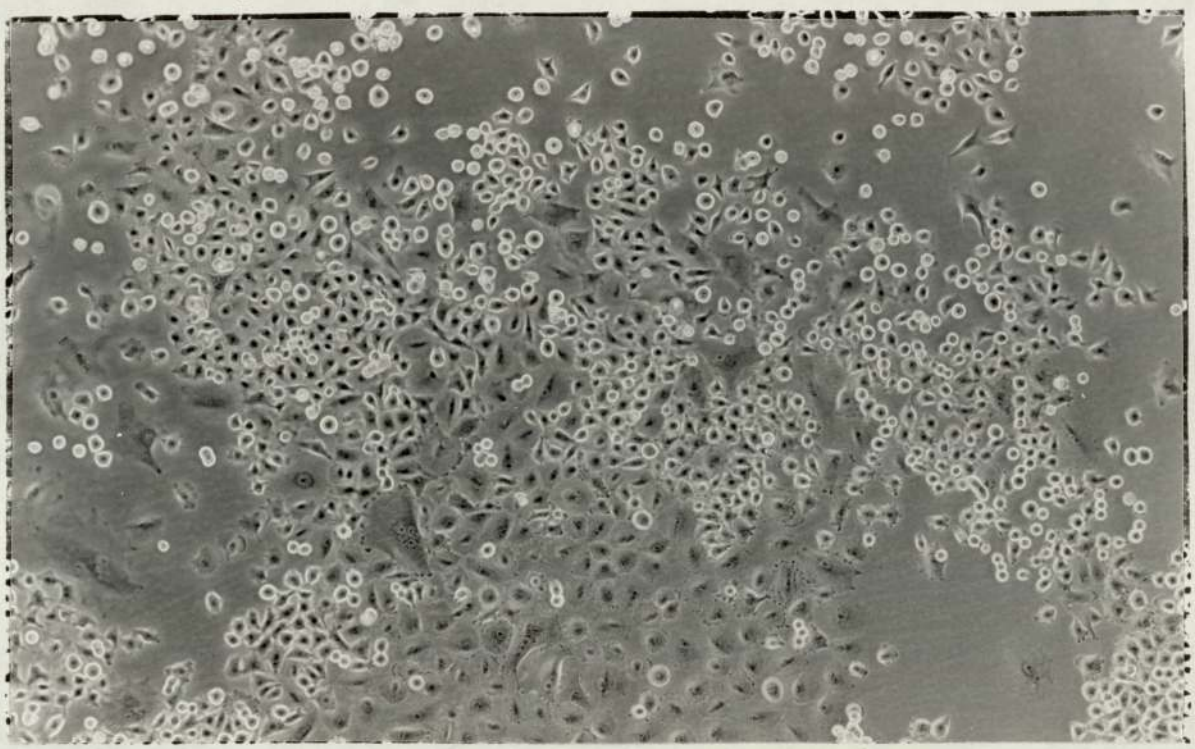


Figure 24, continued

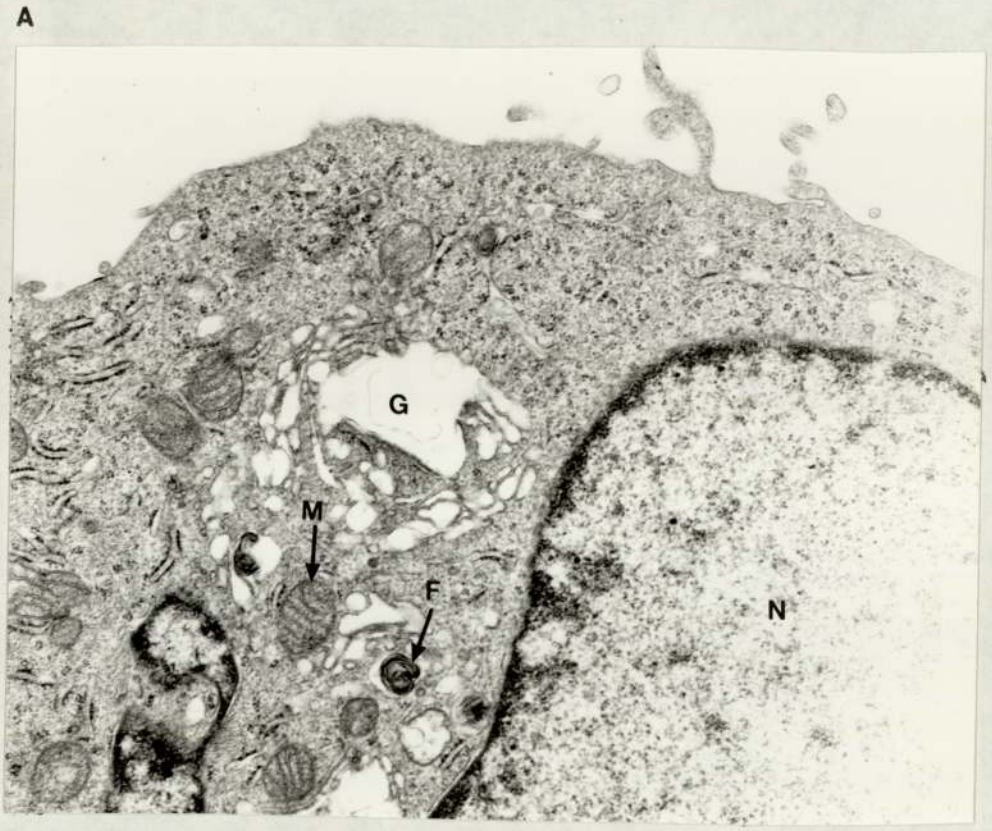


Figure 25. Transmission electron micrographs of control A549 cells and following treatment with 10 nM TPA for 24 h. (A) Control (magnification x 18,600), (B) control (magnification x 4,600), (C) 10 nM TPA (magnification x 4,600). N = nucleus; F = myelin figure; G = dilated golgi; M = mitochondrion.

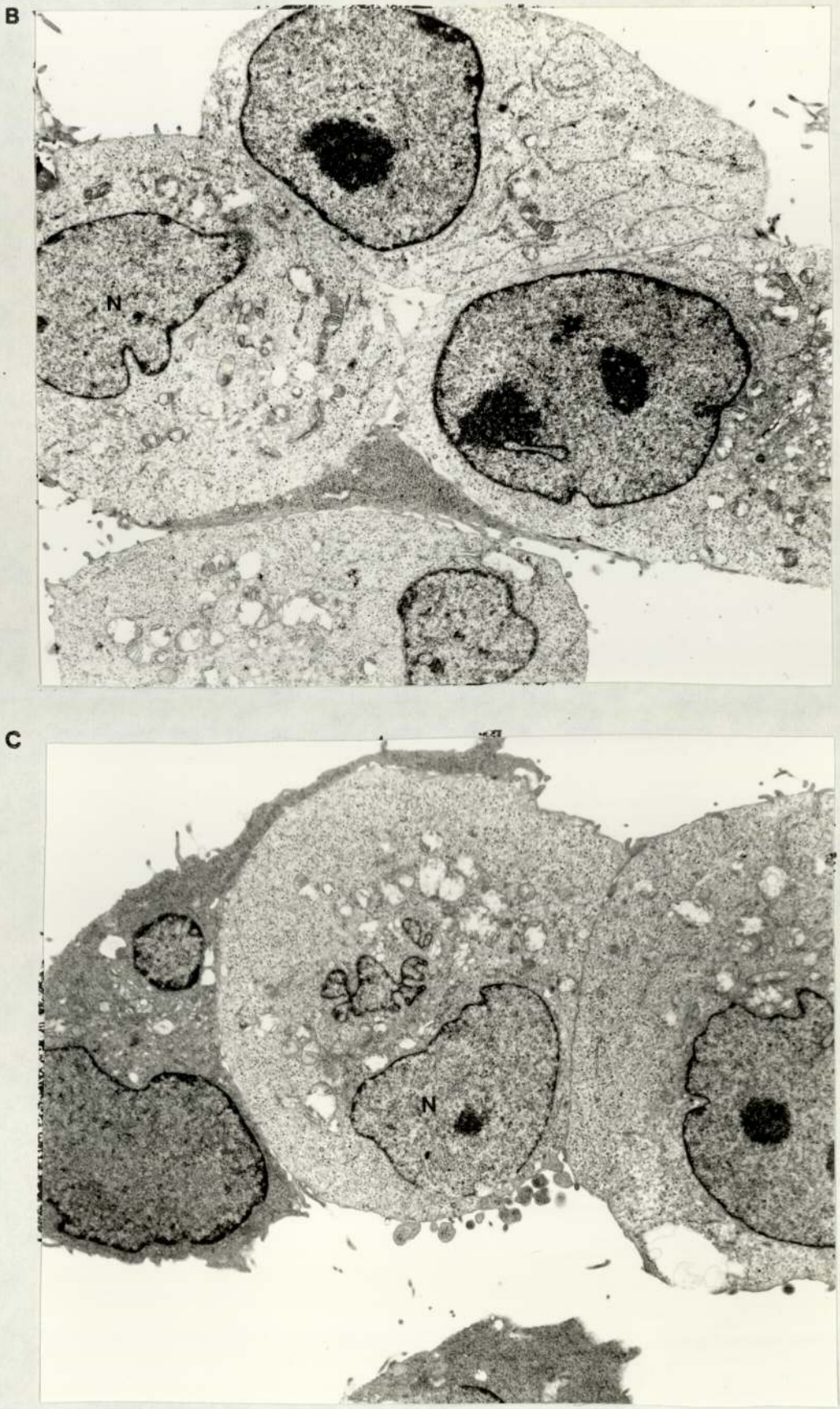


Figure 25, continued

Section 4.1.8. Study of the blockage of the growth-inhibitory effects of compounds by bryostatins

4.1.8.1. Results

When A549 cells were coincubated for 24 h with 10 nM TPA and bryostatin 1 at concentrations > 10 nM (Fig. 26A), the growth-inhibitory influence of the phorbol ester as assessed by the incorporation of [^{3}H]TdR was diminished or abolished with an IC_{50} of 45 nM. The morphological change associated with the inhibition of growth by the phorbol ester was also not apparent. In a parallel experiment, a similar concentration-response curve was obtained, IC_{50} 50 nM, when cell numbers were counted after 5 days coincubation (Fig. 26B). Inhibition of incorporation of [^{3}H]TdR induced by 200 nM TPA was blocked by bryostatin 1 in a concentration-dependent manner with an IC_{50} of 35 nM (Fig. 26C).

At concentrations > 10 nM bryostatin 1 also blocked the growth-inhibitory properties of 100 nM PDBu (Fig. 27A), 100 nM mezerein (Fig. 27B) and 100 nM bryostatin 2 (Fig. 27C) with respective IC_{50} values of 56 nM, 63 nM and 56 nM, and prevented the morphological change associated with growth inhibition.

At concentrations > 10 nM bryostatin 2 blocked the ability of 10 nM TPA (Fig. 28A), 100 nM PDBu (Fig. 28B) and 10 nM bryostatin 1 (Fig. 28C) to induce the growth arrest and morphological change of A549 cells, although less potently than did bryostatin 1.

The results presented in Table 5 demonstrate that the

ability of bryostatins 1 and 2 to block TPA-induced growth arrest was still apparent even following 4 days coincubation with 10 nM TPA and bryostatin. The observed growth characteristics of cells treated with bryostatin and phorbol ester reflected those of cells cultured in the presence of bryostatin alone.

The ability of the phorbol ester PDA, which is only a weak inhibitor of A549 cell growth (Section 4.1.2), to block the antiproliferative influence of 10 nM TPA and 100 nM mezerein was investigated. Unlike the bryostatins, at concentrations as high as 1 µM PDA did not block the action of TPA or mezerein and itself only exerted an antiproliferative effect at micromolar concentrations typical of phorbol esters (Fig. 29).

4.1.8.2. Discussion

As already discussed in Section 1.7, in certain cell systems the bryostatins possess the remarkable ability to inhibit phorbol ester actions, such as the blockage of phorbol ester-induced differentiation of HL-60 cells (Kraft et al, 1986).

In A549 cells the ability of bryostatins 1 and 2 to block the phorbol ester and mezerein-induced inhibition of growth in a concentration-dependent manner has been clearly demonstrated. Further, the growth-inhibitory influence of 10 nM bryostatin 1 could be blocked by concomitant treatment with high non growth-inhibitory concentrations of bryostatin 2 and inhibition of growth by 100 nM bryostatin 2 blocked by high concentrations of bryostatin 1. This

latter observation appears to indicate that the antiproliferative influence of the bryostatins proceeds through a mechanism similar to the phorbol esters and mezerein. The ability of a bryostatin to block the "phorbol ester-like" influence of another bryostatin has also been noted in the CH3 10T½ murine fibroblast line, where in the presence of high concentrations of bryostatin 1 the ability of bryostatin 3 to mimic phorbol ester-induced arachidonic acid release was completely blocked (Dell'Aquila et al, 1988).

Bryostatin 1 blocked growth arrest by phorbol esters, mezerein and bryostatin 2 with an IC_{50} value of approximately 50 nM, suggesting that this effect of bryostatin 1 was mediated via the same receptor(s). Bryostatin 2 also blocked growth arrest by phorbol esters, mezerein and bryostatin 1 with similar efficacy, although with less potency than bryostatin 1. The ability of the bryostatins to completely block TPA-induced growth arrest even after 4-5 days coincubation (Fig. 26B and Table 5) is in contrast to the transient nature of the growth-inhibitory influence of the bryostatins themselves.

There are two possible mechanisms which could explain the blockage of phorbol ester action by the bryostatins. The bryostatins may act directly on PKC at the phorbol ester binding site but evoke a different response to that of the phorbol esters. Alternatively, they may act at an independent target which phorbol esters do not recognize but which feeds back on the PKC pathway. If the former suggested model were correct, a high concentration of TPA

would displace the bryostatin and therefore reverse the bryostatin effect. In the second model, which supposes an independent site of action, the bryostatins would act noncompetitively and their effect would not be reversed by a high concentration of phorbol ester.

In A549 cells it was shown that a similar concentration of bryostatin 1 was required to inhibit the growth-inhibitory effect of TPA at either 10 or 200 nM, suggesting that the bryostatins are acting at at a second site in addition to the phorbol ester receptor/PKC. Further evidence supporting this conclusion has been reported by Dell'Aquila et al (1987), who examined the ability of bryostatin 1 to restore differentiation in the Friend erythroleukaemia cell line as a function of phorbol ester concentration. As was the case in A549 cells, a similar concentration of bryostatin 1 was required to inhibit the effect of PDBu at either 20 or 200 nM PDBu. Warren et al (1987) compared the pattern of protein phosphorylation induced in the HL-60 cell system by bryostatin 1 and PDBu. Bryostatin 1 induced the phosphorylation of the same substrates to be seen in response to PDBu. However, a family of proteins of molecular weight 70 kDa which were phosphorylated by bryostatin 1 but not by PDBu were identified, suggesting that the bryostatins have an additional target in cells.

It has been reported in the literature that weak tumour-promoting phorbol esters such as PDA are able to inhibit tumour promotion by TPA and mezerein (Czerniecki et al, 1986; Slaga et al, 1980). However, it has been shown in this study that PDA was unable to influence TPA- and

mezerein-induced growth arrest of A549 cells and exerted only an antireplicative effect at sufficiently high concentrations. This seems to indicate that PDA cannot interact with the proreplicative receptor(s) modulated by the bryostatins and behaves only as a weak agonist of the antireplicative receptor(s) through which TPA, mezerein and the bryostatins exert their influence on growth.

The observation that in A549 cells the growth-inhibitory influence of 10 nM bryostatin 1 and 100 nM bryostatin 2 could be blocked by concomitant treatment with micromolar concentrations of bryostatin 2 and bryostatin 1, respectively, is highly intriguing. As phorbol esters, mezerein and the bryostatins are all PKC activators one might speculate that the growth inhibition of A549 cells could be mediated, at least in part, via the activation of PKC. At higher concentrations the unique ability of the bryostatins to overcome the block in growth caused by nanomolar concentrations of phorbol esters, mezerein and bryostatins themselves would appear to be mediated by a cellular receptor, as yet unidentified, distinct from PKC. However, in the light of recent knowledge about the existence of a family of PKC genes and the complexity of subcellular localization of PKC (see Section 1.5), a more direct effect on PKC cannot be excluded.

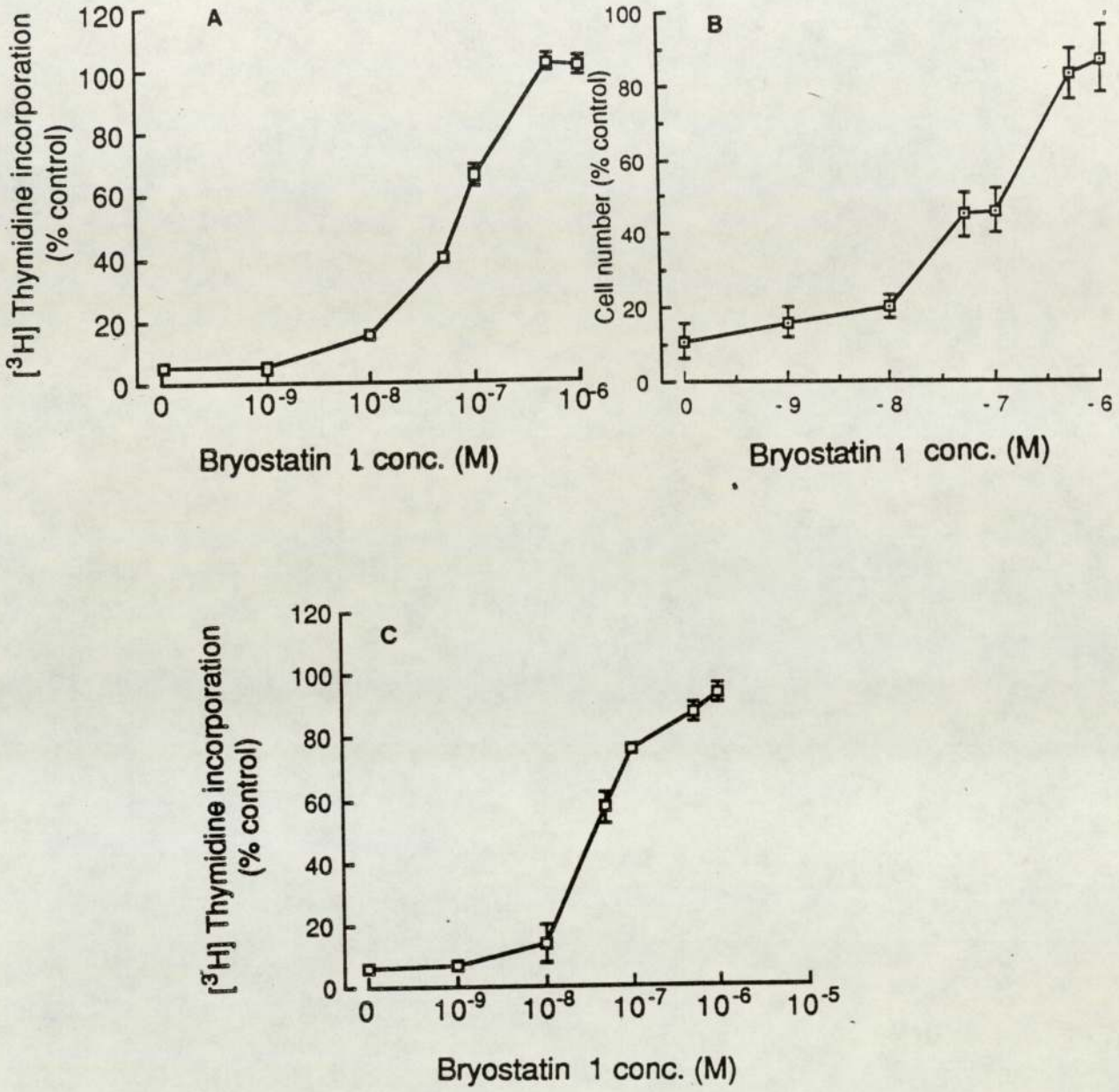


Figure 26. Blockage of the growth-inhibitory influence of TPA in A549 cells by bryostatin 1.

(A) Cells (2×10^5) were treated concomitantly with 10 nM TPA and various concentrations of bryostatin 1 for 24 h before measurement of [^{3}H]TdR incorporation (mean $\pm$ SD (n=4) in one experiment representative of 3).

(B) Cells (2×10^4) were treated concomitantly with 10 nM TPA and various concentrations of bryostatin 1 for 5 days before counting cell number (mean + SD (n=4)).

(C) Treatment as described in (A), except cells treated with 200 nM TPA. (mean $\pm$ SD (n=4) in one experiment representative of 3).

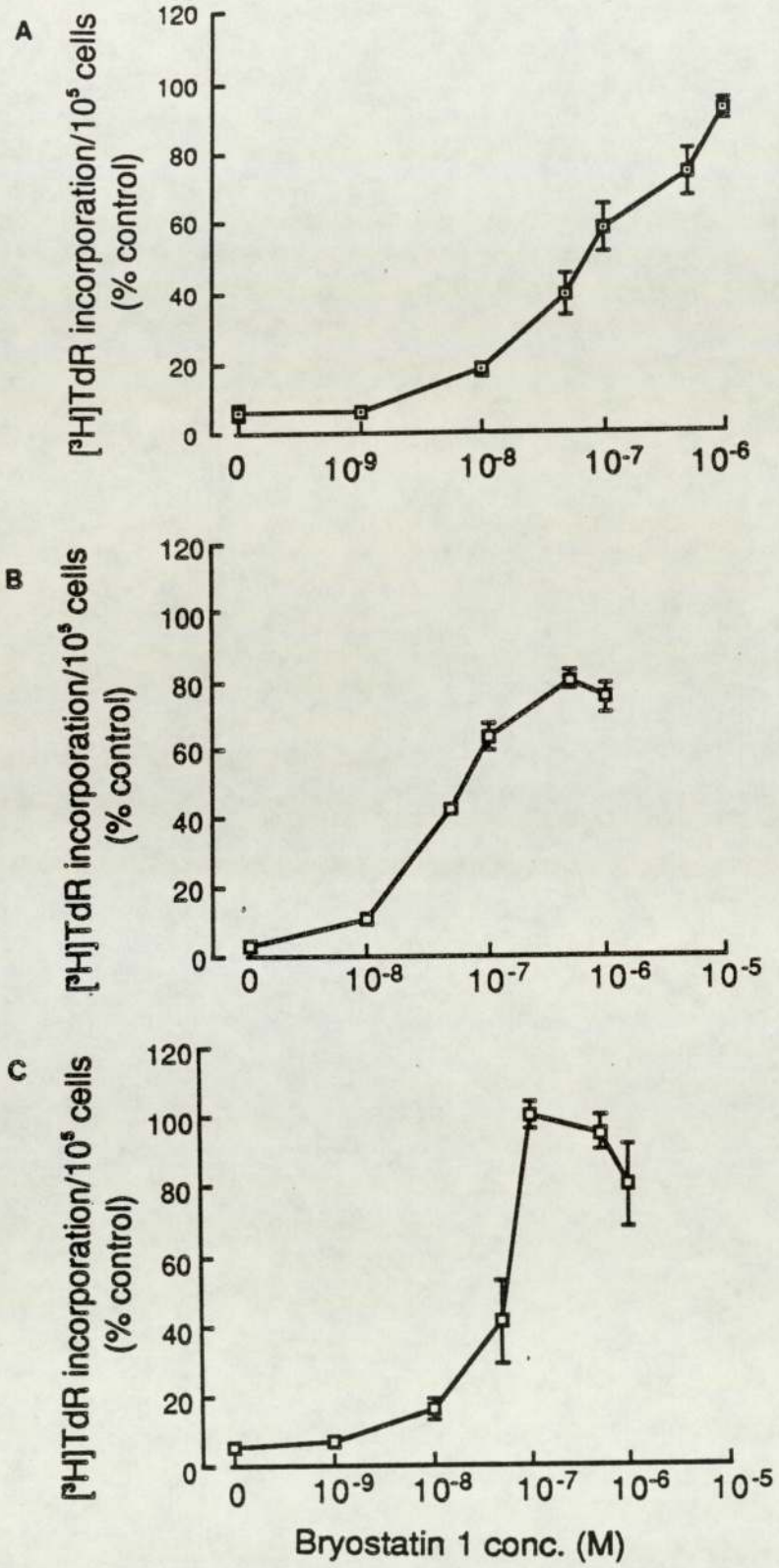


Figure 27. Blockage of the growth-inhibitory influence of (A) 100 nM PDBu, (B) 100 nM mezerein and (C) 100 nM bryostatin 2 in A549 cells by bryostatin 1. Cells (2 x 10^5) were treated concomitantly with compounds as indicated and various concentrations of bryostatin 1 for 24 h before measurement of [^{3}H]TdR incorporation (mean ± SD (n=4) in one experiment representative of 3).

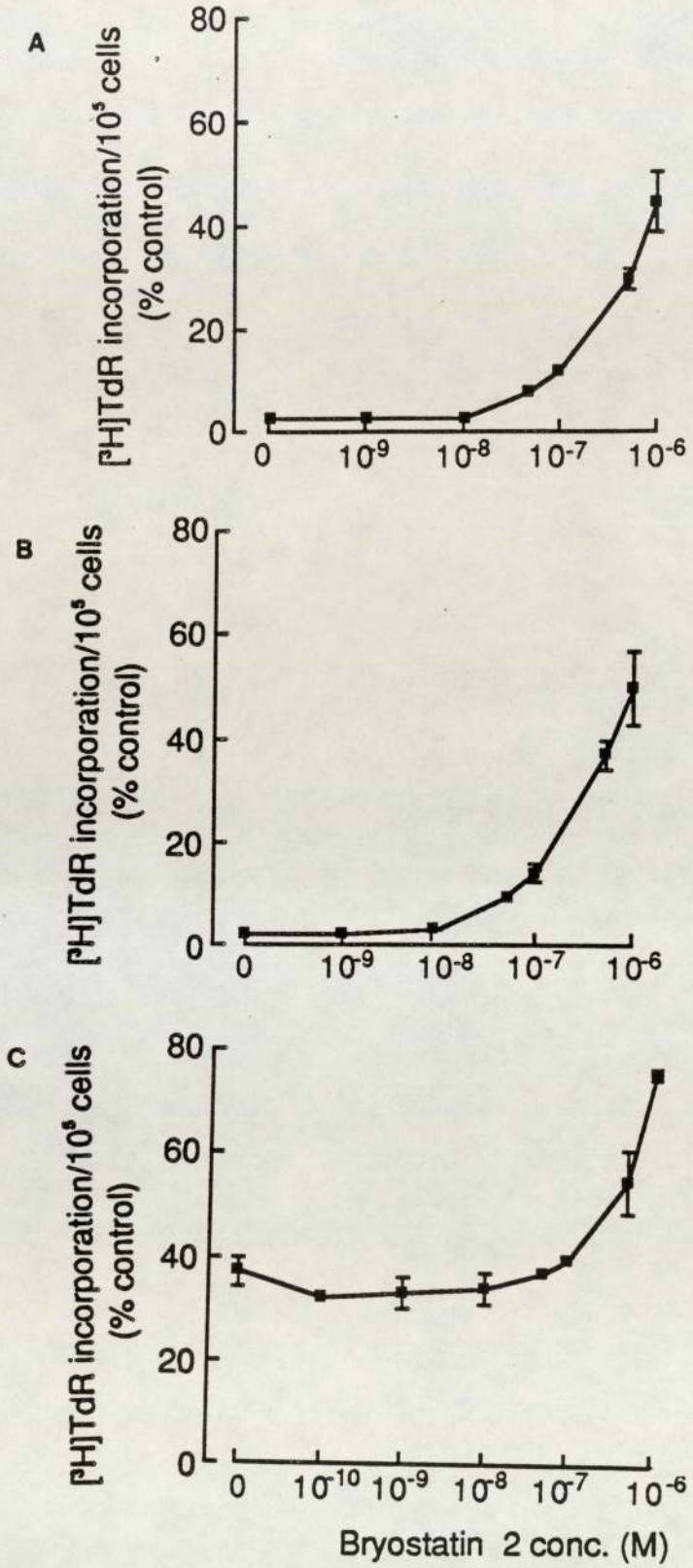


Figure 28. Blockage of the growth-inhibitory influence of (A) 10 nM TPA, (B) 100 nM mezerein and (C) 10 nM bryostatin 1 in A549 cells by bryostatin 2. Cells (2 x 10^5) were treated concomitantly with compounds as indicated and various concentrations of bryostatin 2 for 24 h before measurement of [^{3}H]TdR incorporation (mean ± SD (n=3-4) in one experiment representative of 3).

Treatment	Cell number (x 10^{-5})
None	4.6 ± 0.5
10 nM TPA	0.4 ± 0.1
10 nM Bryostatin 1	1.7 ± 0.3
10 nM Bryostatin 1 + 10 nM TPA	0.9 ± 0.1
1 μM Bryostatin 1	5.0 ± 0.4
1 μM Bryostatin 1 + 10 nM TPA	5.1 ± 0.4
100 nM Bryostatin 2	1.1 ± 0.2
100 nM Bryostatin 2 + 10 nM TPA	0.7 ± 0.03
1 μM Bryostatin 2	4.3 ± 0.2
1 μM Bryostatin 2 + 10 nM TPA	3.3 ± 0.4

Table 5. The effect of incubation with bryostatin 1 or bryostatin 2 together with 10 nM TPA on cell growth. Cells (2×10^4) were treated with bryostatin and/or 10 nM TPA as indicated and cell numbers counted after 4 days incubation (mean ± SD (n=3-6)). Control cultures were confluent on day 6.

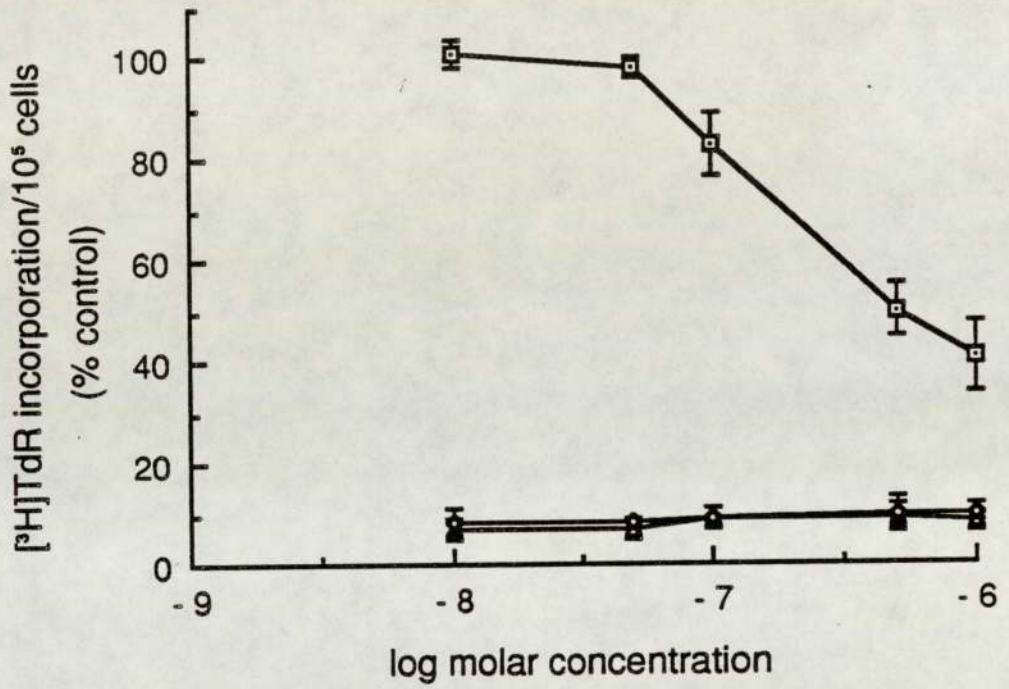


Figure 29. The effect of PDA on the growth-inhibitory influence of 10 nM TPA and 100 nM mezerein in A549 cells. Cells (2 x 10^5) were treated concomitantly with various concentrations of PDA in the presence of 10 nM TPA (▲), 100 nM mezerein (◆) or with PDA only (■) for 24 h before measurement of [^{3}H]TdR incorporation (mean ± SD (n=4) in one experiment representative of 3).

Section 4.2. Study of the growth-inhibitory and cytotoxic properties of compounds synthesized as analogues of phorbol esters or DAGs.

4.2.1. Introduction

Fourteen novel compounds were synthesized in the Cancer Research Campaign Laboratories at Aston University by Dr. Charles A. Laughton. The hypothesis was tested that these compounds, which to varying degrees resemble ring C of the phorbol ester molecule and/or DAGs, mimic the effects of phorbol esters on A549 cells. All were tested for their ability to mimic phorbol ester-induced growth arrest using the A549 cell line as a model system. In order to distinguish any non-toxic, cytostatic influence of the compounds from non-specific effects, cytotoxicities of compounds were determined by assaying LDH release from treated cells.

4.2.2. Growth-inhibitory properties of synthesized compounds

IC_{50} values were determined for compounds 1-12 by treating cultures daily with the test compound for 3 days, before culturing cells in medium only for a further 2 days and counting cell numbers. The DAG analogues, compounds 13 and 14, were evaluated by use of the same protocol as that used to test the synthetic DAGs OAG and diC_8; that is, retreating with compound 3 times daily for 3 days and counting cell numbers after 5 days. The structures of synthesized compounds are shown in Figures 30 and 31 and

their systematic names listed in Appendix 1.

Compounds 1-4, the four diastereoisomers of (±) 1,2-di-O-octanoylcyclohexane-1,2,4-triol, were observed to inhibit cell growth with IC_{50} values near 50 µM (Table 6).

Compounds 5-7 were cyclohexane-1,4-diol monoesters. Compound 5, an octanoyl ester derivative, and compounds 6 and 7, tetradecanoyl ester derivatives, gave respective IC_{50} values of 132, 82 and 85 µM (Table 6).

Compounds 8 and 9 were identical to compounds 1 and 2, respectively, except that the 4-hydroxy group was replaced by a 4-hydroxymethyl group. The IC_{50} values of these compounds were determined to be 49 and 57 µM, respectively (Table 7). Compounds 10 and 11 differed structurally from 8 and 9 only in that the octanoyl functions were replaced by tetradecanoyl groups. The IC_{50} values of 10 and 11 were 52 and 56 µM, respectively (Table 7).

Compound 12, a norbornane-2,3,5-triol diester derivative, exhibited biological properties similar to those of the octanoyl derivatives of the cyclohexane-1,2,4-triol diesters, with an IC_{50} value of 39 µM (Table 7).

Compounds 13 and 14 were DAG analogues. Compound 13, in which the ester function was replaced by a keto group, gave an IC_{50} of 52 µM while the 3-glycinylglycinate derivative of diC_8 had an IC_{50} value of 8 µM (Table 7).

4.2.3. Cytotoxic influence of synthesized compounds measured by the release of LDH from treated cells

LC_{50} values were estimated after treatment of cells with the test compound twice over a 24 h period, as described in

Section 3.1.10. Measurement of LDH release from cells as an indicator of cytotoxicity was preferred to the counting of cells excluding trypan blue, as the former method allowed the mimicking of multiple additions used to determine IC_{50} values.

All compounds were similar in their cytotoxic properties, exhibiting LC_{50} values in the range of 28-90 µM (Tables 6 and 7), with the exceptions of compound 5, LC_{50} 145 µM (Table 6), and compound 14, LC_{50} 11 µM (Table 7). However, in all cases the LC_{50} values of compounds did not differ significantly from their determined IC_{50} values.

It is important to note that visual inspection of cultures treated with growth-inhibitory concentrations of compound suggested a rapid cytotoxic influence within 15 min of the first addition. At non-cytotoxic concentrations, a morphology change such as that observed on treatment with 10 nM TPA was not observed. These results mirror those obtained when cultures were treated with the synthetic DAGs diC_8 and OAG (Section 4.1.2).

4.2.4. Discussion

Few publications have described in detail the structural requirements for activators of PKC. Some studies have focused on the structural requirements of DAGs for activation (Ganong et al, 1986; Boni and Rando, 1985; Kerr et al, 1987; Bonser et al, 1988), while other workers have used computer modelling techniques in an attempt to identify structural features common to PKC-activating natural products (Jeffrey and Liskamp, 1986; Wender et al, 1986;

Itai et al, 1988).

We have been particularly interested in the ability of phorbol esters to cause growth-inhibition at non-toxic concentrations. This property suggests that the interactions between phorbol esters and some of their biochemical targets might be exploited for the therapeutic intervention with proliferative diseases. It has been hypothesized that activation of PKC by phorbol esters is important in the biological responses such as growth inhibition elicited by these compounds. Nishizuka originally proposed that the structural resemblance of ring C of the phorbol ester molecule to DAGs enabled phorbol esters to substitute for DAG in the activation of PKC (Nishizuka, 1984). The structural simplicity of DAGs suggests that, firstly, only a few of the functional groups present in phorbol esters are involved in the binding interaction with PKC and that, secondly, these functionalities lie close to each other.

To investigate the hypothesis that modulation of PKC activity is critical in the induction of growth arrest of A549 cells, a number of cyclohexyl derivatives (compounds 1-11) and a bicyclo [2.2.1] heptyl derivative (compound 12) were synthesized by Dr. C.A. Laughton to mimic structurally both ring C of the phorbol ester molecule and a DAG molecule upon which severe conformational restrictions have been imposed .

Of the 14 compounds tested for their growth-inhibitory properties in A549 cells, compounds 1-4 most closely resemble ring C of the phorbol ester molecule. However, the

results described, in which the compounds only reduced cell numbers at cytotoxic concentrations, indicate that these compounds exert only a non-specific cytotoxic effect on cells. This indicates that either the structural similarity with phorbol esters achieved in these compounds is inconsequential for activity or, alternatively, that the hypothesis which guided the design of the cyclohexanetriol diesters does not take important elements of the phorbol ester pharmacophore into account.

Computer assisted modelling by Dr. C.A. Laughton investigated the degree of resemblance between compounds 1-4 and ring C of the phorbol ester molecule (see also Appendix 2). The O-1, O-2 and O-4 oxygen atoms of the cyclohexane-1,2,4-triol diesters were superimposed over the O-13, O-12 and O-9 oxygen atoms respectively of the phorbol ester molecule. This treatment revealed differences in the goodness of fit depending on the cyclohexanetriol diester isomer and on the conformation chosen. Compound 3 exhibited the best fit, presumably because the stereochemistry of its chiral centres matches that of the corresponding centres in phorbol. However, the analysis revealed that in all cases there were conformational dissimilarities between the phorbol moiety and the cyclohexanetriol diesters. This discrepancy could account for the lack of biological similarity between the compounds and phorbol esters.

Compounds 5-11 mimic ring C to a lesser degree than do the cyclohexanetriol diesters. The growth-inhibitory properties of the cyclohexanediol monoester derivatives, compounds 5-7, indicate that it is the lipophilicity of the compounds which

determines their biological activity. The IC_{50} and LC_{50} values of the octanoyl derivative, compound 5, are approximately twice those of the more lipophilic tetradecanoyl derivatives, compounds 6 and 7. Furthermore, the IC_{50} values of the latter two compounds are similar to compounds 1-4, which possess two octanoyl functions, and are therefore presumably of similar lipophilicity. Therefore, it would seem that the observed growth-inhibitory influence of these compounds is a result of nonspecific cellular damage, apparently influenced by the lipophilicity of the compounds.

Replacement of the 4-hydroxy group of compounds 1 and 2 by a 4-hydroxymethyl group resulted in compounds 8 and 9, respectively. This modification imparted a greater degree of conformational freedom to the molecule. No significant enhancement in growth-inhibitory properties was observed, indicating that this minor structural modification was insufficient for the compounds to mimic the cytostatic properties of phorbol esters. Substitution of the octanoyl functions of compounds 8 and 9 by tetradecanoyl functions resulted in compounds 10 and 11, respectively. Again, no change in the growth-inhibitory influence of the compounds was observed. As compounds 10 and 11 are presumably more lipophilic than compounds 8 and 9, it would appear that the latter are sufficiently lipophilic to elicit the same cytotoxic effects as the tetradecanoyl derivatives.

Compound 12, a norbornanetriol diester derivative, has severe conformational restrictions imposed on it by virtue of the "bridge" across the molecule. Not surprisingly, the

compound displayed biological properties similar to that of the cyclohexanetriol diester derivatives.

It is interesting to note that two recent publications report the synthesis and evaluation of restricted ring diacylglycerol analogues with cyclohexyl or cyclopentyl groups (Bonser et al, 1988; Rando, 1988). These compounds were evaluated by assessment of their ability to activate purified PKC, or alternatively to displace [^{3}H]PDBu binding to purified PKC in a mixed-micellar assay (Section 4.5). All analogues proved to be inactive when compared to DAGs, which are active in these assay systems, and exerted only nonspecific effects at high concentrations.

Two DAG analogues, compounds 13 and 14, exhibited a nonspecific cytotoxic effect similar to that of OAG and diC$_8$ (Section 4.1.2). Compound 13 is resistant to attack by esterases due to the replacement of the ester function by a keto group. However, the compound displayed biological characteristics similar to that of diC$_8$. Compound 14 was synthesized as a putative prodrug which should release 1,2-diC$_8$ by hydrolysis. The compound was found to be marginally more growth-inhibitory than 1,2-diC$_8$ (Section 4.1.2), correlated with an increase in cytotoxicity, possibly because the introduction of a hydrophilic group increased the detergent-like properties of the molecule by combining a polar head group with the hydrophobic DAG tail group.

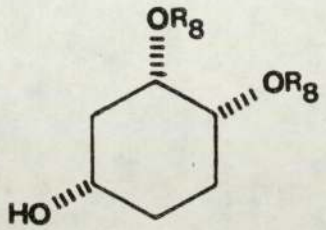
All the evidence presented in this section seems to support the conclusion that the synthesized compounds share insufficient common structural features with the phorbol

ester pharmacophore to induce the same cellular responses. The observation that these compounds exert the same biological effects in the A549 model system as DAGs, the endogenous ligands of PKC, might indicate that activation of PKC is insufficient for the induction of growth arrest, although the ability of the compounds to activate PKC remains to be investigated.

It is conceivable that the rapid metabolism of DAGs and the analogues is responsible for their inability to mimic phorbol esters in the A549 model. Recent work in our laboratories has concentrated on the study of the metabolism of DAGs and the analogues described above in cultures of A549 cells using a gas liquid chromatography method for analytical detection (Laughton, Bradshaw and Gescher, unpublished). Preliminary experiments have compared the metabolic stability of 1,2-diC_8 and a cyclohexanetriol diester, compound 3, in cultures maintained in 1% FCS. 1,2-diC_8 was metabolised by both cells and FCS, and after a 2 h incubation in the presence of both cells and FCS approximately 50% of the DAG had been metabolised. In contrast, the cyclohexanetriol diester was poorly metabolised by either FCS or cells over the 2 hour incubation period, as approximately 90% of the compound was determined unchanged. Therefore, one may tentatively conclude that compound 3 is unable to mimic the effects of TPA on A549 cells because it does not possess the correct elements of the phorbol ester pharmacophore rather than because it is rapidly removed by metabolism. This conclusion cannot be drawn for the synthetic DAG 1,2-diC_8,

as metabolism was apparent.

It is possible that the A549 cell line is a poor model for the screening of molecules with TPA-like activity, especially those which mimic the DAG-like region of the molecule, as DAGs themselves were found to be unable to induce growth arrest. Clearly a much better screening method would be to determine the ability of compounds to bind to purified PKC. This technique is further discussed in Section 4.5.



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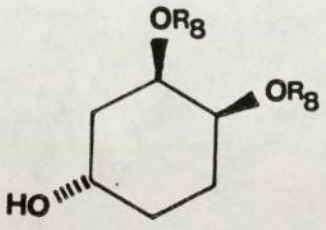

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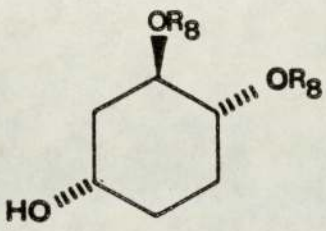

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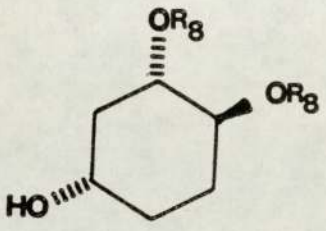

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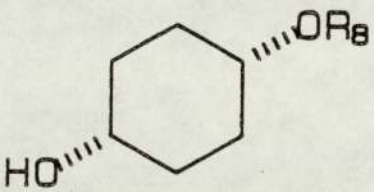

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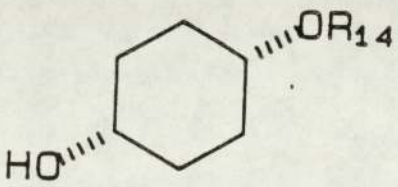

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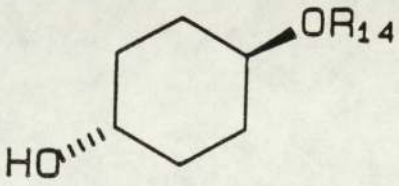

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Figure 30. Structures of compounds 1-7 synthesized to mimic structural features of the phorbol ester molecule and/or DAGs. Growth-inhibitory and cytotoxic properties of compounds 1-7 are described in Table 7. For systematic chemical names of compounds, see Appendix 2.

R_8= $-CO(CH_2)_6CH_3$, R_{14}= $-CO(CH_2)_{12}(CH_3)$

Compound	Growth inhibition IC_{50} (μM)[a]	Cytotoxicity LC_{50} (μM)[b]
(a) Cyclohexanetriol diesters		
1	21 (17, 24)[c]	36 (30, 41)
2	49 (47, 53)	31 (9, 47)
3	43 (27, 66)	36 (30, 41)
4	58 (50, 69)	46 (43, 49)
(b) Cyclohexanetriol monoesters		
5	132 (120, 145)	145 (140, 151)
6	82 (74, 89)	90 (83, 97)
7	85 (80, 90)	84 (80, 87)

Table 6. Growth-inhibitory properties and cytotoxicities of compounds 1-7 synthesized as phorbol ester/DAG analogues. Cells (5×10^4) were treated with compounds as described in Methods and cell numbers counted after 5 days incubation.

[a] Concentration which caused half-maximal inhibition of growth determined by cell counting.

[b] Concentration which caused half-maximal LDH release from cells.

[c] Values were obtained by linear regression analysis of the linear portion of the concentration-response curve constructed with values measured at at least 5 concentrations in each of 4 separate experiments. Values in parenthesis are the 90% confidence limits.

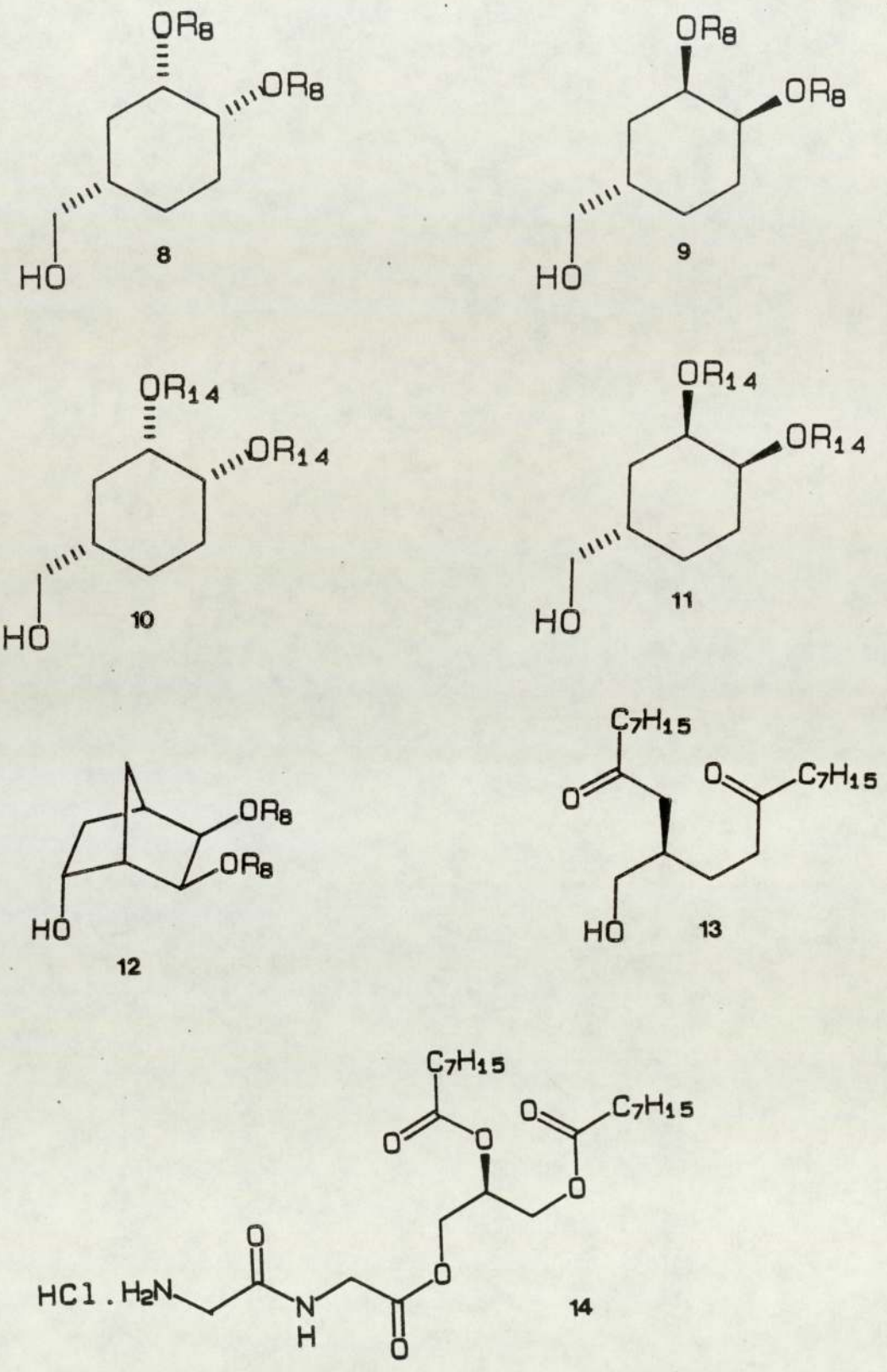


Figure 31. Structures of compounds 8-14 synthesized to mimic structural features of the phorbol ester molecule and/or DAGs. Growth-inhibitory and cytotoxic properties of compounds 8-14 are described in Table 7. For systematic chemical names of compounds, see Appendix 2.

R_8= $-CO(CH_2)_6CH_3$, R_{14}= $-CO(CH_2)_{12}(CH_3)$

Compound	Growth inhibition $IC_{50}(\mu M)$[a]	Cytotoxicity $LC_{50}(\mu M)$[b]
(a) Cyclohexylmethanol diesters		
8	49 (33, 66)[c]	55 (48, 63)
9	57 (47, 68)	57 (51, 62)
10	52 (48, 55)	57 (40, 77)
11	56 (41, 70)	63 (57, 69)
(b) Norbornanetriol diester		
12	39 (27, 50)	28 (19, 36)
(c) DAG analogues		
13	52 (59, 46)	53 (57, 48)
14	8 (10, 6)	11 (6, 17)

Table 7. Growth-inhibitory properties and cytotoxicities of compounds 8-14 synthesized as phorbol ester and or/DAG analogues. Cells (5×10^4) were treated with compounds as described in Methods and cell numbers counted after 5 days incubation.

[a] Concentration which caused half-maximal inhibition of growth determined by cell counting.

[b] Concentration which caused half-maximal LDH release from cells.

[c] Values were obtained by linear regression analysis of the linear portion of the concentration-response curve constructed with values measured at at least 5 concentrations in each of 4 separate experiments. Values in parenthesis are the 90% confidence limits.

Section 4.3. Investigation of the role of PKC in the inhibition of A549 cell growth by phorbol esters and other compounds.

4.3.1. Introduction

Phorbol esters and other active tumour promoters are known to specifically bind to PKC and to activate the enzyme. They substitute for DAG, which is generated during phosphoinositide breakdown in response to various hormonal stimuli. Importantly, the tumour promoters bypass normal physiological regulatory mechanisms, resulting in a permanent activation of the enzyme. This seems to explain, at least in part, their effects on a variety of cellular processes such as growth arrest and differentiation (see Sections 1.5 and 1.6). However, despite intense research effort the precise role of PKC in the modulation of growth remains unclear.

The direct determination of PKC activity in crude cellular extracts is difficult due to the presence of other kinase activities and undefined endogenous inhibitors. Eppenberger and coworkers have developed a method for the quantitation of PKC activity after partial purification by non-denaturing PAGE (Fabbro et al, 1985). We have used this assay technique to investigate the ability of phorbol esters and other PKC activators to influence PKC subcellular distribution and activity in the A549 cell line, previously reported to be potently growth inhibited by phorbol esters (Gescher and Reed, 1985).

4.3.2. Subcellular distribution of PKC activity

PAGE of crude cytosol and particulate subcellular fractions of A549 cells resolved a single peak of Ca^{2+} and phospholipid-dependent protein kinase activity when assayed with histone H1 (Fig. 32). Alternatively, PKC activity was assayed in the absence of Ca^{2+} and phospholipids but with protamine sulphate as exogenous substrate (Figure 32) (Kikkawa et al, 1983). The latter was employed as the method of choice in all cases except where otherwise indicated due to its ability to detect the activity of proteolytic fragments of PKC (Fabbro et al, 1986) and also its ease and cheapness when compared to the former method. The kinase activity was found to coelute with specific [^{3}H]PDBu binding activity (not shown), further confirming the identity of the activity as PKC.

Eppenberger and coworkers report that PKC activity elutes in slices 10-15 when 10% polyacrylamide gels are employed (Fabbro et al, 1985; Regazzi et al, 1986). However, in our hands PKC activity in A549 cells usually elutes in slices 18-25. This discrepancy seems to reflect slightly different electrophoresis running conditions rather than any differences in the nature of PKC in the different cell lines.

Subcellular fractionation of untreated A549 cells revealed that 94 $\pm$ 5% (SD; n=17) of total PKC activity was recovered in the cytosol with the remaining activity located in cellular membranes. Utilising protamine sulphate as substrate the specific activity of PKC in subcellular fractions was: cytosol 1375 $\pm$ 103 units/mg protein, membrane

106 ± 36 units/mg protein and total cellular activity 1494 ± 113 units/mg protein (SD; n=17). As previously reported by Fabbro et al (1986), the incorporation of ^{32}P into protamine sulphate substrate was threefold greater than that with histone substrate (specific activity; cytosol 497 units/mg protein, membrane 33 units/mg protein and total cellular activity 530 units/mg protein (n=2)).

4.3.3. Effect of tumour promoters, DAGs and bryostatins on the subcellular distribution of PKC activity

The relative potencies of phorbol esters, mezerein, DAGs and bryostatins in inducing translocation of cytosolic PKC activity into membranes was investigated by incubating intact A549 cells with various concentrations of compounds for 30 min at 37°C.

TPA was able to induce a concentration-dependent shift of cytosolic PKC activity into membranes, with a half-maximal decrease in total cytosolic PKC activity observed at a concentration of 50 nM. Treatment with concentrations of > 1 nM resulted in loss of cytosolic PKC activity with a corresponding increase of membrane-associated PKC activities, such that treatment with 1 µM TPA for 30 min caused almost complete transfer of PKC activity to the membrane (Fig. 33A). The TPA analogue 4-α-PDD, which is inactive as a tumour promoter and unable to induce growth arrest in A549 cells (Section 4.1.2), did not elicit detectable PKC translocation at concentrations of up to 1 µM (Fig. 34A).

Although less potent than TPA, the structurally related

diterpenoid tumour promoter mezerein was able to induce significant PKC translocation. A decrease in total cytosolic PKC activity was observable at concentrations > 1 nM, while activity detected in the membrane fraction was increased at concentrations > 10 nM. A half-maximal decrease in total cytosolic PKC activity was seen at a concentration of approximately 300 nM (Fig. 33B).

The synthetic DAGs OAG and 1,2-diC_8 did not induce a gross translocation of PKC activity, although 300 µM 1,2-diC_8 was able to cause a small but detectable shift of PKC from cytosol to membrane (Fig. 34B). OAG at concentrations of up to 300 µM did not have any observable influence on the subcellular distribution of PKC activity (Fig. 34C).

Bryostatins 1 and 2 were equipotent in their ability in inducing enzyme translocation to membranes. In both cases, a significant decrease in total cytosolic PKC activity was observed at concentrations > 1 nM, paralleled by an increase in membrane-associated activity at concentrations > 10 nM. At a concentration of 1 µM, both bryostatins induced a loss of approximately 50% of cytosolic PKC activity and a concomitant increase in membrane-associated activity (Figs. 35Aand 35B). In an experiment performed only once, concomitant treatment of cells with 10 nM TPA and 1 µM bryostatin 1 for 30 mins resulted in a large shift of PKC activity from the cytosol to cellular membranes similar to that observed on treatment with 1 µM bryostatin alone, such that 45% of total PKC activity was cytosolic and the remaining 55% was localised in cellular membranes.

4.3.4. Effect of TPA and bryostatins on down-regulation of PKC activity

The ability of various PKC activators to cause time-dependent down-regulation of PKC activity was investigated by assaying enzyme activity after treatment with the compound for various time intervals up to 24 h.

TPA was found to induce a time- and concentration-dependent down-regulation of PKC activity.. Treatment with 300 nM TPA resulted in a rapid and dramatic decrease in cytosolic PKC specific activity within 30 min, with a concomitant increase in its specific activity in the corresponding membrane fraction. However, there was a time-dependent loss of total PKC activity (50% at 30 min) such that PKC activity was not detectable after treatment for 1 h and did not return on continued exposure to the phorbol ester (Fig. 36A). A complete loss of cytosolic phorbol ester receptor binding activity in cells was also noted in parallel with the loss of cytosolic kinase activity (not shown).

Treatment of A549 cells with 100 nM TPA was also observed to elicit gross enzyme translocation which was virtually maximal within 5 min. However, no loss of total specific PKC activity was seen after 30 min and a loss of approximately 50% of total activity was not noted until after incubation for 2 h. After this time period, little or no PKC activity was detectable in the cytosol and all cellular PKC was associated with the membrane fraction, whilst after 6 h down-regulation of total PKC activity was virtually complete (Fig. 36B).

On incubation with 10 nM TPA, the rate of PKC

translocation and down-regulation was slow when compared with 300 nM and 100 nM TPA. Maximal translocation of cytosolic PKC specific activity to membranes was achieved only after 1 h incubation, and total cellular PKC activity did not begin to decrease until after at least 2 h incubation. Correspondingly, after 6 h incubation cytosolic PKC specific activity, although lower than membrane activity, was still observable and total cellular PKC activity was only reduced to 43% of control levels. However, after 24 h incubation complete down-regulation of PKC activity was observed (Fig. 36C).

The ability of bryostatins 1 and 2 to cause down-regulation of PKC activity at their respective maximally inhibitory concentrations of 10 nM and 100 nM, was examined. Both bryostatins induced translocation such that maximal PKC specific activity in cellular membranes was observed after incubation for 2 h. Similar to 10 nM TPA, total cellular PKC activity did not begin to decrease until after at least 2 h incubation, was still apparent after 6 h but down-regulation was maximal after 24 h (Figs. 37A, 37B).

It should be noted that the treatment of cells with tumour promoters or bryostatins resulted in the resolution of three protein kinase activities by PAGE in both the cytosolic and membrane fractions, as previously described by Fabbro et al (1986). This phenomenon is illustrated in Figure 38, which illustrates an experiment in which cells were treated with 100 nM TPA for time intervals of up to 24 h. The major protein kinase activity peak was phospholipid/Ca^{2+} dependent and corresponded to the PKC holoenzyme (Fabbro et al, 1986).

The two minor activities, Ca^{2+} and phospholipid independent, were proteolytic fragments of PKC since they were only present in treated cells (Fig. 38). The appearance and loss of the two minor PKC peaks was more rapid when cells were treated with high concentrations of compounds. No difference was noted in the ability of the tumour promoters and bryostatins to induce the proteolysis of the holoenzyme. PKC activity in A549 cells desensitised to the growth-inhibitory effects of TPA was also examined. No PKC activity was detectable in cells which had been exposed to 10 nM TPA for 6 or 10 days (n=3). Similarly, A549-TPA cells more permanently desensitised to growth inhibition by TPA did not exhibit any PKC enzymic activity (n=3).

4.3.5. Discussion

Growth inhibition by phorbol esters and other tumour promoters has been reported in a wide range of cell types (see Section 1.6). Treatment of A549 cells with nanomolar concentrations of biologically active phorbol esters, mezerein or bryostatins results in the inhibition of proliferation (Section 4.1). However, the cell line is unusual in that the growth-inhibitory influence of these compounds is only transient and that cells regain rapidly their proliferative potential even when cultured in the continued presence of the compound. The A549 cell line has been considered to provide a useful model system to study growth inhibition mediated by tumour promoters and related compounds at the level of PKC, their presumed intracellular target.

In 1983 it was reported that after treatment of EL4 thymoma cells with phorbol esters, PKC activity was found in a form tightly associated with the particulate fraction (Kraft and Anderson, 1983). It has been proposed that translocation of PKC activity to membranes is an early event associated with phorbol ester actions which finally lead to specific biological effects in cells. We have therefore examined the ability of a number of PKC activators to induce enzyme translocation in an attempt to correlate changes in PKC subcellular distribution with inhibition of A549 cell growth.

In untreated A549 cells PKC activity was predominantly localised in the cytosol. As is the case in many other cell lines, exposure to TPA induced a time- and concentration-dependent shift of cytosolic PKC activity to cellular membranes. Mezerein and bryostatins 1 and 2 were also able to induce a similar translocation of enzyme activity, albeit at higher concentrations than TPA. Concomitant treatment of cells with 10 nM TPA and 1 µM bryostatin 1 resulted in a gross translocation of PKC activity from the cytosol to membranes as was observed on treatment with the bryostatin alone. It is therefore apparent that the ability of the bryostatins to block TPA-induced growth arrest is not as a result of any blockage of PKC translocation.

The ability of these compounds to induce a rapid subcellular redistribution of PKC activity strongly supports the hypothesis that translocation and activation of PKC by these compounds is crucial in mediating growth inhibition of

A549 cells. This conclusion is further strengthened by the finding that 4-α-PDD, an inactive TPA analogue, and the synthetic DAGs OAG and 1,2-diC$_8$, which were unable to induce growth arrest at non-toxic concentrations (see Section 4.1.2), did not seem to stimulate significant subcellular redistribution of PKC activity, although it is possible that a small shift of activity to the membrane following treatment with 300 μM 1,2-diC$_8$ for 30 min had occurred. Although the lack of effect of 4-α-PDD can be explained by its inability to bind to and to activate PKC, it is perhaps surprising that DAGs, the presumed physiological ligands of the enzyme, could not induce significant translocation. However, the lack of influence of DAGs on PKC cellular distribution can be explained in a number of ways. Halsey et al (1987) reported that treatment of 3T3-L1 fibroblasts with 200 μM 1,2-diC$_8$ resulted in a modest and transient membrane association of PKC. Reduction of cytosolic PKC activity was maximal after 5 min but after 30 min enzyme activity had returned to control levels. Similarly, in MCF-7 breast carcinoma cells treated with 124 μM 1,2-diC$_8$ a small but significant shift of PKC activity from the cytosol to membranes was noted which was maximal after 5 min. However, after 60 min cytosolic activity had returned to control levels (Issandou and Darbon, 1988; Issandou et al, 1988). In A549 cells, PKC translocation was only assessed after 30 min incubation with concentrations of up to 300 μM OAG or 1,2-diC$_8$, so the possibility that a transient activation of PKC had occurred cannot be ruled out. An alternative explanation for the lack of effect of DAGs is

the unfavourable pharmacological properties of DAGs when compared to phorbol esters (see Section 1.5). A third alternative, that A549 cells express PKC isozyme(s) responsive to phorbol esters but insensitive to DAGs, seems unlikely as recent experiments indicate that 1,2-diC_8 can compete with [^{3}H]PDBu for binding to PKC in A549 cells (Bradshaw and Gescher, unpublished). However, it is possible that a PKC isozyme in A549 cells which does not respond to DAGs is responsible for the growth-inhibitory influence of the tumour promoters and bryostatins.

Many other studies provide strong support for the hypothesis that PKC translocation is necessary for the action of phorbol esters and other compounds. The possible role of PKC in the inhibition of proliferation by phorbol esters in human breast cancer cell lines has been investigated by a number of laboratories. Treatment of the MCF-7 breast cancer cell line with TPA resulted in the complete loss of proliferative potential, although cells began to grow again when the phorbol ester was removed from the medium (Osborne et al, 1981). It was shown that TPA induced rapid translocation followed by a progressive decline of both PKC activity (Darbon et al, 1986; Fabbro et al, 1986) and phorbol ester binding activity (Darbon et al, 1987). Eppenberger and coworkers also observed a similar translocation and down-regulation of PKC activity following treatment of cells with phorbol esters and other tumour promoters in other breast cancer cell lines (Fabbro et al, 1986; Regazzi et al, 1986; Roos et al, 1986). As is the case in A549 cells, TPA-dependent translocation was observed

in the TPA-sensitive MCF-7, ZR-75-1, MDA-MB-231, HBL-100 and BT-20 lines (Fabbro et al, 1986). A TPA-unresponsive line, T-47-D, exhibited only very low levels of PKC activity, suggesting that the enzyme is important in the growth inhibition of breast cancer cell lines by TPA (Fabbro et al, 1986).

Translocation of PKC activity from the cytosolic fraction to cellular membranes can be induced by a number of growth factors and hormones which generate DAG, including interleukin 2 (Farrar and Anderson, 1985), interleukin 3 (Farrar et al, 1985), gonadotropin releasing hormone (Naor et al, 1985) and adrenocorticotropin (Vilgrain et al, 1984). However, these responses are usually transient and of lower magnitude than those induced by phorbol esters.

Results of investigations on the subcellular distribution of PKC activity in relation to the proliferative potential of cells also support the argument that activation of PKC is dependent on its translocation to the particulate fraction. For example, in rat colonic epithelial cells with differing growth characteristics, PKC activity was higher in the particulate fraction in proliferative cell types than nonproliferative ones (Craven and DeRubertis, 1987). Similarly, particulate PKC activity in rapidly growing and transformed 3T3-L1 fibroblasts (Halsey et al, 1987) and CH3 10T½ cells (Miloszewska et al, 1986) was greater than in confluent cultures. Readdition of serum to serum-deprived U937 monoblastic cells resulted in the resumption of growth, associated with a two-fold increase in particulate PKC activity (Ways et al, 1986).

Other workers have attempted to analyse the role of PKC translocation by using TPA-resistant cell variants. In human promyelocytic leukaemia HL-60 cell variants resistant to the induction of differentiation elicited by TPA, treatment with the phorbol ester did not cause the subcellular redistribution of PKC observed in the parent cell line (Homma et al, 1987). In a T-lymphocyte mutant which did not respond to the mitogenic influence of TPA seen in wild type cells no detectable PKC activity was found (Mills et al, 1988). However, other TPA-resistant cell lines appear to contain normally responsive PKC and may have abnormal responses to PKC activation or altered PKC-independent effects of TPA (Forsbeck et al, 1985; Leftwich et al, 1987; Shoji et al, 1987).

It has been suggested that the direction of translocation of PKC by phorbol esters and other compounds is important in determining cellular responses. Girard et al (1987) reported that in HL-60 cells TPA induced a rapid, marked and sustained translocation of PKC to the plasma membrane, as determined immunocytochemically, immunologically and by assaying PKC activity in the subcellular fractions. In contrast, the phorbol ester caused translocation to the perinuclear and nuclear structures in leukaemic K562 and fibroblastic CHO and E7SKS cells, all of which are resistant to TPA-induced differentiation (Girard et al, 1987). Shoji et al (1987) have studied the effect of TPA on PKC translocation and down-regulation in the KG-1 human acute myeloid leukaemia cell line, which differentiates in response to treatment with the phorbol ester. TPA induced

translocation to the plasma membrane in an adherent subpopulation of cells, whereas the enzyme remained largely in the cytoplasm and perinuclear area of nonadherent cells. However, in a subline resistant to the differentiating effect of TPA, PKC translocation to the perinuclear region was observed (Shoji et al, 1987). In HL-60 cells, bryostatin 1 was observed to stimulate specific PKC translocation to the nucleus whereas TPA treatment induced translocation to the plasma membrane (Fields et al, 1988).

However, despite the wealth of evidence supporting an important role for PKC translocation in the biological effects of phorbol esters and other compounds, a number of reports seem to indicate that this phenomenon is not essential for the cellular response. Indeed, our results indicate that a significant shift of PKC activity to membranes is not necessary in the growth inhibition of A549 cells. Treatment of cells with 0.1 nM TPA, the concentration required to inhibit A549 cell proliferation by 50%, did not induce any detectable shift in PKC activity to membranes and half-maximal translocation of PKC was only noted at a concentration of 50 nM TPA. Similarly, mezerein and the bryostatins were able to induce growth arrest at concentrations at which subcellular redistribution of PKC activity was not observed. The induction of biological responses by TPA at concentrations below those required for PKC translocation has also been observed in a number of other cell systems. In the monoblastoid U937 cell line, which is terminally differentiated by treatment with phorbol esters, translocation of PKC was only apparent at

concentrations of TPA 100-fold those required to cause inhibition of DNA synthesis and 10-fold those necessary to elicit adherence of cells to plastic (Forsbeck et al, 1985; Skoglund et al, 1988). Treatment of 3T3-L1 fibroblasts with the peptide mitogens PDGF, FGF and bombesin, which all stimulate phosphoinositide turnover and are therefore believed to act via the activation of PKC (Berridge et al, 1985), induced proliferation without any detectable increase in the membrane association of the kinase (Halsey et al, 1987). Similarly, in mast cells the concentration of TPA required to induce a half-maximal subcellular redistribution of immunodetectable PKC activity was an order of magnitude greater than the half-maximal dose required to inhibit the antigen-mediated increase in $[Ca^{2+}]_i$ (Erne et al, 1987). However, it is conceivable that the ability of PKC to amplify the phosphoinositide signal cascade by phosphorylating a large number of proteins when activated means that only a small fraction of the total cellular PKC needs to be activated to induce a biological response.

Early studies showed that chronic exposure of cells to phorbol esters resulted in the loss or down-regulation of phorbol ester binding sites (Collins and Rozengurt, 1982; Solanki et al, 1981). Following the identification of PKC as the phorbol ester receptor, Rodriguez-Pena and Rozengurt reported that treatment of 3T3 fibroblasts with TPA induced the down-regulation of PKC activity (Rodriguez-Pena and Rozengurt, 1984). Down-regulation of PKC activity possibly accounts for the widespread observation that prolonged incubation of many cell types with phorbol esters results in

the depletion of total cellular PKC activity and in resistance to many phorbol ester responses (Gainer and Murray, 1985; Glynn et al, 1986; Katakami et al, 1986; Rodriguez-Pena and Rozengurt, 1984). Down-regulation is due to the activity of a specific protease, calpain I, that preferentially proteolyses the membrane-bound enzyme (Kishimoto et al, 1983), resulting in a phospholipid- and Ca^{2+}-independent catalytic (50 kDa) and a regulatory phorbol ester-binding unit (30 kDa) (Kishimoto et al, 1983; Lee and Bell, 1986). Although the physiological significance of down-regulation of PKC is unclear (reviewed by Murray et al, 1987), it is possible that it represents a level of control that may play a role in determining cellular responsiveness towards phorbol esters and also towards hormones and growth factors.

In A549 cells, TPA has been demonstrated to induce down-regulation of PKC activity in a concentration- and time-dependent manner. The observation that treatment with a high concentration of TPA (300 nM) resulted in a more rapid translocation, proteolysis and loss of PKC activity than lower concentrations (100 nM and 10 nM) supports the suggestion that the membrane-associated form of the enzyme is susceptible to proteolysis. Chronic exposure of cells to bryostatins 1 and 2 also resulted in the proteolysis and loss of PKC activity in a manner similar to that observed with TPA. There was no evidence to suggest that down-regulation of PKC activity was more rapid in cells treated with bryostatins, as has been shown in some other cell types (Blumberg, 1988; Kraft et al, 1988). The ability

of the bryostatins, which seem to inhibit A549 cell growth only transiently, to induce a similar translocation and down-regulation of PKC activity as TPA, which inhibits proliferation for a number of days, suggests that the molecular mechanism(s) responsible for the resumption of growth are not related to the down-regulation of PKC.

The loss of PKC activity in A549 cells induced by 10 nM TPA was shown to be sustained even after cells had regained their proliferative potential after 6 and 10 days incubation in the presence of the phorbol ester. Similarly, the more permanently TPA-resistant cell variant A549-TPA showed no detectable PKC activity. This again seems to suggest that PKC plays no role in the mechanism(s) which result in the recovery of the proliferative capacity of the cells. It should perhaps be noted that the initial study of the down-regulation of the phorbol ester receptor/PKC by Gescher and Reed (1985) indicated that receptor down-regulation in phorbol ester-desensitised cells was never greater than 25% This is in contrast to the present studies, in which complete loss of functional PKC activity was observed. As PDBu rather than TPA was used in the receptor down-regulation studies, one may speculate that this discrepancy is the result of the differential abilities of TPA and PDBu to induce the down-regulation of phorbol ester binding or PKC activity, as has been reported in certain other cell lines (Collins and Rozengurt, 1984; Kariya and Takai, 1987; Mattingley et al, 1987; Phillips and Jaken, 1983). The recent observation by Nishizuka's group that TPA induces differential down-regulation of PKC subspecies in

the clonal pre-B pre-T KM3 cell line (Ase et al, 1988) indicates that the varying ability of phorbol esters and other PKC activators to induce down-regulation is as a result of the differential resistances of these subspecies to down-regulation.

In view of the finding that A549-TPA cells completely lack functional PKC activity, the inability of mezerein and bryostatin 1, like TPA, to induce growth arrest in this cell variant (Section 4.1.4) would seem to argue strongly that PKC plays a crucial role in the antiproliferative influence of these compounds. However, the possibility that TPA causes other cellular changes, as yet unidentified, which result in the loss of responsiveness to these other compounds cannot be discounted.

The fate of PKC following down-regulation is unclear. Proteolysis of the holoenzyme by calpain I results in the production of a catalytic fragment which is not regulated by DAG, phospholipid or Ca^{2+} (Kishimoto et al, 1983). It is possible that the ability of this catalytic fragment to phosphorylate cytosolic substrate proteins which are inaccessible to the holoenzyme is responsible, at least in part, for some of the effects of phorbol esters (reviewed by Murray et al, 1987). Indeed, in A549 cells phospholipid- and Ca^{2+}-independent kinase activity was observed in both the cytosol and membrane fractions following treatment with tumour promoters and bryostatins, although the activity of these catalytic fragments themselves was rapidly lost.

The appearance and subsequent loss of phospholipid- and

Ca^{2+}-independent kinase activity has also been observed following TPA treatment of breast cancer cell lines growth inhibited by the phorbol ester (Fabbro et al, 1986; Regazzi et al, 1986). As has been previously mentioned, the kinetics and down-regulation of PKC activity in these cell lines was homologous. PKC synthesis was unaffected by TPA treatment but functional PKC (77/80-kDa) activity was found to be replaced by membrane-bound 74- and 80-kDa PKC-related polypeptides (Borner et al, 1988). These two proteins lacked protein kinase and phorbol ester binding activity. Interestingly, the amounts of the membrane-bound immunoprecipitable 80-kDa PKC-related polypeptide in long-term TPA-treated cells appeared to be inversely related to the degree of TPA-induced growth inhibition of the respective cell lines (Borner et al, 1988). As removal of TPA led to growth resumption of human breast cancer cells concomitant with the reappearance of functional PKC (Fabbro et al, 1986), it was suggested that the variable turnovers of these putative PKC precursors might play a role in the different growth response to phorbol esters. One might speculate that a similar sequence of events occurs in A549 cells, as a preliminary Western blot experiment performed during a study visit to the laboratories of Professor Eppenberger indicated that a PKC-related polypeptide was present in the membrane fraction after 6 h treatment with 300 nM TPA (result not shown), at which time functional PKC activity was not detectable.

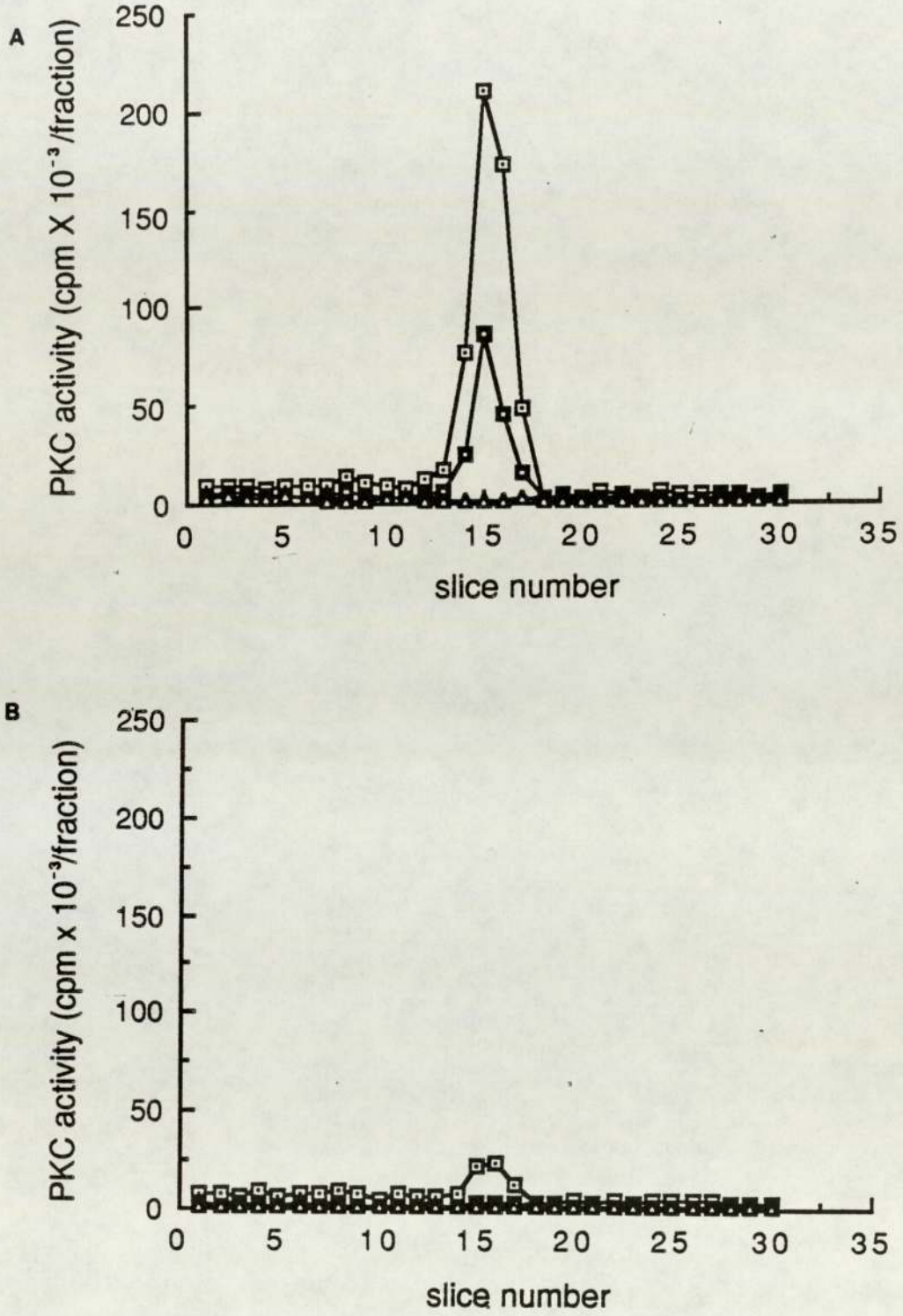


Figure 32. Resolution of PKC activity from (A) cytosolic and (B) membrane fractions of A549 cells by non-denaturing PAGE. PKC activity eluted from gel slices as described in Methods was assayed using protamine sulphate (□) or lysine-rich histone H1 (■) as substrate. When using histone as substrate, basal activity was determined in the absence of Ca^{2+}, PS and diolein but in the presence of 0.5 mM EGTA (▲).

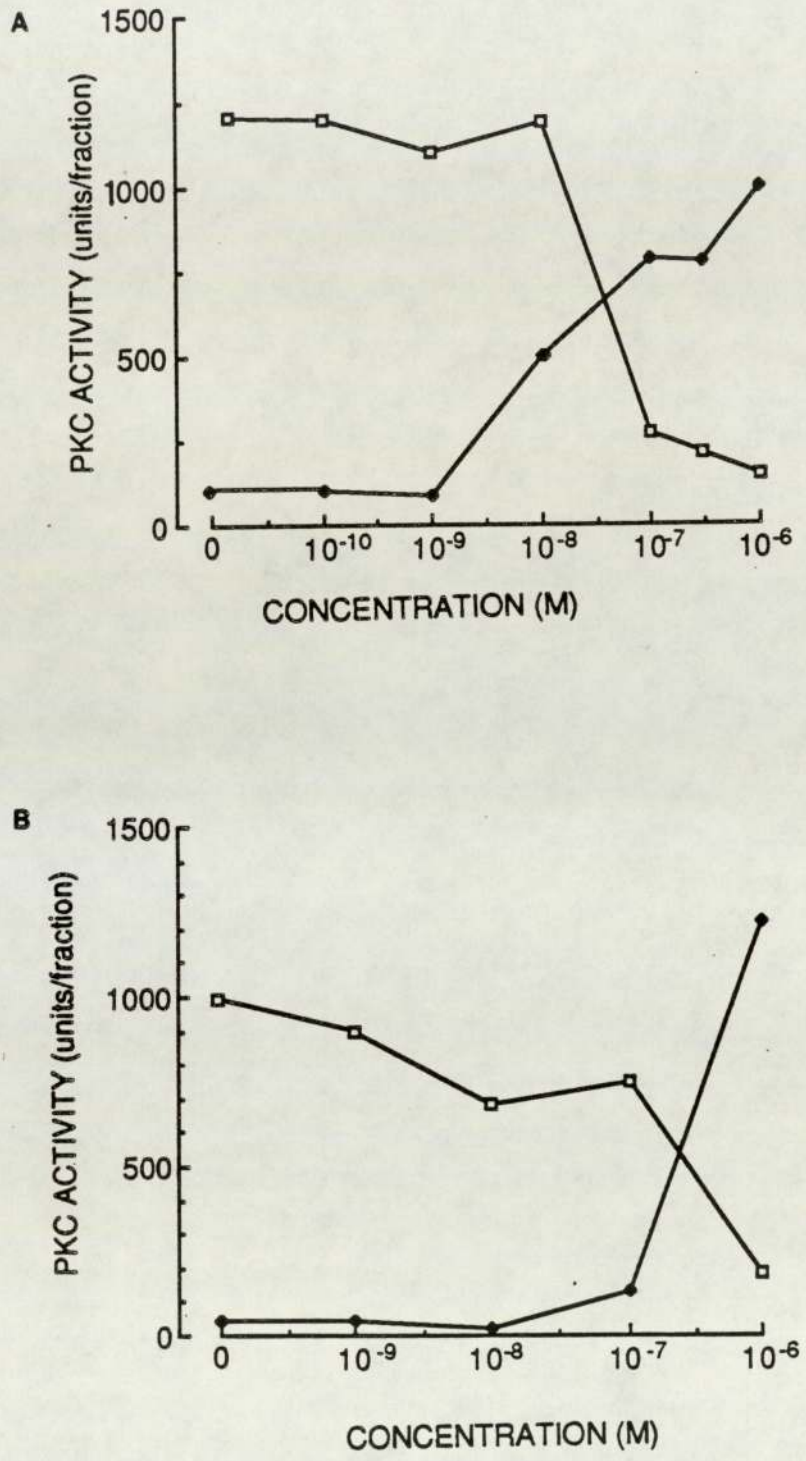


Figure 33. The effect of different concentrations of (A) TPA and (B) mezerein on the subcellular distribution of PKC activity in A549 cells following incubation for 30 min. PKC activity in the cytosol (□) and the particulate fraction (◆) was determined after incubation of cells for 30 min with different concentrations of compounds. Values are the the mean of 2 or 3 experiments. In the case of the mean values calculated from 3 experiments the SD was ≤ 18% of the mean.

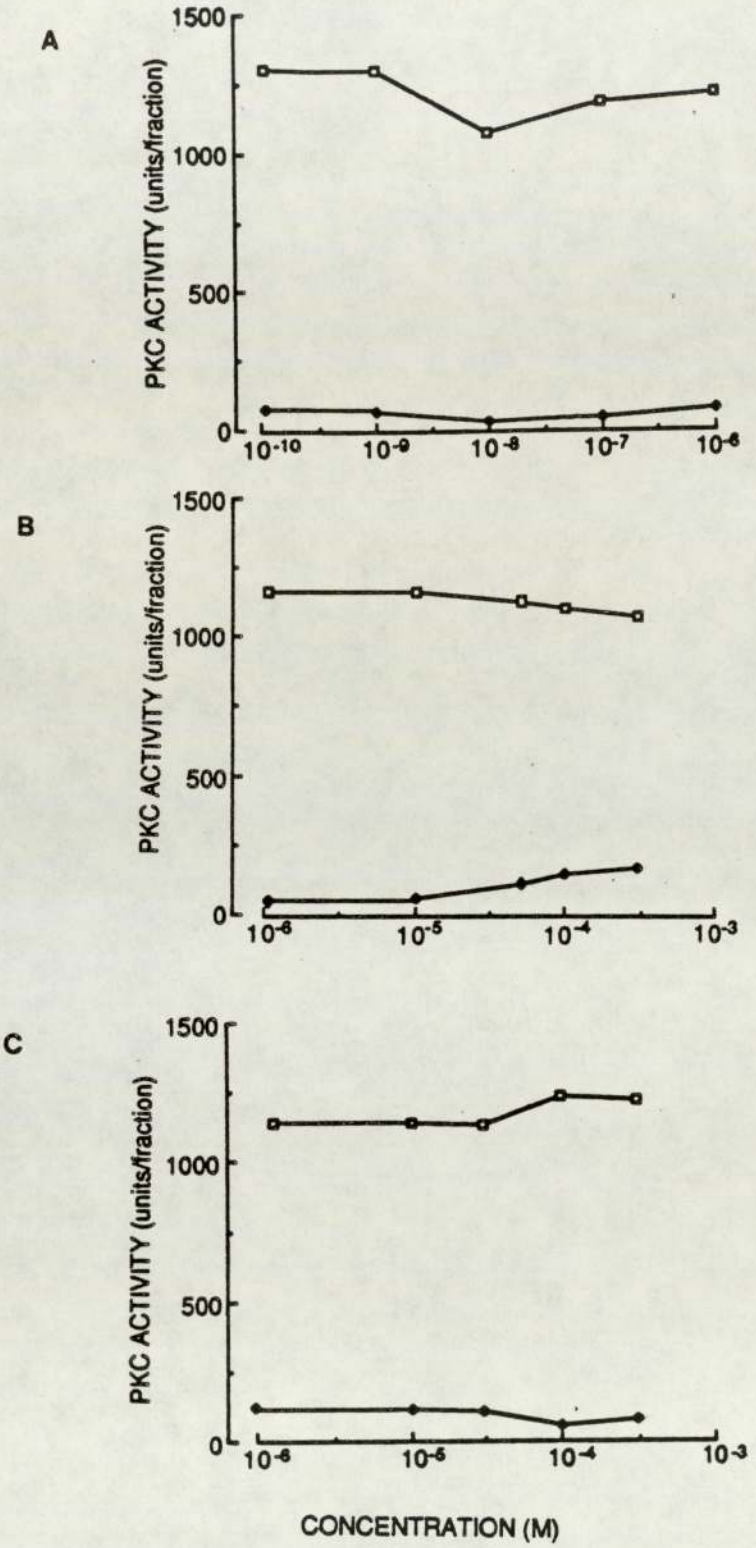


Figure 34. The effect of different concentrations of (A) 4-α-PDD, (B) 1,2-diC$_8$ and (C) OAG on the subcellular distribution of PKC activity in A549 cells following incubation for 30 min. PKC activity in the cytosol (□) and the particulate fraction (◆) was determined after incubation of cells for 30 min with different concentrations of compounds. Values are the mean of 2 or 3 experiments. In the case of the mean values calculated from 3 experiments the SD was ≤ 18% of the mean.

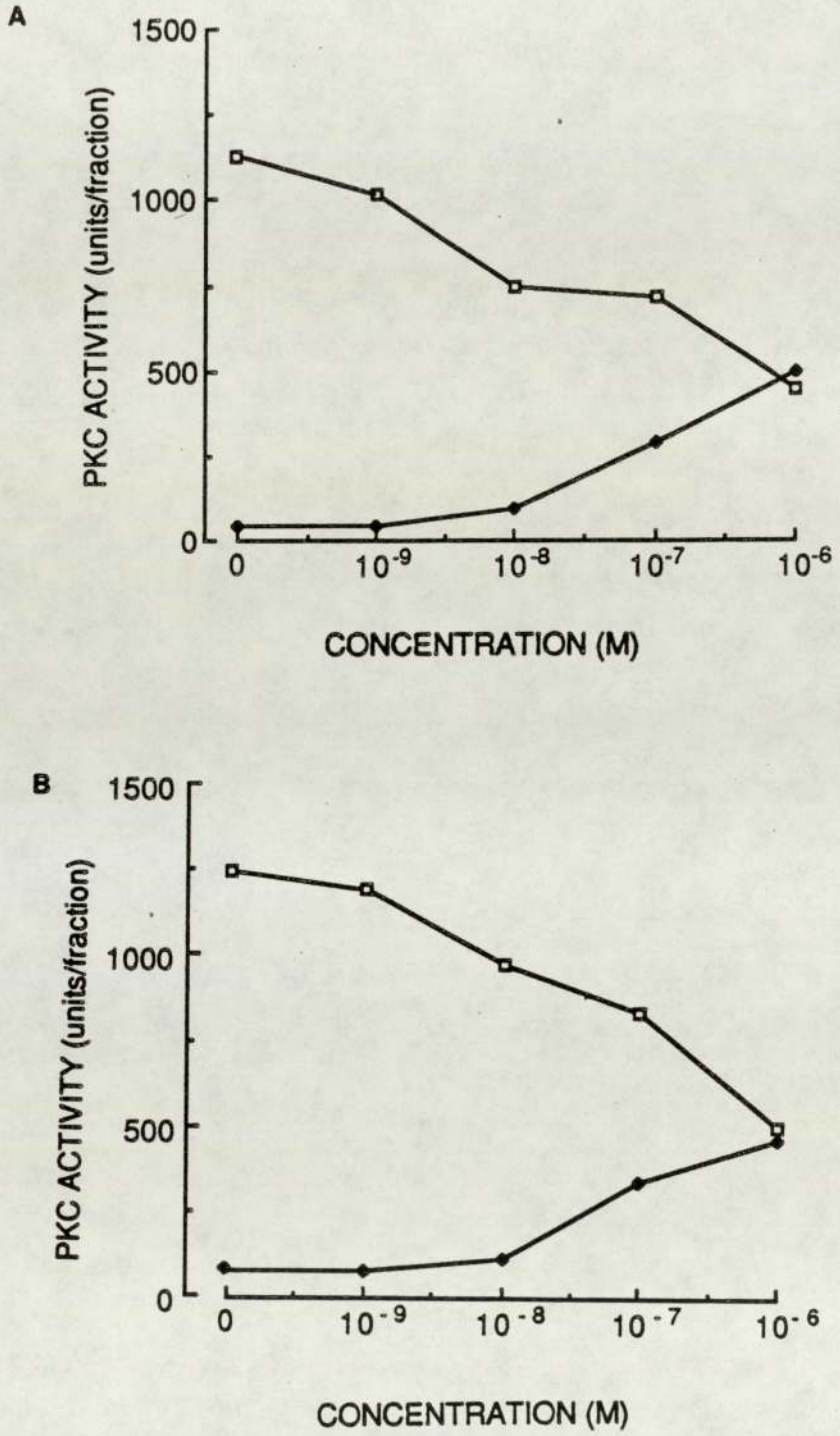


Figure 35. The effect of different concentrations of (A) bryostatin 1 and (B) bryostatin 2 on the subcellular distribution of PKC activity in A549 cells following incubation for 30 min. PKC activity in the cytosol (□) and the particulate fraction was determined after incubation of cells for 30 mins with different concentrations of bryostatin. Values are the mean of 2 or 3 experiments. In the case of the mean values calculated from 3 experiments the SD was ≤ 18% of the mean.

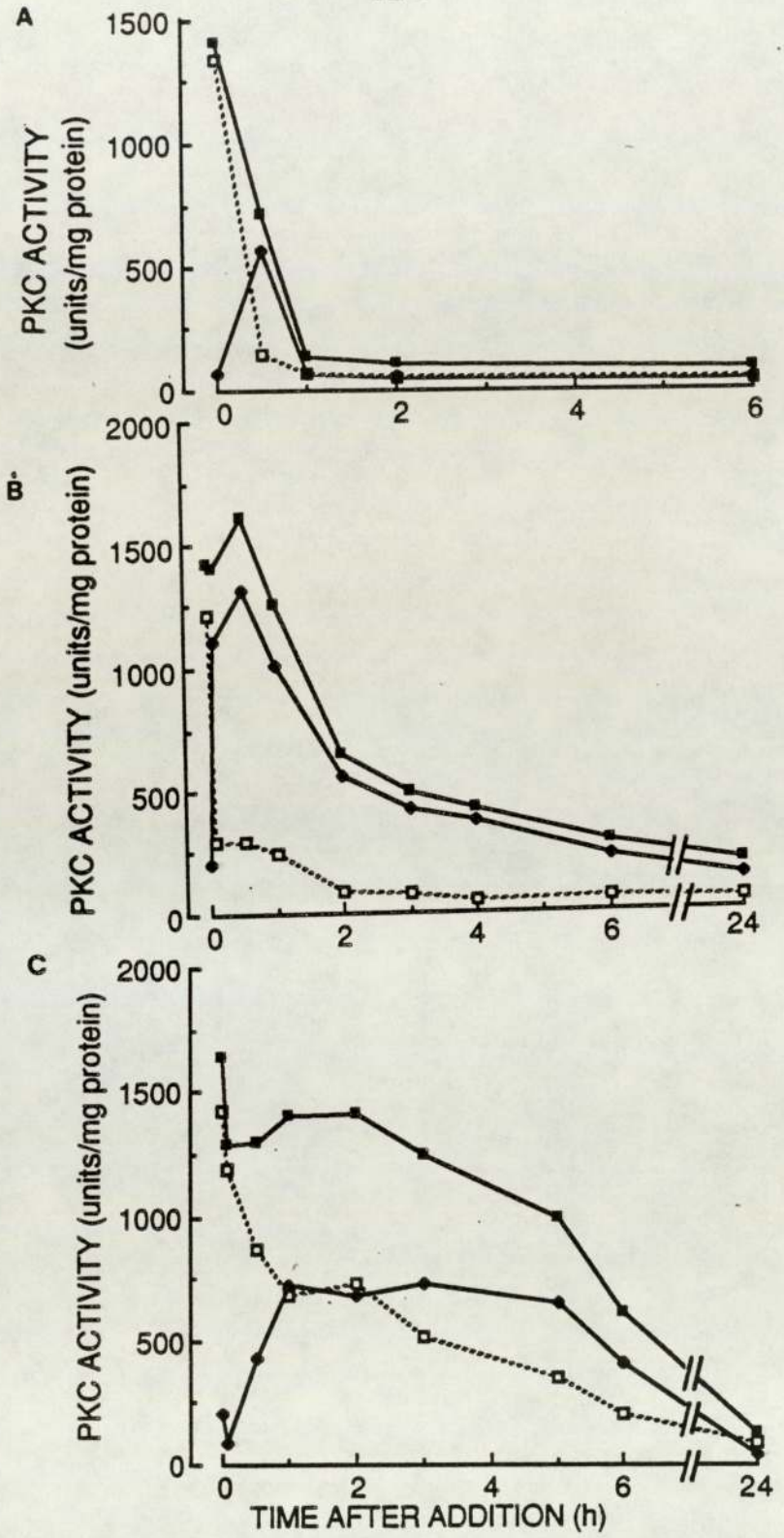


Figure 36. Down-regulation of PKC activity following treatment of A549 cells with different concentrations of TPA. Total cellular PKC specific activity (■) and PKC specific activity in the cytosol (□) and particulate fraction (◆) of A549 cells after incubation with TPA; (A) 300 nM, (B) 100 nM or (C) 10 nM for different time intervals. Values are the mean of 2 or 3 experiments. In the case of the mean values calculated from 3 experiments the SD was $\leq$ 15% of the mean.

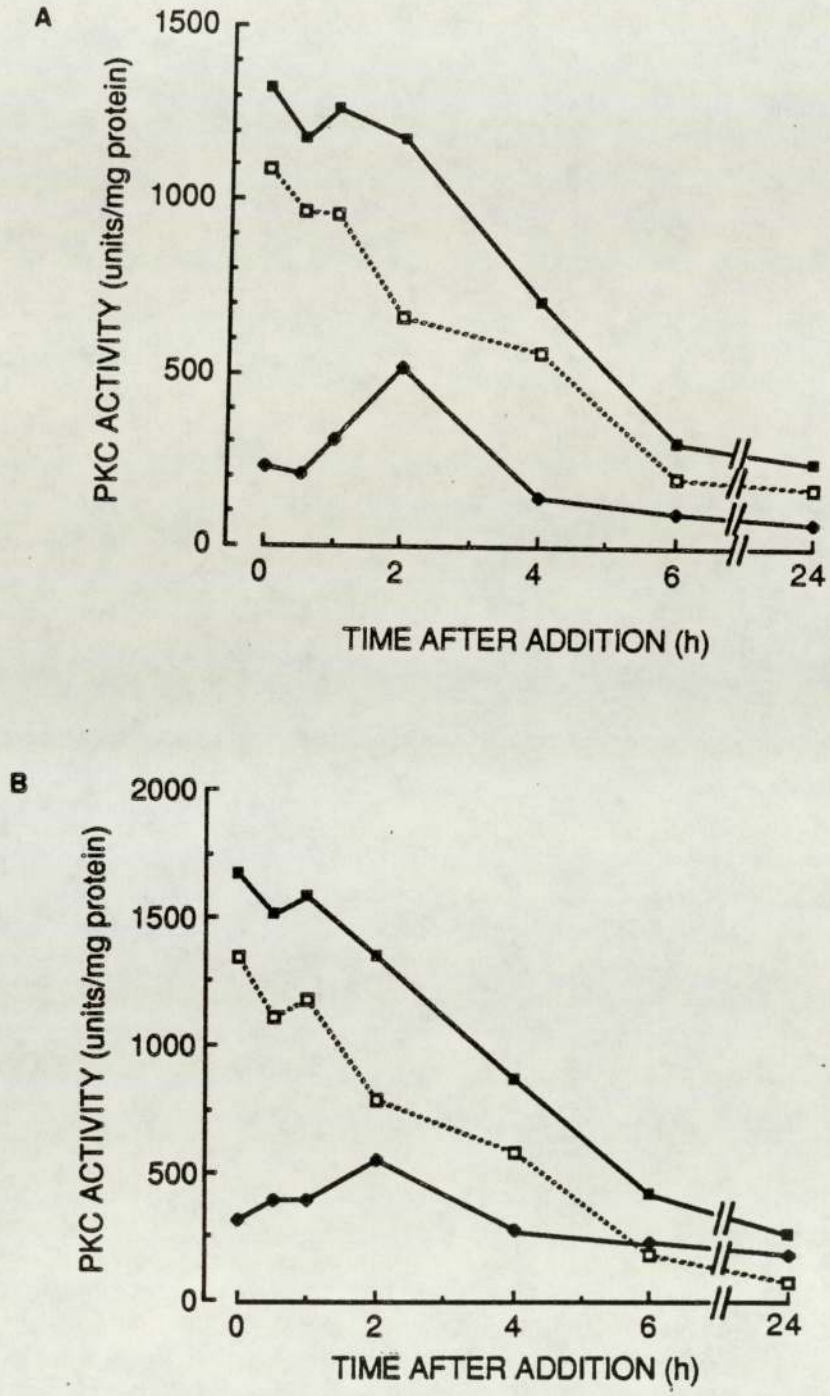


Figure 37. Down-regulation of PKC activity following treatment of A549 cells with (A) 10 nM bryostatin 1 and (B) 100 nM bryostatin 2. Total cellular PKC specific activity (■) and PKC specific activity in the cytosol (□) and particulate fraction (◆) of A549 cells after incubation with bryostatin for different time intervals. Values are the mean of 2 or 3 experiments. In the case of the mean values calculated from 3 experiments the SD was $\leq$ 15% of the mean.

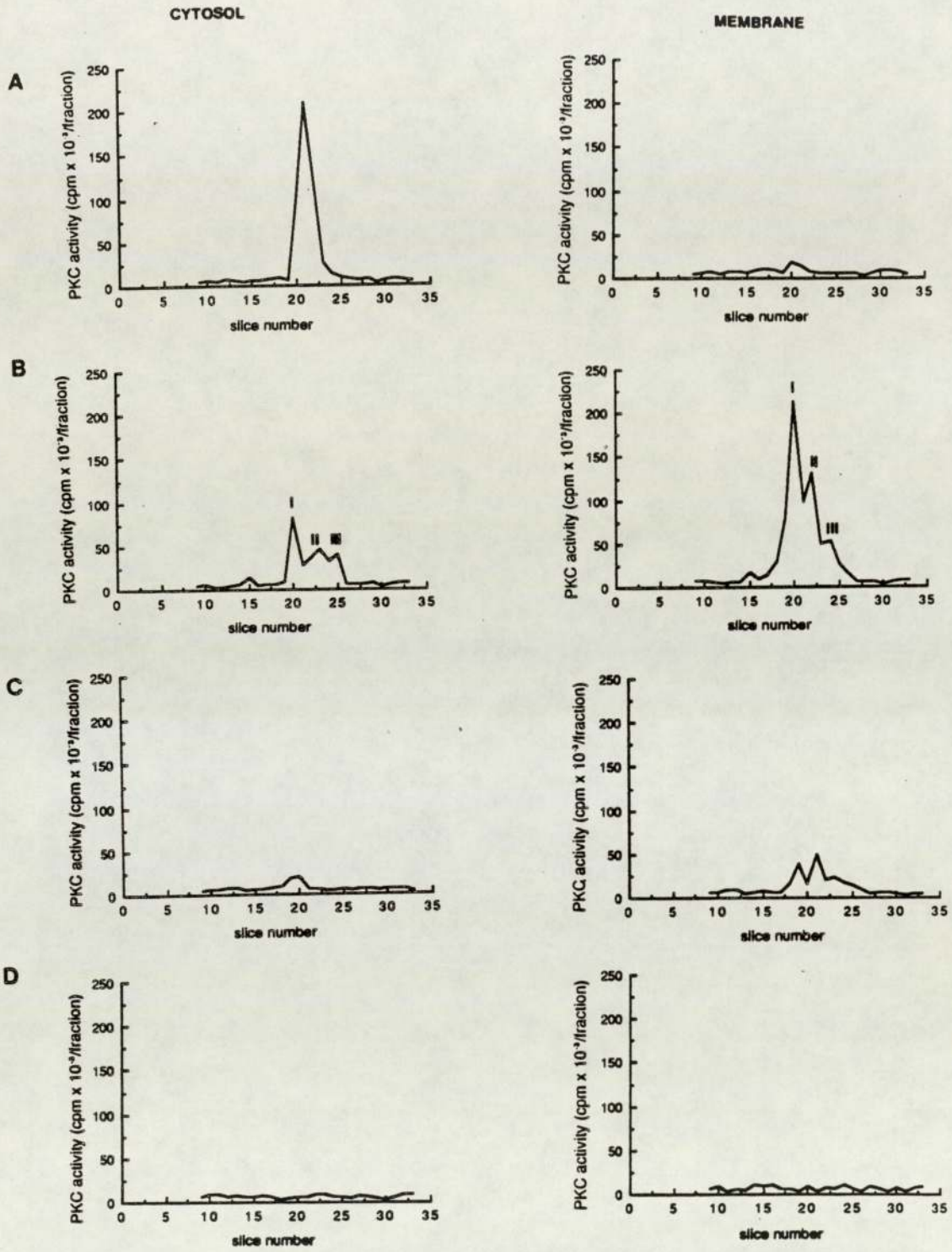


Figure 38. Illustration of the down-regulation and proteolytic degradation of PKC activity in A549 cells following treatment with 100 nM TPA. Cells were treated with 100 nM TPA and PKC activity in cytosolic and membrane fractions determined at times indicated; (A) control (time 0), (B) 1 h, (C) 4 h and (D) 24 h. A representative experiment is shown to illustrate the proteolysis of the PKC holoenzyme (I), the appearance of 2 minor protein kinase activity peaks (II and III) and the complete loss of PKC activity by 24 h.

Section 4.4. Measurement of $[Ca^{2+}]_i$ and its modulation by PKC activators and the cytotoxic ether lipid SRI 62-834 on A549 cells cultured on Cytodex 1 microcarrier beads

4.4.1. Introduction

A key feature of the phosphoinositide signalling pathway is that the breakdown of PtdIns4,5P_2 generates two second messengers, DAG and Ins1,4,5P_3 (Berridge, 1985; Nishizuka, 1984). The dual signalling hypothesis (reviewed by Nishizuka, 1984) suggests that the two "arms" of this pathway cooperate with each other to control a whole host of intracellular activities. As has been previously discussed (Section 1.4), Ins 1,4,5P_3 functions to release Ca^{2+} from intracellular stores. It has been recognized for a number of years that Ca^{2+} serves as a major intracellular messenger for stimulating a wide range of processes such as secretion, contraction, metabolism and mitogenesis (Campbell, 1983). Typically for a second messenger, a variety of control mechanisms operate to maintain intracellular cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) at nanomolar concentrations, even though extracellular Ca^{2+} concentrations ($[Ca^{2+}]_e$) are millimolar (reviewed by Carafoli, 1987).

Much evidence suggests that PKC plays a major role in the modulation of both positive and negative feedback interactions within the phosphoinositide pathway, such as Ca^{2+} signalling, and also in other second messenger pathways, particularly the cyclic nucleotides (Berridge, 1987; Nishizuka, 1984; Nishizuka, 1986). For example, it is thought that in secretory cells the DAG/PKC pathway can

sensitize the exocytic process to the stimulatory effect of Ca^{2+} (Harris et al, 1986; Katakami et al, 1984; Knight and Baker, 1983). In view of the link between PKC and $[Ca^{2+}]_i$, a method for the determination of $[Ca^{2+}]_i$ in A549 cells has been developed in order to probe the ability of PKC activators to modulate Ca^{2+} fluxes and determine the role of Ca^{2+}, if any, in the mechanism(s) by which these growth-inhibitory compounds exert their influence.

The fluorescent Ca^{2+} chelators quin-2 (Tsien et al, 1982) and, more recently, fura-2 (Grynkiewicz et al, 1985) have been widely employed in the measurement of $[Ca^{2+}]_i$ (reviewed by Cobbold and Rink, 1987). In suspension cultures, cells are simply "loaded" with dye, washed and aliquots transferred to quartz cuvettes for the measurement of fluorescence in a spectrofluorimeter. However, measurement of $[Ca^{2+}]_i$ in monolayer cultures such as A549 cells is more difficult. Usually cells are grown on coverslips that must be inserted into a quartz cuvette using a special holder. An alternative method for the measurement of $[Ca^{2+}]_i$ in anchorage-dependent cells, first described by Morris et al (1984), utilises cells cultured on Cytodex microcarrier beads and effectively allows the experimenter to treat anchorage-dependent cells as suspension cultures. We have therefore developed a method for the culture of A549 cells on Cytodex 1 microcarrier beads in order to determine $[Ca^{2+}]_i$ changes in these cells.

In addition, the influence of the cytotoxic ether lipid SRI 62-834 on $[Ca^{2+}]_i$ in A549 cells has been investigated. Previous work in our laboratories has shown that SRI 62-834

causes a rapid elevation in $[Ca^{2+}]_i$ in HL-60 cells which is antagonised by pretreatment with TPA (Thompson and Hickman, 1988). It was suggested that the elevation of $[Ca^{2+}]_i$ by the ether lipid is responsible for its cytotoxic influence. Here we have investigated the influence of SRI 62-834 on $[Ca^{2+}]_i$ in A549 cells.

4.4.2. Assessment of the viability of A549 cells cultured on microcarriers

A method for the culture of A549 cells on Cytodex 1 microcarriers was developed such that when the final culture method described in Section 3.3 was employed, cells grown for 24 h displayed 100% viability as assessed by trypan blue exclusion (not shown) and counting of released nuclei stained by crystal violet following cell lysis indicated that cell numbers had approximately doubled during the culture period (not shown). Figure 39 illustrates the appearance of cells cultured on Cytodex 1 for 24 h. Cells exhibited the morphology typical of cells cultured on plastic and there was little evidence of cell damage, as non-viable cells presumably are lost from beads as a result of the shear forces associated with stirring of the culture. It was therefore concluded that cells cultured by this method were both viable and proliferating.

Initial studies using quin-2 for the determination of $[Ca^{2+}]_i$ yielded unsatisfactory results as the high intracellular concentrations of quin-2 required to obtain a measurable signal also caused a rapid loss in cell viability, particularly in cells already stressed by

overnight culture in sub-optimal conditions. The use of fura-2, which can be used at lower intracellular concentrations due to its higher fluorescent signal, coupled with the refinement of conditions for the culture of cells on Cytodex 1 allowed the maintenance of fura-2 loaded cultures for 2-3 h. Typically, 4-6 traces could be obtained in an experiment before the reduction in cell viability caused by fura-2 became significant. Cells were designated as non-viable when calibration indicated that $[Ca^{2+}]_i$ was in excess of 200 nM.

4.4.3. Modulation of $[Ca^{2+}]_i$ by PKC activators

The $[Ca^{2+}]_i$ of fura-2-loaded A549 cells cultured on microcarriers was determined to be 117 $\pm$ 37 nM (SD; n=15). This can be compared with a $[Ca^{2+}]_i$ of 154 $\pm$ 62 nM (SD; n=32) for quiescent 3T3 fibroblasts cultured on microcarriers (Morris et al, 1984).

The ability of TPA, mezerein and bryostatins 1 and 2 to modulate $[Ca^{2+}]_i$ was assessed by incubating cells in the presence of the compound for 10 min before calibration. The addition of TPA produced a concentration-dependent rise in $[Ca^{2+}]_i$ (Fig. 40A), such that 10 min incubation with 200 nM TPA resulted in a rise of $[Ca^{2+}]_i$ by 140 $\pm$ 17 nM (SD; n=5). A representative trace illustrating the rise in $[Ca^{2+}]_i$ resulting from the incubation of cells with 100 nM TPA is shown in Figure 40B.

A concentration-dependent rise in $[Ca^{2+}]_i$ was also observed on incubation of cells with mezerein (Fig. 41A), bryostatin 1 (Fig. 41B) and bryostatin 2 (Fig. 41C). Incubation for

10 min with 200 nM mezerein, 1 µM bryostatin 1 or 1 µM bryostatin 2 resulted in a rise in $[Ca^{2+}]_i$ of 123 ± 14, 126 ± 15 and 119 ± 21 nM (SD; n=4), respectively.

4.4.4. Modulation of $[Ca^{2+}]_i$ by the cytotoxic ether lipid SRI 62-834

Incubation of cells with a range of concentrations of SRI 62-834 (10-100 µM) for 10 min resulted in a concentration-dependent increase in $[Ca^{2+}]_i$. However, between experiments much variation was noted in the ability of the ether lipid to elevate $[Ca^{2+}]_i$, with the consequence that in many cases comparison between experiments was not possible. A representative experiment is therefore illustrated in Figure 42. In this experiment, a maximal elevation in $[Ca^{2+}]_i$ of 153 nM was observed on incubation with 100 µM SRI 62-834.

Although it appeared that the maximal response of cells to the ether lipid had not been achieved at 100 µM, higher concentrations were observed to result in the detachment of cells from beads, presumably as a result of cytotoxicity.

An intriguing finding was that the rise in $[Ca^{2+}]_i$ observed in the presence of the ether lipid, although usually maximal after 4-5 min incubation, was only transient and after 10 min $[Ca^{2+}]_i$ had returned to resting levels. Traces demonstrating this phenomenon are shown in Figure 42B.

4.4.5. Discussion

The dual signalling hypothesis proposes that the activity of PKC in many cells is intimately associated with changes

in $[Ca^{2+}]_i$. In view of this cooperative mechanism, a method for the measurement of $[Ca^{2+}]_i$ in A549 cells cultured on Cytodex 1 microcarrier beads has been developed and utilised in this study in order to determine the influence of the PKC activators TPA, mezerein and bryostatins on $[Ca^{2+}]_i$ in cells.

Measurement of $[Ca^{2+}]_i$ in anchorage-dependent cells grown on microcarrier beads has two significant advantages over cells grown on glass coverslips: (i) no special equipment is required for the positioning of cells in the fluorimeter beam as cells can be treated as suspension cultures and, (ii) "bleaching" of dye, often a problem associated with the coverslip method due to the continual illumination of the same cells by the excitation beam, is avoided due to the constant movement of the microcarriers through the path of the excitation beam. However, bleaching of fura-2 is minimal when compared to quin-2 (Cobbold and Rink, 1987) so in the final method using fura-2 this would not have been a significant advantage.

Numerous difficulties were encountered during the development of the microcarrier method, usually as a result of inefficient stirring. Stirring at high speeds subjected cells to excessive shear forces whereas low speeds were insufficient to keep the microcarriers in suspension. Quin-2 was also found to be unsuitable as the high intracellular concentrations of the dye caused a rapid decrease in cell viability, presumably as a result of interference with $[Ca^{2+}]_i$. Fura-2, which gave a much stronger fluorescence signal than quin-2 and could therefore

be used at much lower loading concentrations, was found to be much less toxic to cells.

The observation that the potency of TPA, mezerein and the bryostatins in elevating $[Ca^{2+}]_i$ in A549 cells correlated with their ability to translocate PKC from the cytosol to membranes (Section 4.3) strongly indicates that PKC modulates Ca^{2+} fluxes within the cell, presumably *via* the phosphorylation of Ca^{2+} channels. Possible future studies could be aimed at further characterisation of the nature of these Ca^{2+} channels by the use of selective Ca^{2+} channel blockers such as verapamil and identification of the source of the Ca^{2+} released by PKC activators as either intra- or extracellular.

SRI 62-834 is a tetrahydrofuran analogue of the antitumour ether lysophospholipid Et-18-OCH_3. Although the the mechanism(s) by which ether lipids such as SRI 62-834 exert their antitumour activity is unclear, their cytotoxic influence is believed to be the result of the inhibition of normal phospholipid metabolism and the subsequent perturbation of membrane function (Herrman and Neumann, 1986). It has been suggested that ether lipids may act, at least in part, *via* the inhibition of PKC activity (Helfman *et al*, 1983b). Et-18-OCH_3 also inhibits the phosphorylation of various endogenous proteins and differentiation of HL-60 cells promoted by TPA, strongly suggesting that inhibition of PKC-dependent reactions may be responsible for the influence of the ether lipids. Very recently, Shoji *et al* (1988) have reported that the synthetic thioether phospholipid BM 41.440 inhibited PKC activity by competing

with PS for binding to the enzyme. The compound also inhibited the TPA-induced differentiation of HL-60 and KG-1 cells at a concentration inhibitory to PKC, suggesting that inhibition of PKC activity might partly account for the antineoplastic effect of BM 41.440 (Shoji et al, 1988).

Recent work in our laboratories has shown that SRI 62-834 rapidly elevates $[Ca^{2+}]_i$ in HL-60 cells, but that this rise can be blocked by preincubation with TPA (Thompson and Hickman, 1988). It was therefore hypothesised that the imbalance in Ca^{2+} homeostasis caused by the prolonged elevation of $[Ca^{2+}]_i$ by SRI 62-834, possibly by the opening of a Ca^{2+} channel whose activity is regulated by PKC, may be responsible for the cytotoxic effect of the ether lipid.

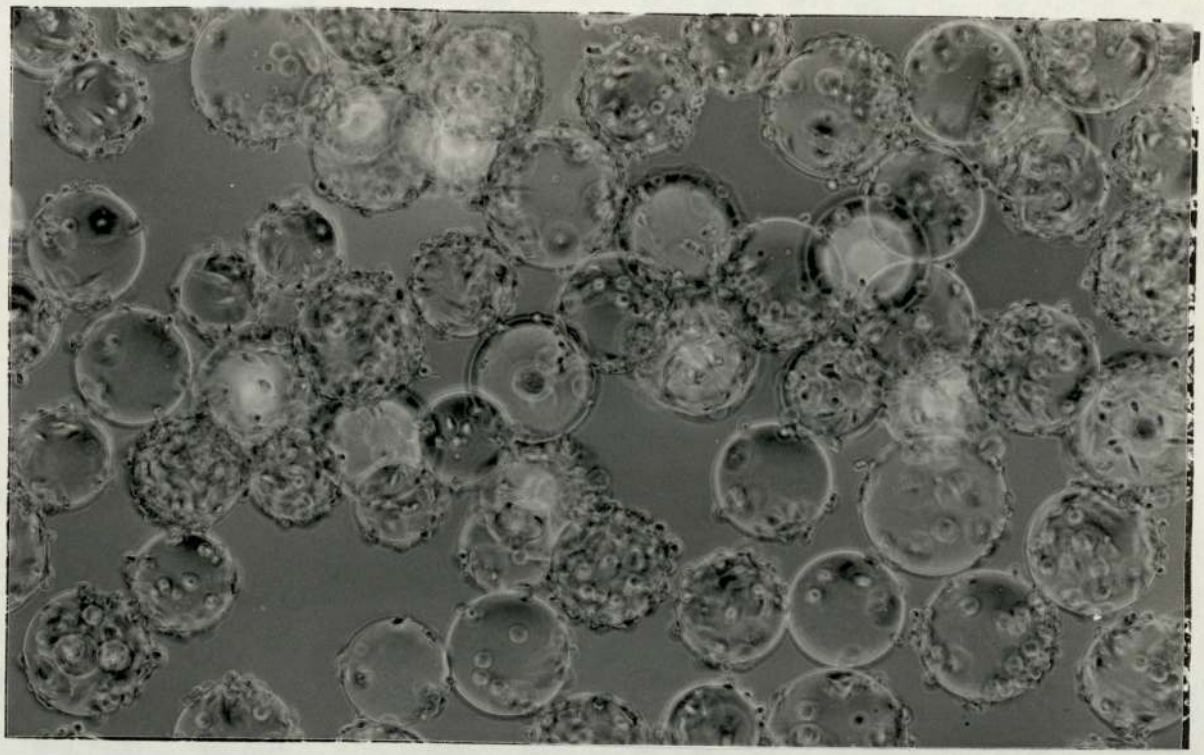
The influence of SRI 62-834 on $[Ca^{2+}]_i$ in A549 cells was investigated in order to extend studies on the ether lipid to other cell lines. Unfortunately, results obtained were rather limited due to problems with cell viability for reasons which have been discussed above. In addition, there was some doubt as to the validity of results obtained on treatment of cells with high concentrations (> 100 µM) of SRI 62-834 as significant cell detachment was noted, presumably as a result of non-specific effects. However, in cells treated with concentrations of SRI 62-834 < 100 µM, two observations seem to indicate that cells remained viable; (i) $[Ca^{2+}]_i$ did not equilibriate with $[Ca^{2+}]_e$ and, (ii) $[Ca^{2+}]_i$ rapidly returned to resting levels, suggesting that cells could still regulate $[Ca^{2+}]_i$. This fall in Ca^{2+} could be the result of either extrusion of Ca^{2+} from the cell or alternatively sequestration into intracellular

stores.

The ability of A549 cells to restore $[Ca^{2+}]_i$ to resting levels was not observed in SRI 62-834 treated HL-60 cells (Thompson and Hickman, 1988). If the hypothesis that the elevation of $[Ca^{2+}]_i$ is responsible for the cytotoxic influence of SRI 62-834 one would therefore expect that the ether lipid would not be toxic in A549 cells. This possibility remains to be tested in future studies.

In summary, although little data was obtained from these studies due to time limitations, the feasibility of the method for the measurement of $[Ca^{2+}]_i$ has been demonstrated. In addition to the investigation of $[Ca^{2+}]_i$, the technique can also be extended to the measurement of intracellular pH using the fluorescent pH indicator BCECF (Rink et al, 1982). Intracellular alkalinization following the activation of the Na^+/H^+ antiporter by PKC is believed to be important in the mechanism of action of a number of mitogenic growth factors such as EGF and vasopressin (Hesketh et al, 1985; Moolenaar et al, 1984).

A



B

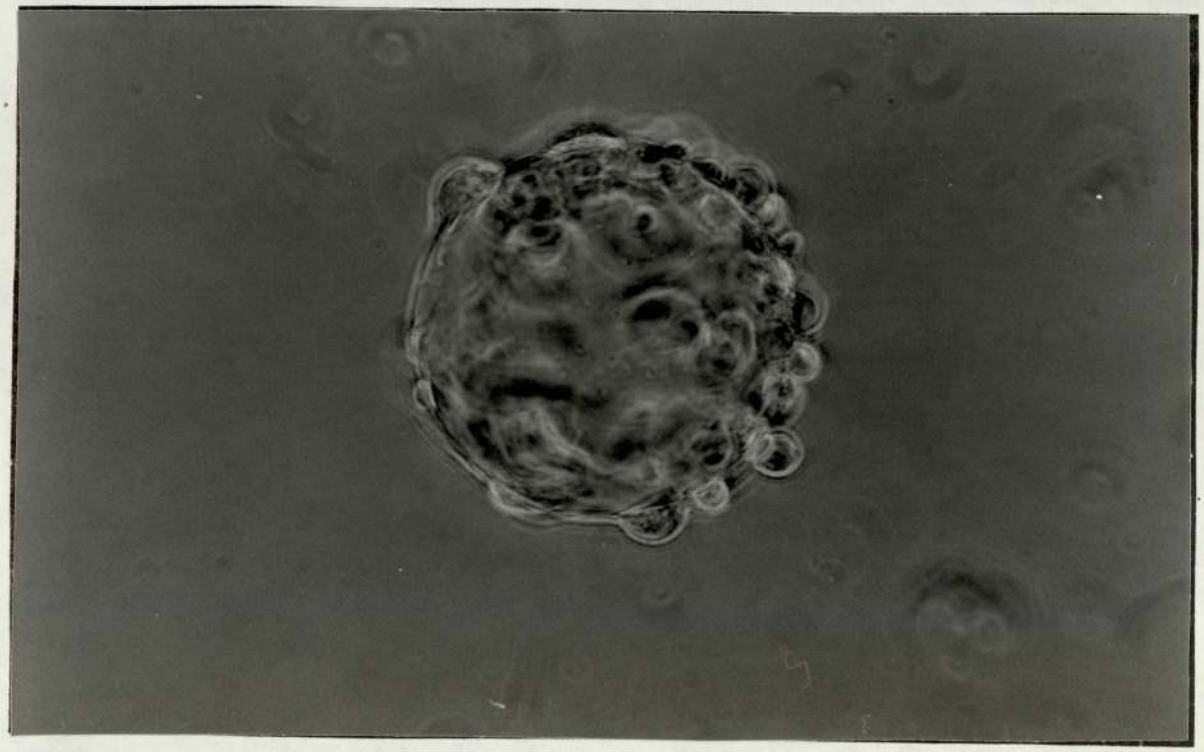


Figure 39. Phase contrast micrographs of A549 cells cultured on Cytodex 1 microcarrier beads.
(A) magnification x 20, (B) magnification x 80.

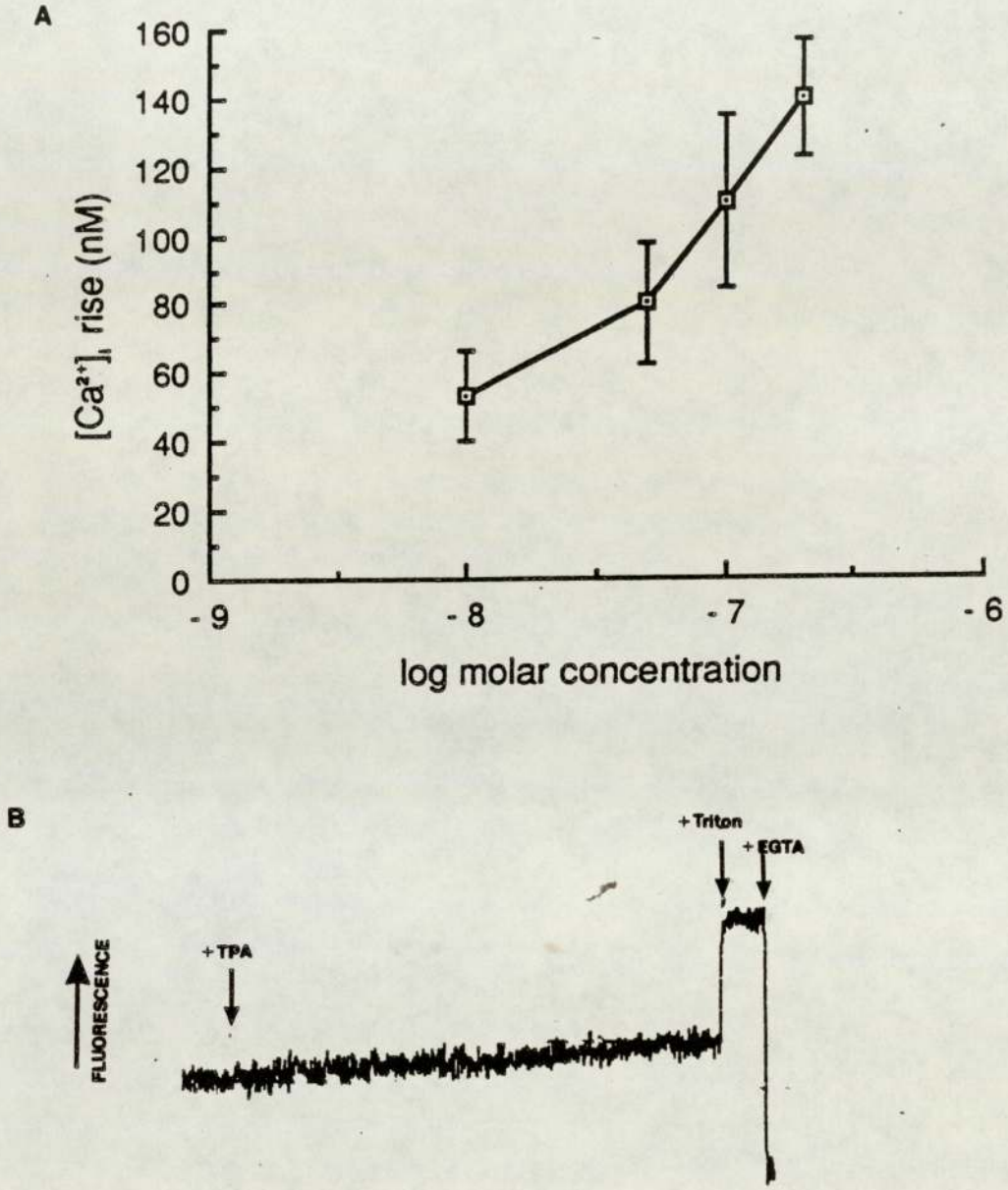


Figure 40. Elevation of $[Ca^{2+}]_i$ in A549 cells cultured on microcarriers induced by treatment with different concentrations of TPA.

(A) Fura-2 loaded cells were incubated for 10 min with different concentrations of TPA before calibration (mean ± SD (n=3-9)).

(B) Representative trace illustrating $[Ca^{2+}]_i$ rise stimulated by treatment of fura-2 loaded cells with 100 nM TPA for 10 min. In this determination, resting and stimulated $[Ca^{2+}]_i$ levels were 123 and 244 nM, respectively.

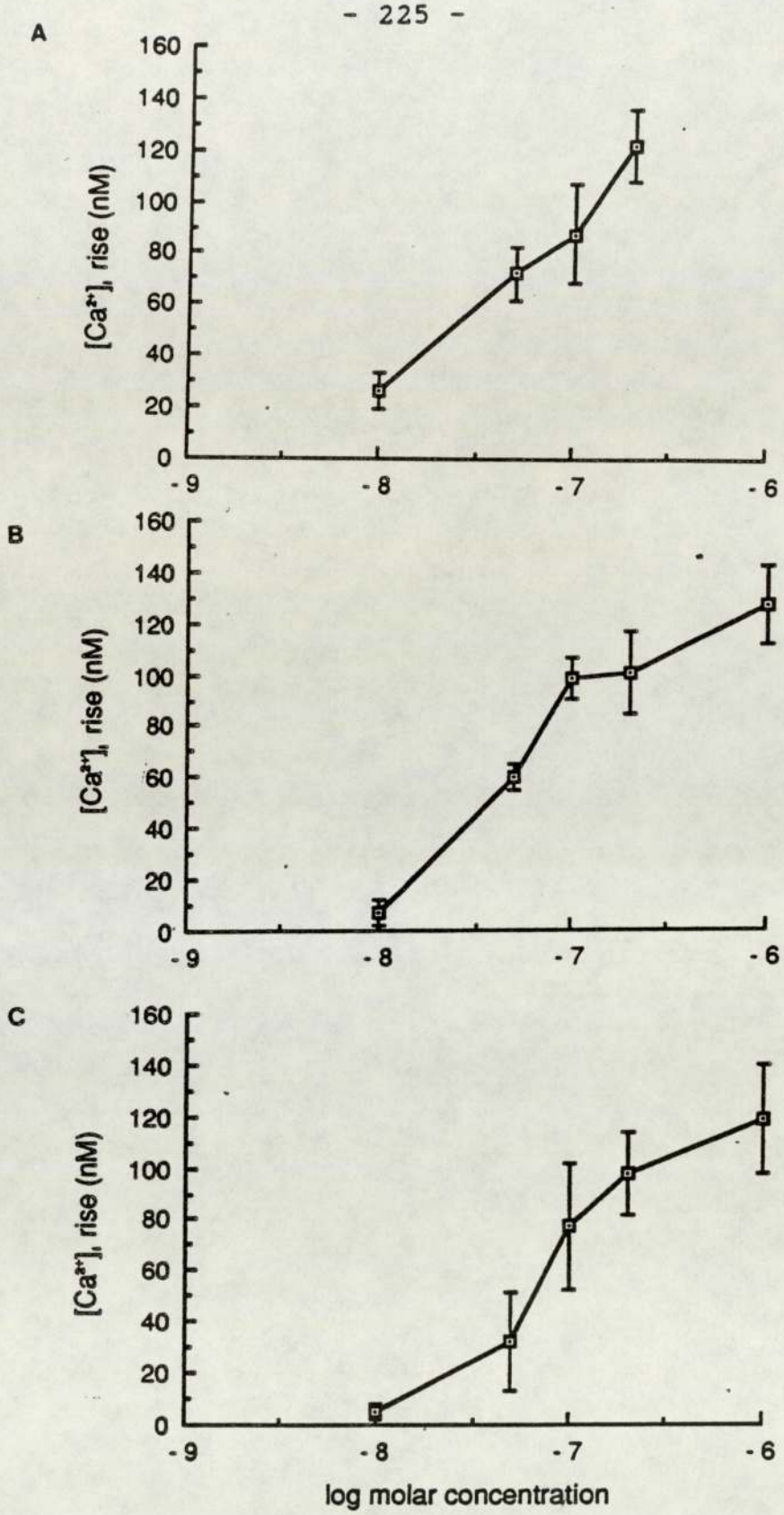


Figure 41. Elevation of $[Ca^{2+}]_i$ in A549 cells cultured on microcarriers induced by treatment with different concentrations of (A) mezerein, (B) bryostatin 1 and (C) bryostatin 2. Fura-2 loaded cells were incubated for 10 min with different concentrations of compound before calibration (mean ± SD (n=4-6)).

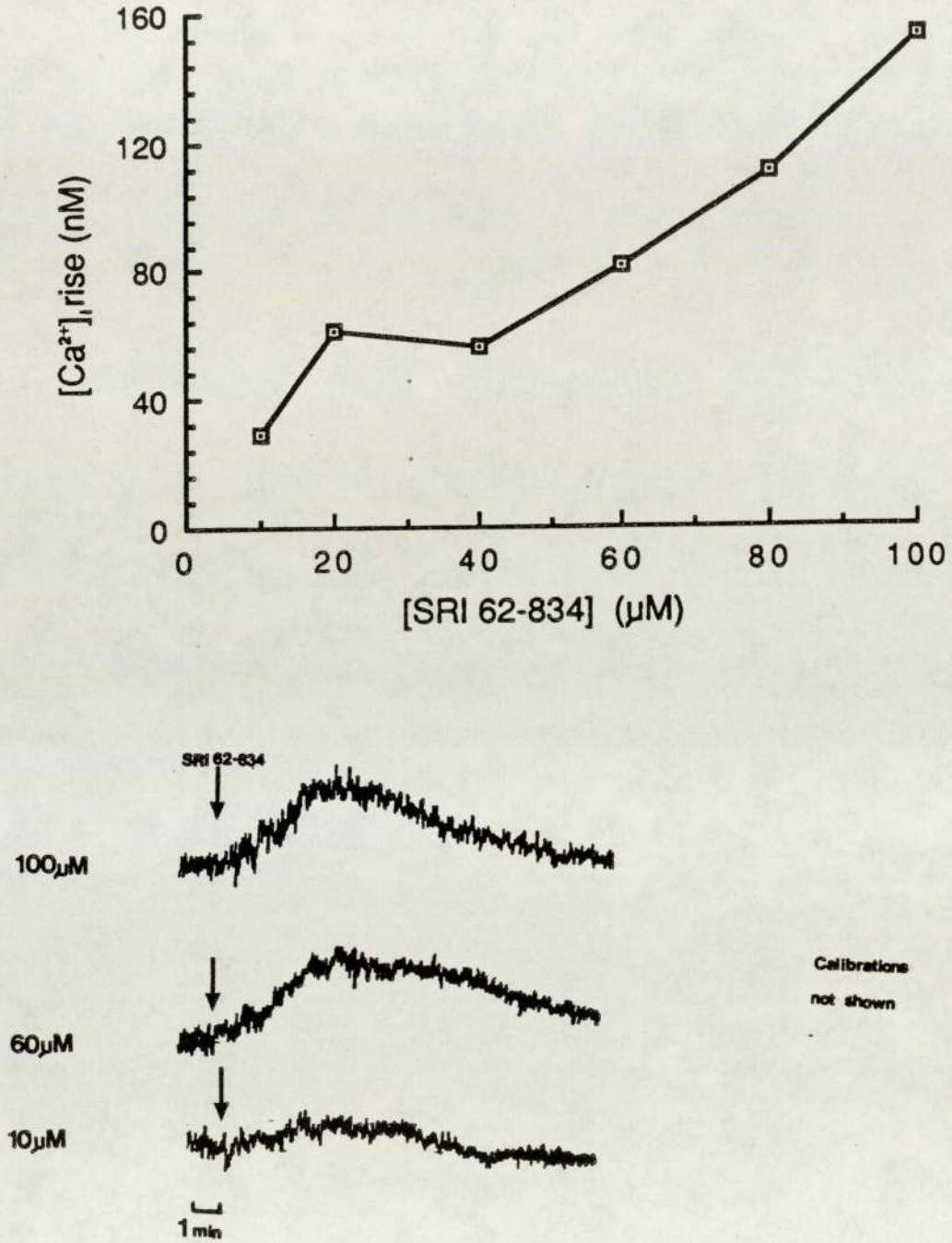


Figure 42. Elevation of $[Ca^{2+}]_i$ in A549 cells cultured on microcarriers induced by treatment with different concentrations of the cytotoxic ether lipid SRI 62-834.
(A) Fura-2 loaded cells were incubated for 10 min with different concentrations of SRI 62-834 before calibration. One experiment, representative of five, is illustrated.
(B) Traces used to plot (A).

Section 4.5. Mixed micellar assay for phorbol ester binding in crude cytosolic fractions of A549 cells

4.5.1. Introduction

Understanding of the molecular mechanisms of phorbol ester action were advanced with the development of a phorbol ester receptor ligand binding assay utilising [^{3}H]PDBu (Driedger and Blumberg, 1980b). Using this technique, specific and high affinity cellular receptors for biologically active phorbol esters were demonstrated.

The binding of phorbol esters to the phorbol ester receptor/PKC is dependent on Ca^{2+} and phospholipids (see Section 1.5.1). Binding studies have usually employed sonically dispersed phospholipids and DAGs (Leach et al, 1983). However, the physical properties of these lipid cofactors result in multilamellar and unilamellar vesicles which are not homogeneous, hampering study of the mechanistic interactions of the receptor with its cofactors. Recently, Hannun et al have reported the development of a Triton X-100/PS mixed micellar assay system which is physically defined and therefore allows the investigation of the specificity and stoichiometry of PKC-lipid interactions (Hannun and Bell, 1986; Hannun and Bell, 1987; Hannun et al, 1985).

In this section the feasibility of the mixed micellar binding assay has been demonstrated using crude cytosolic extracts of A549 cells, with the future objective of screening compounds synthesized as phorbol ester/DAG analogues for their ability to compete with [^{3}H]PDBu for

binding to the phorbol ester receptor.

4.5.2. Results

In all experiments, unpurified cytosolic preparations derived as described in Section 3.3 were used as the source of phorbol ester receptor/PKC activity.

The specific binding of [^{3}H]PDBu was investigated by incubating various concentrations of [^{3}H]PDBu with Triton X-100 mixed micelles containing 20 mol% PS in the presence of 200 µM Ca^{2+}. Figure 43A shows that [^{3}H]PDBu was saturable and specific, with saturation reached at approximately 50 nM.

The dependence of binding on PS was demonstrated by preparing Triton X-100/PS micelles with varying amounts of PS ranging from 0-25 mol% and measuring specific binding in the presence of 75 nM [^{3}H]PDBu and 200 µM Ca^{2+}. Binding was poor with PS less than 10 mol%, and reached saturation at approximately 20 mol% (Fig. 43B).

The dependence of binding on [^{3}H]PDBu and PS in crude cytosolic extracts of A549 cells were in good agreement with previous findings which utilized PKC purified from rat brain (Hannun and Bell, 1986; Hannun and Bell, 1987).

4.5.3. Discussion

The screening of compounds synthesized to mimic features of the phorbol ester pharmacophore by assessment of their ability to inhibit the proliferation of A549 cells (Section 4.2) presents a number of difficulties. In particular, the lack of influence of a screened compound could conceivably

be due to metabolic inactivation and/or the inability to reach their presumed target, PKC, rather than an inability to bind to and to activate the enzyme. These problems are also applicable to DAGs, the presumed "endogenous phorbols", which are rapidly metabolised by cells (Welsh and Cabot, 1987). In addition, the inability of the synthetic DAGs OAG and 1,2-diC_8 to mimic phorbol ester-induced growth arrest in A549 cells at non-toxic concentrations (Section 4.1.2) possibly indicates that the activation of PKC is insufficient for the induction of growth arrest and that the cell line is therefore a poor model for the screening of putative PKC modulators.

The use of a cell-free screening method in order to determine the ability of compounds to compete with [^{3}H]PDBu for the phorbol ester receptor/PKC circumvents both problems of delivery and metabolism, as compounds are able to interact directly with the receptor site. Wender et al (1986) screened compounds synthesized to mimic putative features of the phorbol ester pharmacophore predicted by computer modelling studies. All the tested compounds inhibited [^{3}H]PDBu binding at high concentrations (10-60 µM) and further analysis of one of these compounds, decylhydroxylindole (DHI), demonstrated that this inhibition was of a competitive nature. In addition, DHI inhibited EGF binding in Swiss 3T3 cells, a characteristic in vivo effect of phorbol esters (Wender et al, 1986).

Previous studies have indicated that the interaction of DAGs and phorbol ester/DAG analogues with PKC in cell membranes can be reproduced in the PS/Triton mixed micelle

assay, which provides a physically defined system for the study of PKC-cofactor interactions (Hannun and Bell, 1986; Hannun and Bell, 1987). Indeed, in a recent publication this assay method has been used to further extend understanding of the structure/activity relationships for activation of PKC by DAGs and DAG-related molecules (Bonser et al, 1988).

In this section the PS/Triton mixed micellar assay has been used to demonstrate specific phorbol ester receptors in a crude cytosolic extract of A549 cells. Although few results are presented, it is important to note that the technique has since been further refined and is now routinely used in the screening of synthesized compounds (Bradshaw and Gescher, unpublished).

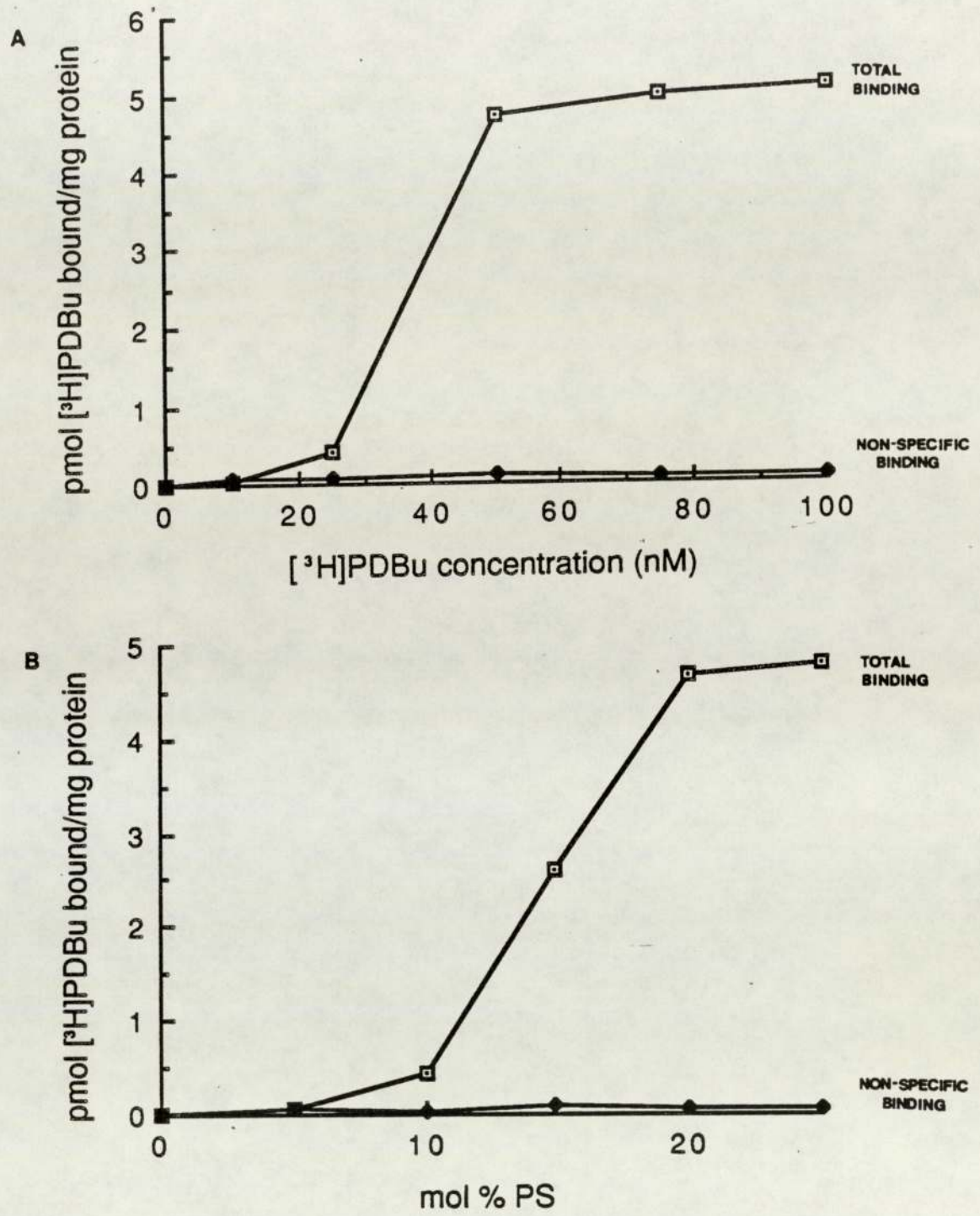


Figure 43. Dependence of phorbol ester binding in A549 cell cytosolic fractions on (A) [³H]PDBu and (B) PS.
(A) Crude cytosolic fractions were incubated with various concentrations (0-100 nM) of [³H]PDBu with Triton X-100 mixed micelles containing 20 mol% PS in the presence of 200 µM Ca^{2+}. Values are the mean of 2 experiments.
(B) Crude cytosolic fractions were incubated with Triton X-100 mixed micelles containing varying amounts (0-25 mol%) of PS in the presence of 75 nM [³H]PDBu and 200 µM Ca^{2+}. Values are the mean of 2 experiments.

SECTION 5: GENERAL DISCUSSION

SECTION 5: GENERAL DISCUSSION

The results presented in this thesis have further characterised the growth-inhibitory action of phorbol esters in the A549 lung carcinoma cell line. In addition, the influence of other classes of PKC activator, namely DAGs, mezerein and bryostatins, have been investigated. It has been demonstrated that some, but not all, PKC activators are able to arrest the growth of A549 cells, seemingly *via* the same mechanism(s). Further, the unique ability of the bryostatins to block the antireplicative action of other PKC activators, including other bryostatins, has been observed.

One of the major aims of this project was to investigate changes in PKC activity and distribution in response to treatment with PKC activators and its relation to the proliferative status of A549 cells. Evidence presented supports a role for PKC activation in the induction of growth arrest as TPA, mezerein and bryostatins were able to cause a shift of PKC activity from the cytosol to membranes. Down-regulation of PKC activity by TPA and the bryostatins occurred in a similar manner. DAGs, which did not mimic phorbol ester-induced growth arrest, were unable to induce a dramatic translocation of PKC activity.

The demonstration that PKC exists as a family of polypeptides with varying biological properties has suggested possible explanations for the many observed differences in the biological effects of the different classes of PKC activator. It is possible that different PKC isoforms exist with distinct patterns of sensitivity to

inputs from cell-signalling pathways (reviewed by Dreher and Hanley, 1988). The indication that novel messengers such as arachidonic acid and its metabolites may be important in the regulation of PKC activity is also made attractive by the existence of multiple PKC isozymes.

An implication of the observed heterogeneity of the PKC family is that the isozymes respond differently to various activators in vivo and that these subspecies are differentially distributed in various cell types and even within the same cell, greatly complicating the interpretation of experimental observations. For example, one could suggest that the inability of the synthetic DAGs OAG and 1,2-diC_8 to mimic TPA-induced growth inhibition in A549 cells (Section 4.1.2) is not because PKC activation is unnecessary for the induction of growth arrest, but rather that the PKC isozyme(s) responsible for the antiproliferative influence of phorbol esters is insensitive to these DAGs. Indeed, the preliminary investigations performed by Nishizuka and collaborators have shown that the α-and γ-subspecies of PKC are much less activated by DAGs than are the β_I and β_{II} isoforms (reviewed by Nishizuka, 1988). Similarly, the observation that bryostatins 1 and 2 blocked the ability of phorbol esters and mezerein to inhibit growth of A549 cells in a non-competitive manner does not rule out the involvement of PKC in this blockage One could argue that the bryostatins are able to modulate the activity of a PKC isozyme (or isozymes) insensitive to phorbol esters which is able to block the antiproliferative action of the phorbol esters and mezerein, which in turn is

mediated *via* another PKC isozyme (or isozymes). Thus, although studies which model the bryostatins to the phorbol ester pharmacophore on PKC (Wender *et al*, 1988) provide information about the common features of PKC activators which are apparently structurally unrelated, they do not clarify why the bryostatins are able to induce a mixture of phorbol ester agonistic and antagonistic properties. These structural features which account for the unique bryostatin effects remain to be explored.

In addition to the complexity of the phosphoinositide signalling pathway introduced by the demonstration of the existence of multiple PKC isozymes, other workers have challenged the classical pathway of PKC activation *via* phosphoinositide hydrolysis as the sole source of DAG. Recent research has revealed a cellular pathway for the agonist-induced generation of DAG from phosphatidylcholine (PC) *via* the activation of a PC-specific PLC (Besterman *et al*, 1986). The hydrolysis of PC by such a mechanism would result in the generation of DAG without the production of inositol phosphates and hence PKC activation would be possible without an associated increase in $[Ca^{2+}]_i$. Indeed, Lacal *et al* (1987) have recently reported that Ha-ras oncogene transformed NIH/3T3 fibroblasts displayed elevated levels of DAG in the absence of a detectable increase in inositol phosphates. Rather, evidence suggested that the DAG was derived from the hydrolysis of PC and phosphatidylethanolamine (PE) by the action of PLC, as an increase in the release of both phosphorylcholine and phosphorylethanolamine was noted (Lacal *et al*, 1987). As

DAG generated from PC is likely to have a different fatty acid composition from that of the DAG generated from the phosphoinositides, it is possible that preferential activation of particular PKC isozyme(s) occurs according to the source of the DAG. To add further to this highly complex and confusing story, a recent report has demonstrated that in the rat embryo-derived REF52 cell line phorbol esters stimulated DAG formation from PC, not *via* PLC, but *via* the sequential actions of phospholipase D and phosphatidate phosphohydrolase (Cabot *et al*, 1988).

The evidence supporting a role for PKC in the regulation of cellular proliferation is now overwhelming. A direct involvement has been indicated by studies using fibroblastic cell lines. Persons *et al* (1988) reported that transfection of NIH 3T3 cells with plasmids containing rat brain type I (γ) cDNA resulted in altered growth regulation; transfectants exhibited a reduced dependence on serum for growth, higher growth saturation densities and enhanced tumorigenicity when inoculated into nude mice (Persons *et al*, 1988). Housey *et al* (1988) also noted disordered growth when rat fibroblastic cell lines were transfected with the β_I form of PKC. The perturbation of PKC activity in cells, resulting in unregulated proliferation, reflects the central role of the enzyme in the mechanism(s) by which growth is controlled.

Although this thesis has concentrated on the direct influence of PKC on the proliferation of A549 cells, it is clear that cellular events distal to this enzyme are also crucial in the induction of growth arrest. In particular,

a wealth of evidence has implicated a role for oncogenes in the control of proliferation (reviewed by Bishop, 1987). Studies using phorbol esters and serum growth factors have linked PKC activation with the modulation of the expression of oncogenes such as c-myc and c-fos. Although the role of myc in growth control remains unclear, much evidence seems to support an important role for the c-myc product during proliferation as a mediator of growth factor signalling (Campisi et al, 1984). In U-937 monoblastic cells, the induction of differentiation and consequent loss of proliferative capacity is associated with a rapid decrease in the expression of the endogenous myc gene (Einat et al, 1985). In order to determine if this reduction was a prerequisite for differentiation, Larsson et al (1988) introduced a constitutively expressed v-myc gene into U-937 cells. It was found that the expression of the v-myc oncogene did not interfere with TPA-induced differentiation for the first 24 h, and the ability of cells to incorporate [^{3}H]TdR was greatly decreased. However, after this time point the differentiation of cells was blocked, resulting in a regained proliferative potential and a reexpression of the immature phenotype (Larsson et al, 1988). As a transient inhibition of growth was noted similar to that described here as the response of A549 cells to tumour promoters and bryostatins, the investigation of changes in myc expression in A549 cells would appear worthy of investigation, particularly in view of the recent finding that bryostatin 1 is unable to modulate myc expression in HL-60 cells in a manner similar to phorbol esters (Kraft et al, 1988).

In addition to the further investigation of changes in PKC activity and distribution following treatment with PKC activators, future studies should be aimed at the characterisation of PKC substrates in A549 cells. Evidence suggests that different PKC activators induce dissimilar phosphorylation patterns in cells (for example: Warren et al, 1987; Yamamoto et al, 1988), which offers a possible explanation for the disparate effects of phorbol esters, DAGs and bryostatins in A549 cells. One could postulate that the apparently paradoxical observation that activators of the same enzyme phosphorylate different target proteins are due to the activation of distinct PKC isozymes. The identification of the substrates which are essential for the biological effects of PKC activators may provide insights into the molecular events which occur following PKC activation Additionally, the study of phosphorylation patterns in TPA-sensitive and -resistant A549 cells might also provide clues in the elucidation of mechanisms of resistance.

In this thesis, compounds synthesized to mimic certain structural features of phorbol esters did not demonstrate phorbol ester-like effects in A549 cells. However, certain antitumour agents such as the bryostatins, adriamycin (Wise and Kuo, 1983), tamoxifen (Su et al, 1985), ether lipids (Helfman et al, 1985b) and sangivamycin (Loomis and Bell, 1988) are able to modulate PKC activity. Further, a number of PKC inhibitors have been reported to enhance the antiproliferative effect of cis-diamminedichloroplatinum(II) and nitrogen mustard (Hofmann et al, 1988), while other

workers have suggested that the pleiotropic resistance of P388 murine leukaemic cells to anticancer compounds such as etoposide is related to decreased levels of phorbol ester receptor and PKC activity (Ido et al, 1987a). Thus, although direct evidence which indicates that the modulation of PKC activity is exploited by anticancer agents is lacking, it is clear that PKC represents a potentially important target for these drugs. The existence of PKC as a family of polypeptides also seems to provide some hope of selectivity in the development of compounds with chemotherapeutic properties.

Very recently, Cohen and coworkers have reported that the potent tumour promoter okadaic acid, which does not activate PKC, is a powerful inhibitor of protein phosphatases-1 and -2A (Haystead et al, 1989). These phosphatases are likely to be the chief enzymes which reverse the actions of PKC. This intriguing finding demonstrates that further putative PKC-related intracellular targets exist which might also be exploited chemotherapeutically.

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SECTION 7: APPENDICES

APPENDIX 1. Systematic chemical names of compounds synthesized as analogues of phorbol esters or DAGs.

Compound	
1	(±)-(1R*,2S*,4S*)-1,2-di-O-octanoylcyclohexane-1,2,4-triol
2	(±)-(1R*,2S*,4R*)-1,2-di-O-octanoylcyclohexane-1,2,4-triol
3	(±)-(1R*,2R*,4S*)-1,2-di-O-octanoylcyclohexane-1,2,4-triol
4	(±)-(1R*,2R*,4R*)-1,2-di-O-octanoylcyclohexane-1,2,4-triol
5	cis-1-O-octanoylcyclohexane-1,4-diol
6	cis-1-O-tetradecanoylcyclohexane-1,4-diol
7	trans-1-O-tetradecanoylcyclohexane-1,4-diol
8	(±)-(1R*,2S*,4S*)-4-hydroxymethyl-1,2-di-O-octanoyl-cyclohexane-1,2-diol
9	(±)-(1R*,2S*,4R*)-4-hydroxymethyl-1,2-di-O-octanoyl-cyclohexane-1,2-diol
10	(±)-(1R*,2S*,4S*)-4-hydroxymethyl-1,2-di-O-tetradecanoylcyclohexane-1,2-diol
11	(±)-(1R*,2S*,4R*)-4-hydroxymethyl-1,2-di-O-tetradecanoylcyclohexane-1,2-diol
12	(±)-(2R*,3S*,5R*)-2,3-di-O-octanoylnorbornane-2,3,5-triol
13	9-hydroxymethyloctadecan-7,12-dione
14	(±)-1,2-di-O-octanoyl-3-O-(glycylglycyl)-glycerol hydrochloride

APPENDIX 2. PUBLICATIONS

ABSTRACTS

Dale, I.L. and Gescher, A. Studies of the effects of phorbol ester analogues on the growth of A549 human carcinoma cells.
(British Society for Cell Biology Autumn Meeting, Norwich, UK, September 1986).

Dale, I.L. and Gescher, A. Investigation of the growth-inhibitory properties of 12-O-tetradecanoylphorbol-13-acetate (TPA) and mezerein (MEZ).
(West Midlands Oncology Association Meeting, Aston University, UK, March 1987).

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(European Organization for Research into the Treatment of Cancer / Pharmacokinetics and Metabolism Winter Meeting, Nice, France, December 1987).

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(British Pharmaceutical Society Annual Meeting, Manchester, UK, September 1987).

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., in press, 1989.

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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[1]

Ian L. Dale, Tracey D. Bradshaw, Andreas Gescher[2] and George R. Pettit

Cancer Research Campaign Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET, England [I.L.D, T.D.B., A.G.] and Cancer Research Institute, Arizona State University, Tempe [G.R.P.]

Running title: Modulation of Protein Kinase C Activity by Bryostatins.

Footnotes:

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[2] To whom requests for reprints should be addressed.

[3]The abbreviations used are: DiC8, 1,2-sn-dioctanoylglycerol; DMSO, dimethylsulfoxide; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PAGE, polyacrylamide gel electrophoresis; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; 4-α-PDD, 4-α-phorbol-12,13-didecanoate.

ABSTRACT

Activators of protein kinase C (PKC), such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and bryostatins 1 and 2, inhibit the growth of A549 cells. At high concentrations the bryostatins do not affect cell growth. Here the hypothesis has been tested that modulation of A549 cell growth is the consequence of agent-induced changes in location or extent of cellular PKC activity. PKC activity was measured after semi-purification with non-denaturing PAGE in the cytosol and the particulate fraction of A549 cells. When cells were exposed to TPA or mezerein, PKC activity underwent rapid and concentration-dependent translocation from the cytosol to the membrane. TPA at 0.1μM or mezerein at 1μM caused almost complete translocation within 30min. Incubation with bryostatins 1 or 2 also led to enzyme translocation, which was however much weaker than that observed with the tumor promotors. Neither 4-α-phorboldidecanoate nor the synthetic diacylglycerols 1,2-sn-dioctanoylglycerol or 1-oleoyl-2-acetyl-sn-glycerol mimicked TPA in this way. Exposure of cells to TPA or the bryostatins for longer than 30min caused the gradual disappearance of total cellular PKC activity. PKC downregulation was concentration-dependent and complete after 24h. A549 cells which had acquired temporary resistance towards the growth-arresting potential of TPA were completely devoid of any measurable PKC activity. The bryostatins were potent inhibitors of the binding of [^{3}H]phorbol-12,13-dibutyrate to its receptors in intact cells and the inhibition was dependent on bryostatin concentration. The results support the contention that PKC is involved in the mediation of growth inhibition caused by TPA or the bryostatins. However the relationship between growth arrest and PKC translocation or downregulation seems to be a complex one.

INTRODUCTION

Tumor-promoting phorbol esters such as TPA[3] elicit a large variety of responses in cultured cells, including stimulatory or inhibitory effects on growth and differentiation (1-3). In A549 human lung carcinoma cells non-toxic concentrations of TPA induce growth arrest accompanied by a change in cell morphology (4). The biological effects of TPA are thought to be mediated, at least in part, by PKC[3] (5,6), a pivotal enzyme in the transmembrane signalling system involving the receptor-coupled breakdown of inositol phospholipids. The bryostatins, macrocyclic lactones isolated from marine bryozoans (7,8), are, like TPA, activators of PKC (9,10). The bryostatins are particularly interesting compounds from a chemotherapeutic point of view because they possess antineoplastic activity against the murine P388 lymphocytic leukemia (12). A puzzling feature of their biological activity is the fact that in many cell lines they are not only agonistic with TPA but also able to antagonize biochemical responses elicited by themselves or by TPA (13-16). We have recently shown that in A549 cells bryostatins 1 and 2 inhibit growth maximally at 10nM and 0.1μM, respectively, but at higher concentrations they block both their own inhibitory effect and the antireplicative action of phorbol esters or of mezerein, another diterpenoid tumor promotor (11).

In many cell types activators of PKC, such as TPA, induce a rapid translocation of PKC from the cytosol to the membrane, followed by a progressive disappearance of enzyme activity (17). In the work described here an attempt has been made to correlate the growth-modulatory activity of TPA and bryostatins with their ability to modulate PKC activity. In particular, the hypothesis has been tested that the change in the growth pattern of A549 cells caused by TPA and

the bryostatins is the result of their ability to elicit enzyme translocation or downregulation. As part of the investigation PKC activity was measured in cells which have been desensitized to the growth-inhibitory action of TPA (4).

MATERIALS AND METHODS

Chemicals. TPA, PDBu, 4-α-PDD, OAG, DiC8, mezerein and biochemicals for the enzyme assay were purchased from Sigma (Poole,UK). Bryostatins 1 and 2 were isolated as described previously (7,8). Tissue culture reagents and media were obtained from Gibco (Paisley, UK) and [^{3}H]PDBu and [γ-^{32}P]ATP from New England Nuclear Division of Dupont Europe (Stevenage, UK). Stock solutions of tumor promotors, bryostatins and diacylglycerols were prepared in DMSO and stored at -20^{0}C. The final concentration of DMSO in the culture medium did not exceed 0.5%. DMSO was added to control cultures and at this concentration it did not affect growth or PKC activity.

Cell Culture Conditions. A549 lung carcinoma cells were obtained from the American Type Culture Collection and routinely cultured in Nutrient Ham's F12 medium supplemented with 10% fetal calf serum as described previously (4). For the PKC assays cells were grown in Petri dishes (14 cm diameter) and used when they were 60 - 90% confluent.

Preparation of Cytosol and Membrane Fraction. Fractions were separated essentially as described by Regazzi et al.(18). Briefly, cells were scraped off the dish and suspended in homogenization buffer (Tris 20mM, EGTA 2mM, EDTA 2mM, aprotinin 2µg/ml, leupeptin 20µg/ml, mercaptoethanol 6mM, pH 7.4) before disruption by sonication. Cytosolic supernatant (designated the "cytosolic fraction") and membrane pellet were obtained by centrifugation (100000xg) at 4^{0}C for 30min. The

membrane pellet was resuspended in homogenization buffer containing 1% (w/v) Nonidet P-40 before renewed sonication and centrifugation. The resulting supernatant (designated the "particulate" or "membrane fraction") or the cytosolic fraction were supplemented with 15% (w/v) glycerol and stored for up to 24h at -20°C.

Polyacrylamide Gel Electrophoresis. PKC in the fractions was partially purified by non-denaturing PAGE according to Fabbro et al. (19) using a Bio-Rad Model 175 tube cell gel apparatus. Conditions for the gel electrophoresis were as follows: Temperature was maintained at 4°C. Voltage was at 100 V whilst the dye (Amaranth) boundary moved through the stacking gel (running time approximately 4h), and at 160-180 V while the boundary moved through the resolving gel (total running time 7-9h). After completion of the electrophoresis the stacking gel was discarded and the resolving gel was placed in a polystyrene tube and frozen on top of dry ice. Gels were sliced into 1 mm sections using a Mickle gel slicer. The gel discs were transferred to tubes containing elution buffer and the enzyme was eluted from slices overnight by gentle shaking at 4°C.

Protein Kinase C Assay. PKC activity was assayed in gel eluates using a microassay technique adapted from the method described by Kikkawa et al. (20) using protamine sulfate as substrate. Aliquots of the gel eluate (150µl) were pipetted into the wells of a 96-well microtiter plate and allowed to equilibriate at 32°C. The enzyme reaction was initiated by the addition of protamine-assay mixture (50µl) using a multichannel pipette. Final concentrations or amounts of assay components in the incubate (200µl) were: 20mM Tris-HCl pH 7.4; 10mM $Mg(NO_3)_2$; 24µM ATP; 0.5µCi [γ-^{32}P]ATP; 100µg protamine sulfate. After 10min incubation aliquots (150µl), seven at a time, were removed with the multichannel

pipette and immediately spotted onto rectangles of Whatman 17Chr filter paper (approximately 2cmx6mm) arranged in a multiwell microtiter plate. The reaction was quenched by immersion in ice-cold 10% (w/v) trichloroacetic acid. Filter paper strips were washed for 1h in 10% (w/v) trichloroacetic acid, the washing solution being changed every 15min. Paper strips were briefly soaked in methanol and dried by heat. Radioactivity was counted in 5ml Luma gel scintillant using a Packard Tricarb CA2000 scintillation counter. One unit of PKC activity is defined as the amount of enzyme which incorporated 1pmol of phosphate from [γ-^{32}P]ATP into protamine sulfate per min at 32^{0}C.

Phorbol Ester Receptor Binding Studies. The conditions of the assay, in which the ability of the bryostatins to compete with the binding of [^{3}H]PDBu to intact A549 cells was measured, were as described before (4). The concentration of [^{3}H]PDBu was 50nM (specific activity 10.2 Ci/mmol) which was complemented with 50μM unlabelled PDBu in case of the determination of non-specific binding. Non-specific binding was between 19% (without bryostatin) and 42% (with bryostatins at 1μM) of total binding.

Protein Determination. Protein was quantitated by the method of Bradford (21) using the Bio-Rad dye reagent and bovine serum albumin as standard.

RESULTS

Translocation of PKC Activity. When A549 cells were incubated with the tumor promotors TPA or mezerein for 30min enzyme activity was translocated in a dose-dependent fashion from the cytosol to the particulate fraction (Fig.1). In contrast, neither 4-α-PDD, which is inactive as a tumor-promotor (1), nor DiC8 or OAG, synthetic analogs of

naturally occurring diacylglycerols, caused significant translocation of PKC. DiC8 was able to elicit a slight decrease in cytosolic PKC activity by 8% at the highest concentration employed (0.3mM) (results not shown). At this concentration DiC8 was cytotoxic. Exposure of cells to bryostatin 1 or 2 resulted in partial translocation of enzyme activity from the cytosol to the membrane (Fig.2). The relative potency of tumor promotors and bryostatins in inducing PKC translocation to the membrane was TPA > mezerein > bryostatin 1 = bryostatin 2.

Downregulation of PKC Activity. Phorbol esters are known to modulate not only the subcellular distribution of PKC activity, but also the total amount of PKC activity in cells (17). In order to test the hypothesis that TPA and the bryostatins cause downregulation of PKC in A549 cells, enzyme activity was measured in cells which had been incubated with TPA or the bryostatins for up to 24hr. TPA elicited the disappearance of enzyme activity in a concentration-dependent manner (Fig.3). On exposure to 10nM TPA, a concentration which causes maximal growth inhibition in these cells (4), downregulation was observed after incubation for 5hr (Fig.3A), whereas at 0.3μM TPA enzyme activity had almost totally disappeared already after 1hr of incubation (Fig.3C). Likewise incubation of cells with bryostatin 1 (Fig.4) or bryostatin 2 (results not shown) led to enzyme downregulation. As in the case of TPA there was a marked concentration dependency in the pattern of enzyme disappearance. The rate at which PKC was downregulated in the presence of 1μM bryostatin 1 (Fig.4B), a concentration at which it abolishes its own growth-arresting activity (11), was considerably faster than that observed with bryostatin 1 at 10nM, the maximal growth-inhibitory concentration (Fig.4A).

Previously we reported that A549 cells acquire reversible

resistance towards the growth-inhibiting properties of TPA under two conditions (4). When incubated with TPA for more than 5 to 6 days cells became desensitized unless they were detached and subcultured, upon which they regained sensitivity. After subculturing cells in the continued presence of TPA for 9 weeks more permanent desensitization was achieved. Cells treated with TPA to acquire either of the described phenotypes of resistance towards the growth-arresting potential of TPA were completely without any measurable PKC activity, whereas naive cells contained 1375±103 units of PKC/mg protein in the cytosol and 106±36 units/mg protein in the particulate fraction (mean±SD, n=17).

Binding of the Bryostatins to the Phorbol Receptor. Bryostatins possess high affinity for the phorbol ester receptor (9). The hypothesis was tested that their ability to inhibit growth at low concentrations or to abolish their growth inhibition at higher concentrations in A549 cells is related to a concentration-dependent change in their pattern of binding to the phorbol ester receptor. To that end the ability of bryostatins 1 and 2 to inhibit the binding of [^{3}H]PDBu to its receptor in intact A549 cells was studied. Fig.5 shows that both bryostatins are potent inhibitors of PDBu binding and that this effect is dependent on bryostatin concentration. At 1μM, the concentration at which they abolish their own growth inhibition, the ability of the bryostatins to compete with [^{3}H]PDBu for the phorbol ester receptor binding site was not decreased but maximal.

DISCUSSION

The relationship between modulation of PKC activity caused by tumor promotors such as TPA or mezerein and their effect on cell proliferation and differentiation is unclear. This issue has been addressed in the present study using A549 cells, one of the cell lines which respond to these agents by cessation of growth (4,11). PKC activity in A549 cells was altered in a manner similar to that observed in certain other cells following treatment with TPA, for example in HL-60 promyelocytic leukemia (22,23) and MCF-7 breast cancer cells (18,24). On exposure of A549 cells to TPA cytosolic PKC activity was rapidly translocated to the particulate fraction and incubation for several hours led to the downregulation of cellular enzyme activity. The inactive analog of TPA, 4-α-PDD, as well as DiC8 and OAG, synthetic analogs of the physiological activators of PKC unable to arrest the growth of A549 cells, did not cause a marked translocation of PKC activity. Thus it appears that the changes in enzyme activity elicited by TPA and mezerein in A549 cells are linked to the causation of growth arrest. Bryostatins 1 and 2 also inhibit the growth of A549 cells at nontoxic concentrations, though less effectively than TPA (11). In this study we have shown that the bryostatins not only possess high affinity for the phorbol ester receptor in these cells but also induce significant enzyme translocation and marked downregulation. Unlike TPA the bryostatins abolish their own growth-inhibitory activity at concentrations which exceed their maximal growth-arresting concentrations by a factor of 10 (11). In view of this difference in effect on cell growth between the bryostatins and TPA it is noteworthy that enzyme translocation caused by the bryostatins was weaker than that elicited by TPA. However, the concentration dependency of the rate at which PKC was downregulated by both agents was similar.

The following three observations render it unlikely that the effects of the tumor promotors and bryostatins on growth are a direct consequence of the observed changes in PKC activity:

(i) The concentrations of agents required to cause detectable enzyme translocation were 10 to 100 fold higher than those necessary to arrest cell growth.

(ii) The time course of TPA-induced disappearance of total cellular enzyme activity demonstrated here reflects the time course of appearance of growth inhibition (11). However, cells which had regained their growth potential after 5 days incubation in the presence of TPA or cells which had acquired resistance to TPA-induced growth inhibition by subculturing them for 3 months with TPA had not regained any measurable PKC activity. This result contrasts with observations using a HL-60 derived cell type made resistant towards the differentiation-inducing action of TPA (25). In these resistant cells the extent of PKC activity was similar to that found in the parent cell line but PKC was not translocated on exposure to TPA. We showed previously that the binding of PDBu to the phorbol ester receptor in intact desensitized A549 cells is 75% of control cells after exposure to PDBu for 5 days and 77% in the more permanently desensitized cells (4). The results obtained here suggest that the receptor population in the desensitized cells is either not functional PKC or a subspecies of the enzyme which does not phosphorylate protamine under the conditions of the assay.

(iii) The third finding presented here which casts doubt on a direct causal relationship between effect on growth of A549 cells and alterations in PKC activity is concerned with the bryostatins: The paradoxical nature of the response of cell growth to different concentrations of the bryostatins was not reflected by a

concentration-dependent reversal in their effects on cellular PKC activity, because their ability at high concentrations to abolish growth inhibition was not paralleled by inhibition of PKC downregulation and consequent maintenance of enzyme activity. The fact that TPA, mezerein and the bryostatins cause PKC translocation and downregulation and induce growth inhibition is consistent with a role for PKC in growth arrest. However, the nature of the relationship between growth potential and PKC activity seems to be complex. Furthermore PKC does not appear to be directly involved in the mediation of the phorbol ester antagonistic effect of the bryostatins at high concentrations.

The inability to explain the inhibition of the growth of A549 cells as the direct consequence of changes in PKC location or PKC activity is in accordance with conclusions drawn from experiments with phorbol esters in other cell lines. For example, neither activation of PKC and the phosphorylation of specific substrates nor the loss of total PKC activity were considered to be directly responsible for the ability of TPA to induce differentiation in HL-60 cells (23). Instead, other specific changes in cellular metabolism which resulted in a decrease in c-myc RNA levels seemed to be involved.

Now it is clear that PKC is not one enzymatic entity but a family of 7 or more related proteins (26). The tissue- and cell-specific expression of the different PKC subspecies suggests that each member of the enzyme family might have a different function in the mediation of cellular responses to external stimuli. Recently it has been shown that on treatment with TPA the different PKC subspecies co-expressed in KM3 cells are downregulated at different rates (27). It remains to be investigated whether the modulation of the activity of a specific, perhaps minor, PKC isoform in A549 cells parallels the growth-modulatory

action of the tumor promoters and bryostatins more closely than do the changes in total PKC activity described here.

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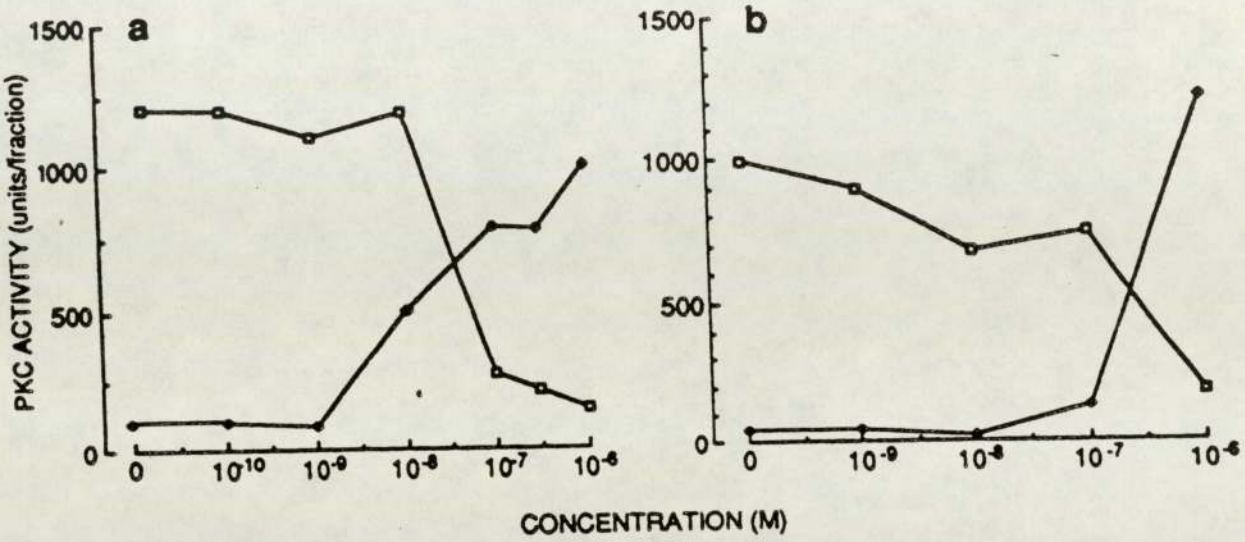

Fig.1. PKC activity in the cytosol (□) and the particulate fraction (◆) of A549 cells after incubation for 30min with different concentrations of TPA (a) or mezerein (b). Values are the mean of 2 or 3 experiments. In case of the mean values calculated from 3 experiments the SD was ≤18% of the mean.

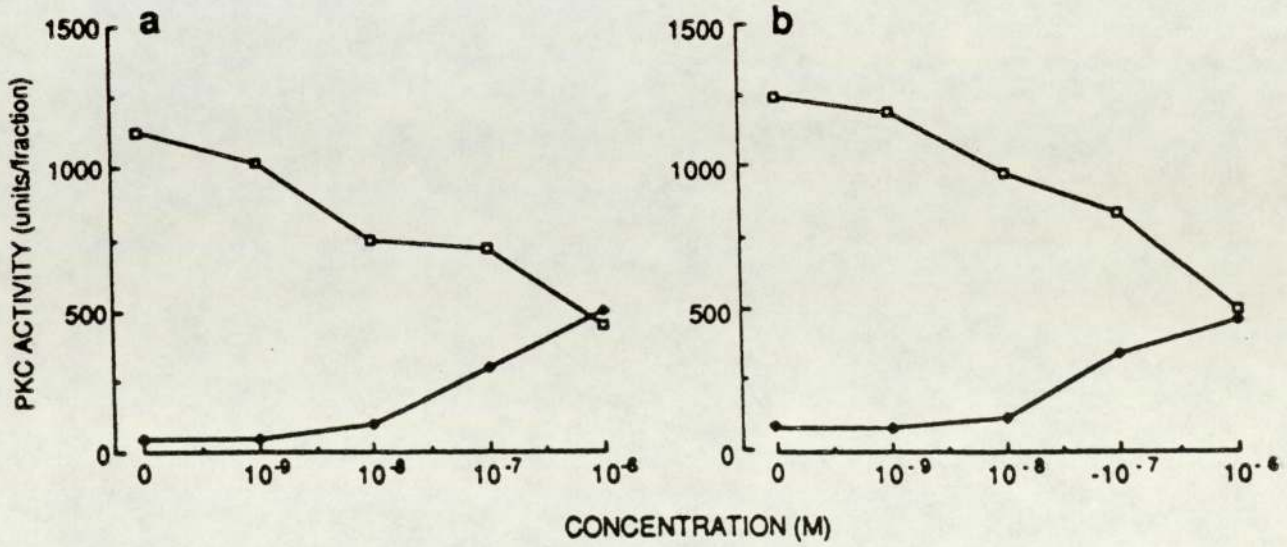

Fig.2. PKC activity in the cytosol (□) and the particulate fraction (◆) of A549 cells after incubation for 30min with different concentrations of bryostatin 1 (a) or bryostatin 2 (b). Values are the mean of 2 or 3 experiments. In case of the mean values calculated from 3 experiments the SD was ≤18% of the mean.

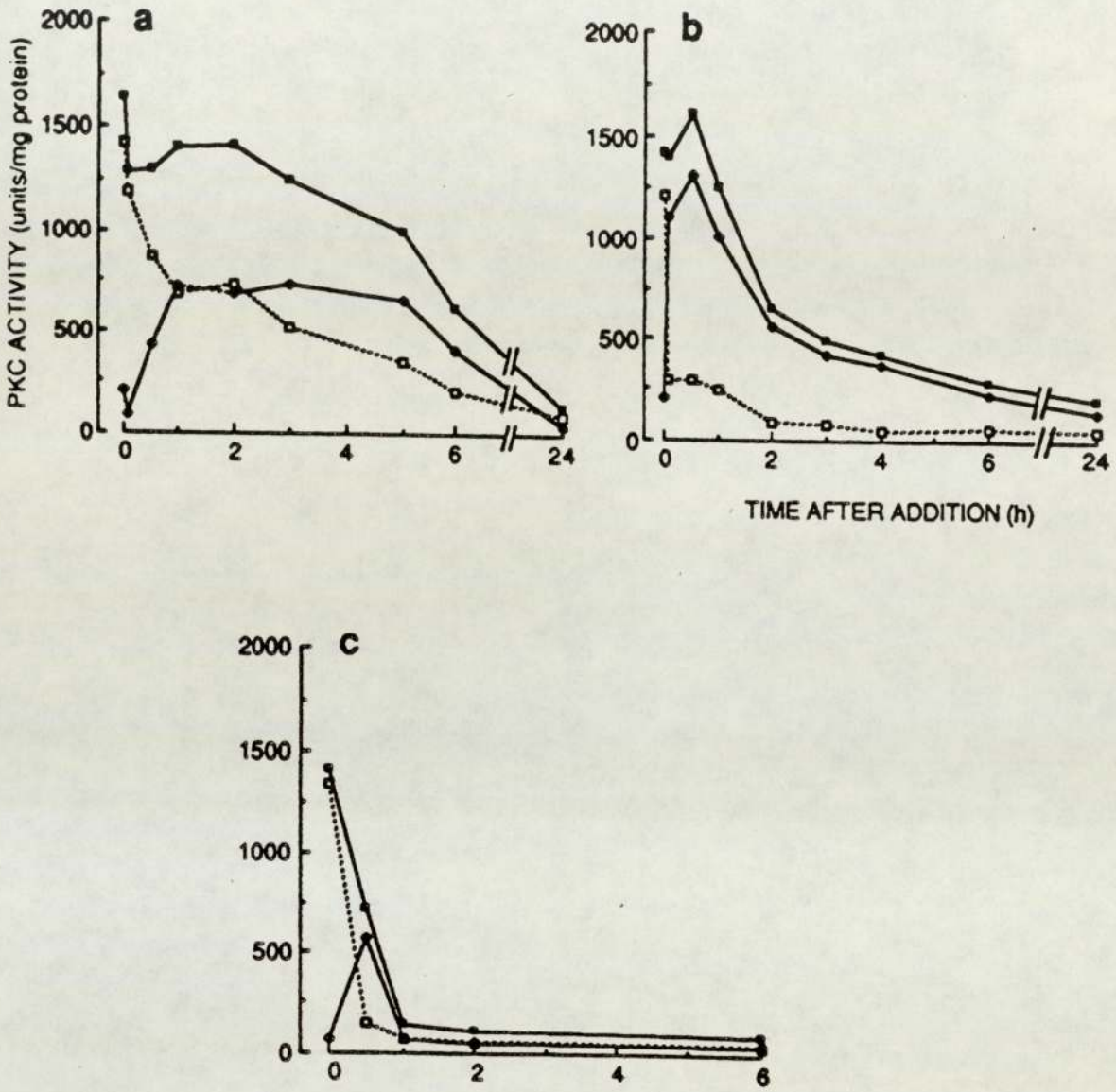

Fig.3. Total cellular PKC activity (■) and PKC activity in the cytosol (□) and particulate fraction (◆) of A549 cells after incubation with TPA 10nM (a), 0.1µM (b) or 0.3µM (c) for different time intervals. Values are the mean of 2 or 3 experiments. In case of the mean values calculated from 3 experiments the SD was ≤15% of the mean.

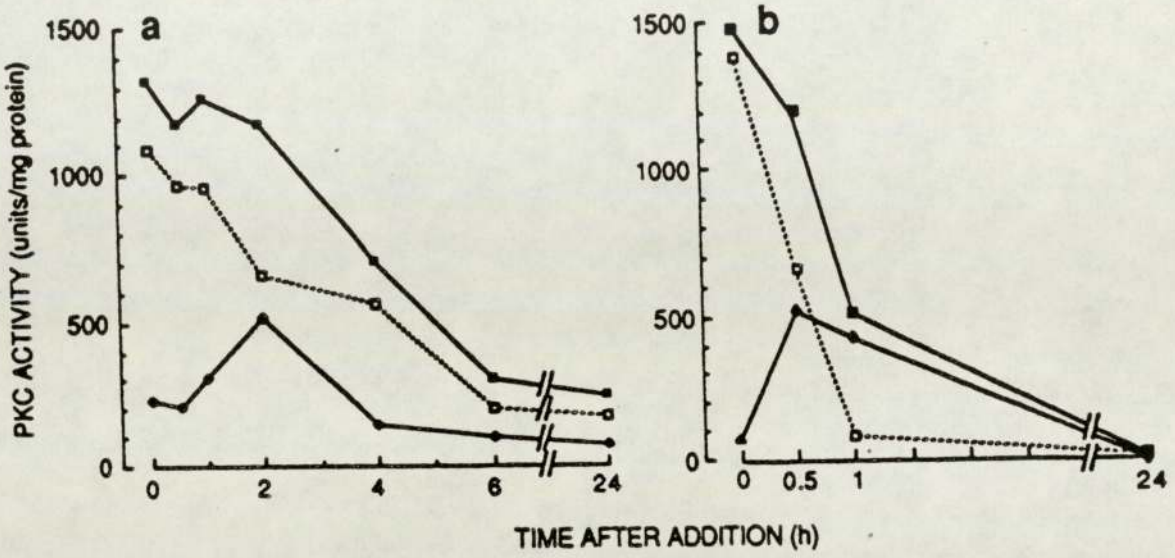

Fig.4. Total cellular PKC activity (■) and PKC activity in the cytosol (□) and particulate fraction (◆) of A549 cells after incubation with bryostatin 1 10nM (a) or 1μM (b) for different time intervals. Values are the mean of 2 or 3 experiments. In case of the mean values calculated from 3 experiments the SD was ≤15% of the mean.

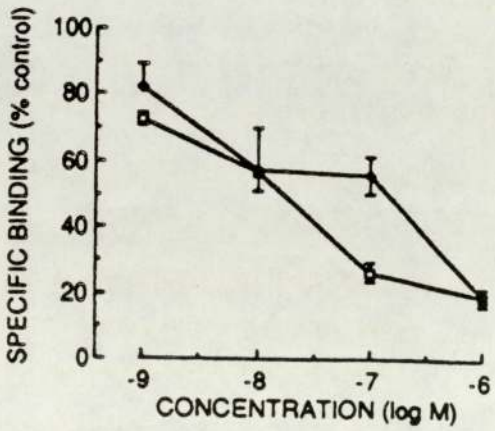

Fig.5. Effect of different concentrations of bryostatin 1 (□) or bryostatin 2 (◆) on the specific binding of [^{3}H]PDBu (50nM) to its receptor in A549 cells. Values are the mean±SD of 3 experiments, each conducted with duplicate samples.

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EFFECTS OF ACTIVATORS OF PROTEIN KINASE C, INCLUDING BRYOSTATINS 1 AND 2, ON THE GROWTH OF A549 HUMAN LUNG CARCINOMA CELLS

Ian L. DALE and Andreas GESCHER[1]

Cancer Research Campaign Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Birmingham, B4 7ET, UK.

Phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibit the growth of A549 human lung carcinoma cells at non-toxic concentrations, whereas 1-oleoyl-2-acetylglycerol and 1,2-dioctanoylglycerol, synthetic analogues of the physiological ligands of protein kinase C (PKC), do not. Experiments were conducted to test the hypothesis that other activators of PKC are capable of interfering with A549 cell growth. The non-phorbold tumour promotor mezerein mimicked the growth-inhibitory effect of TPA in that it arrested growth for 5 days, after which cells proliferated again in the continued presence of the agent. TPA was 20 times more potent as a growth inhibitor than was mezerein. Bryostatin 1 at 10 nM and bryostatin 2 at 100 nM also arrested A549 cell growth and inhibited DNA replication as measured by incorporation of [methyl-^{3}H]-thymidine into cells. Inhibition of DNA synthesis to between 90 and 75% of control values developed during the first hour of incubation of the cells with TPA, mezerein or the bryostatins. The extent of inhibition changed little during the subsequent 5 hr of incubation, after which it increased further to reach maximal values within 12 hr. At concentrations above those which caused maximal growth inhibition, the bryostatins abolished both their own inhibition of DNA synthesis and the anti-replicative effect of TPA and mezerein. The results show that activators of PKC other than phorbol esters are capable of inhibiting the growth of A549 cells. The bryostatins not only interfere with A549 cell growth but can also counter the growth-inhibitory effect of PKC activators, presumably *via* interaction with a target separate from the phorbol ester receptor site.

Growth inhibition is one of the plethora of effects which tumour-promoting phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) can exert on cells in culture (Diamond *et al.*, 1980; Gescher, 1985). In some cell lines inhibition of growth caused by TPA is the consequence of the induction of terminal differentiation (Vandenbark and Niedel, 1984). The growth-modulatory and biochemical effects of phorbol esters are thought to be mediated *via* activation of the ubiquitous phospholipid and Ca^{2+}-dependent enzyme protein kinase C (PKC). PKC is the major or perhaps the only phorbol ester receptor (Nishizuka, 1984, 1986) and a pivotal enzyme in the transduction of physiological signals which influence cell proliferation and differentiation involving the receptor-mediated hydrolysis of inositol phospholipids (Berridge, 1984; Nishizuka, 1984). Recently, evidence has emerged which suggests that PKC may be a target suitable for therapeutic intervention in malignant diseases. This suggestion is primarily based on the finding that PKC is not one enzyme but an enzyme family (Coussens *et al.*, 1986), the individual members of which may possess different substrate specificities and affect different biochemical processes.

The growth of the A549 cell line, which was derived from a human lung carcinoma, is potently inhibited by treatment with phorbol esters (Gescher and Reed, 1985). The specific objective of the work described here is to characterize features of this growth inhibition and to investigate the mechanisms involved. Special attention has been focused on the effects on cell growth of the non-phorboid tumour promotor mezerein and of bryostatins 1 and 2. Bryostatins are naturally-occurring macrocyclic lactones which have been isolated from marine bryozoans (sea mosses) (Pettit *et al.*, 1982). Like TPA and mezerein, bryostatins are potent activators of PKC (Kraft *et al.*, 1986), but they do not promote tumours (Hennings *et al.*, 1987). They are also able to block some of the biochemical responses elicited by phorbol esters, such as the induction of terminal differentiation of HL-60 human promyelocytic leukaemia cells (Kraft *et al.*, 1986).

MATERIAL AND METHODS

Chemicals

TPA, phorbol-12,13-dibutyrate (PDBu) and mezerein were purchased from Sigma (Poole, UK). Bryostatins 1 and 2 isolated from the bryozoan *Bugula neritina* (Linn.) were gifts from Dr. G.R. Pettit, Arizona State University, Tempe, AZ. [Methyl-^{3}H]-labelled thymidine (^{3}H-TdR) (specific activity 5 Ci/mmol) was obtained from Amersham (Little Chalfont, UK). Tissue culture reagents were purchased from Gibco (Paisley, UK).

The tumour promoters and bryostatins were stored at −20°C. Stock solutions were prepared in dimethylsulphoxide. The final concentration of dimethylsulphoxide in the culture medium did not exceed 0.3%. Addition of dimethylsulphoxide to control cultures at this concentration did not affect cell growth.

Cell culture and measurement of cell growth

A549 lung carcinoma cells were obtained from the American Type Culture Collection and routinely cultured as described previously (Gescher and Reed, 1985). When effects of the compounds on cell growth were studied, cells ($2–5 \times 10^4$ per well) were seeded in 6-well Nunclon multi-dishes (35 mm diameter, Gibco) and incubated with 3 ml Nutrient Ham's F12 medium supplemented with 10% foetal calf serum and the test compound in a humidified incubator gassed with 95% air:5% CO_2. Cells and compounds were incubated for up to 13 days. Control cultures reached confluence after 5–6 days. Medium and test compounds were replaced every 2 days. At the end of the incubation period, cells were counted with either a haemocytometer or a Coulter Counter, model ZM, after detachment of cells from the dish by a short incubation with a solution of trypsin (0.1%) in versene. The IC_{50} value quoted in the "Results" is the concentration of compound which inhibited cell growth by 50%. It was obtained by linear regression analysis of the linear portion of the concentration-response curve constructed with values measured at at least 5 concentrations in 3 experiments.

Measurement of incorporation of ^{3}H-TdR into cells

Cells (2×10^5 per well) were seeded and compounds were added after a 4-hr period during which the cells attached to the plastic surface. Cells were incubated routinely for 24 hr or for shorter time periods in the case of the experiments in which the time course of incorporation of ^{3}H-TdR was studied. At the end of the incubation time, cells were washed and kept for

[1]To whom reprint requests should be addressed.

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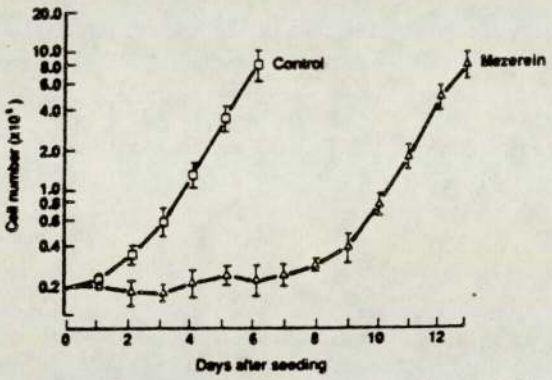

FIGURE 1 - Growth of control cells (□) and of cells incubated with mezerein (100 nM) (△). Values are the mean ± SD of 3 experiments conducted with 3 replicates.

1 hr in medium (1 ml) containing 1 μCi ^{3}H-TdR. Subsequently, cells were treated essentially as described by Besterman *et al.* (1984). After fixing with a solution containing methanol (50%) and acetic acid (10%), acid-insoluble material was solubilized in 2 aliquots (0.5 ml) of 1% SDS and added to Luma gel scintillant (10 ml). Radioactivity was counted in a Packard Tricarb CA 2000 scintillation counter. In parallel with the ^{3}H-TdR incorporation assays, cells in identical cultures were counted and the results shown in Figure 4 are expressed as a percentage of control ^{3}H-TdR incorporation. The IC_{50} values for bryostatin 1 (legend to Fig. 5) are the concentrations required to decrease the inhibition of DNA synthesis caused by PKC activators by 50% and were calculated by linear regression analysis of the linear portion of the log concentration-response curve.

RESULTS

Effect of mezerein and bryostatins 1 and 2 on cell growth and DNA replication

When A549 cells were incubated with mezerein (100 nM), cells were affected in a manner apparently identical to that observed on incubation with TPA (10 nM) (Gescher and Reed, 1985). Within 3 hr of incubation, cells changed their morphology from flat and spread out to rounded and protruding (result not shown). Cell growth was inhibited for 6 days in the continued presence of mezerein (Fig. 1). On incubation for longer time periods, the cells resumed proliferation at a rate similar to that of control cells (Fig. 1). In these experiments medium and mezerein were replenished every 2 days. After incubation with mezerein at varying concentrations for 24 hr, DNA synthesis, as measured by incorporation of ^{3}H-TdR into cells, was inhibited with an IC_{50} of 3.9 nM (90% confidence limits: 2.7 and 5.5 nM). TPA has been previously shown to inhibit DNA synthesis in A549 cells with an IC_{50} of 0.2 nM (Laughton *et al.*, 1988).

Both bryostatin 1 (10 nM) and bryostatin 2 (100 nM) also altered cell morphology (result not shown) and caused growth inhibition in a manner similar to phorbol esters (Fig. 2), even though the extent of this inhibition was not as dramatic as that observed in the case of mezerein (Fig. 1) or TPA (Gescher and Reed, 1985).

The plot describing the concentration-dependence of the effect of the bryostatins on DNA synthesis exhibits 2 distinct phases (Fig. 3). Bryostatin 1 inhibited the incorporation of ^{3}H-TdR maximally at a concentration of 10 nM; however, arrest

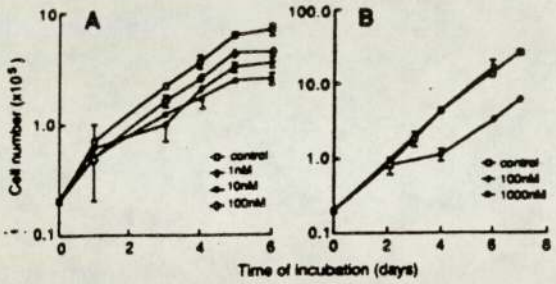

FIGURE 2 - Influence of bryostatin 1 (*a*) and bryostatin 2 (*b*) on the growth of A549 cells. Values are the mean ± SD of 3 experiments conducted with 3 replicates.

of DNA synthesis was diminished or abolished at higher concentrations. Similarly, bryostatin 2 affected incorporation of ^{3}H-TdR into the cells maximally at 100 nM, whereas higher concentrations were less inhibitory (Fig. 3). At the high non-inhibitory concentrations, the bryostatins also caused no change in cell morphology.

Time-course of inhibition of DNA synthesis caused by activators of PKC

In order to study the speed at which the PKC activators affect cell proliferation, their influence on DNA synthesis was measured at different time intervals after commencement of incubation. Figure 4 shows that incorporation of ^{3}H-TdR was inhibited by only 10–25% within the first hour of incubation, and that the level of inhibition changed very little during the subsequent period of 4–5 hr. Upon longer incubation, incorporation of ^{3}H-TdR was increasingly arrested until it reached a minimal value after incubation for 12 hr (Fig. 4).

Effect of bryostatins 1 and 2 on the arrest of DNA replication and of cell growth caused by activators of PKC

According to the results presented in Figure 3, the bryostatins inhibited the incorporation of ^{3}H-TdR only when present at concentrations within a defined window. At higher concentrations, inhibition of DNA replication was only weak or absent. Similarly, bryostatin 1 at concentrations of more than 10 nM decreased or abolished the inhibition of DNA synthesis

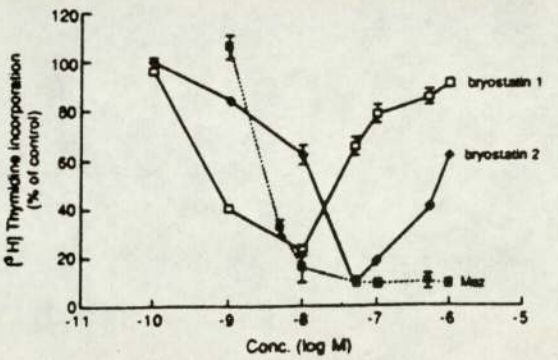

FIGURE 3 - Influence of different concentrations of mezerein (■), bryostatin 1 (□) and bryostatin 2 (♦) on DNA replication. Values are the mean ± SD of 4 observations in one experiment representative of 3.

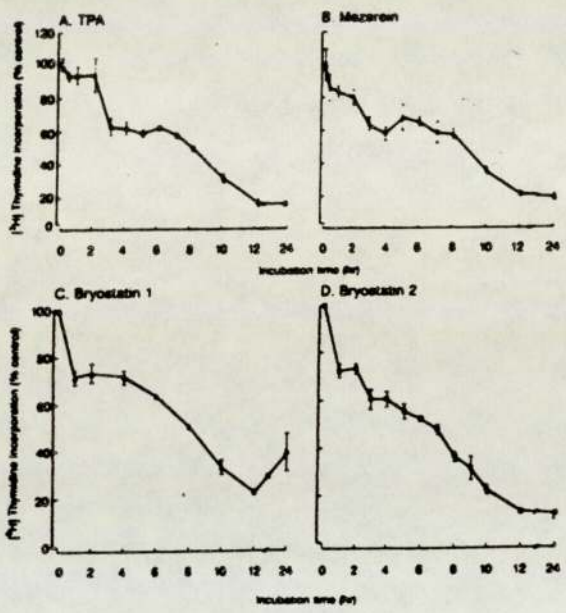

FIGURE 4 - Time course of effect of incubation with PKC activators on DNA replication. Cells exposed to TPA (10 nM) (*a*), mezerein (100 nM) (*b*), bryostatin 1 (10 nM) (*c*) or bryostatin 2 (100 nM) (*d*). Values are the mean ± SD of 4 observations in one experiment representative of 3.

which was observed in the presence of TPA (10 nM) (Fig. 5*a*), mezerein (100 nM) (Fig. 5*c*) or bryostatin 2 (100 nM) (Fig. 5*d*). Even at a concentration of 200 nM, TPA was unable to overcome the ability of bryostatin 1 to eliminate the TPA-induced inhibition of ^{3}H-TdR incorporation (Fig. 5*b*). Results similar to those shown in Figure 5*a* were obtained when TPA was replaced by PDBu (100 nM), another tumor-promoting phorbol ester (results not shown), which inhibits the growth of A549 cells (Gescher and Reed, 1985).

Bryostatin 2 was able to reduce the inhibitory effects of TPA, mezerein and bryostatin 1 on DNA replication (Fig. 6). However, in comparison with the "de-inhibitory" ability of bryostatin 1, higher concentrations of bryostatin 2 had to be added to interfere noticeably with the inhibition of DNA synthesis. When the bryostatins at sufficiently high concentrations were co-incubated with TPA or mezerein, growth characteristics were those of the bryostatins only. This is illustrated by Table I which shows that the bryostatins decreased or abolished the growth arrest caused by 10 nM TPA.

TABLE I - EFFECT OF INCUBATION WITH BRYOSTATIN 1 OR BRYOSTATIN 2, TOGETHER WITH TPA, ON CELL GROWTH

Treatment	Cell number ($\times 10^5$)
None	4.3 ± 0.4
TPA (10 nM)	0.4 ± 0.1
Bryostatin 1 (10 nM)	1.7 ± 0.3
Bryostatin 1 (10 nM) + TPA (10 nM)	0.9 ± 0.1
Bryostatin 1 (1 μM)	5.3 ± 0.2
Bryostatin 1 (1 μM) + TPA (10 nM)	5.6 ± 0.4
Bryostatin 2 (100 nM)	1.1 ± 0.2
Bryostatin 2 (100 nM) + TPA (10 nM)	0.7 ± 0.03
Bryostatin 2 (1 μM)	4.3 ± 0.2
Bryostatin 2 (1 μM) + TPA (10 nM)	3.3 ± 0.4

Cells (2×10^4/well) were seeded and incubations were carried out as described in "Material and Methods". Cells were counted 4 days after seeding and values are the mean ± SD of 3 experiments. Control cells were confluent on day 6.

DISCUSSION

The growth of certain neoplastic cell lines, such as the MCF-7 breast carcinoma line (Roos *et al.*, 1986; Valette *et al.*, 1987) or the A549 lung carcinoma line, is arrested by TPA and other tumour-promoting phorbol esters. There is now little doubt that this growth-inhibitory property of phorbol esters is closely associated with their ability to activate PKC (Nishizuka, 1986). On the basis of its pivotal role in processes mediating cell proliferation and differentiation, it has been suggested that PKC might constitute a target for anti-neoplastic therapy. In accordance with this hypothesis are the findings that the anti-neoplastic purine nucleoside analogue sangivamycin is a potent inhibitor of PKC (Loomis and Bell, 1988), and that the bryostatins, experimental agents with anti-neoplastic activity in the murine P388 lymphocytic leukaemia model (Pettit *et al.*, 1970) are activators of PKC (Kraft *et al.*, 1986). However, it is still far from clear to what extent the ability of these agents to modulate PKC activity is responsible for their anti-neoplastic properties.

In this study, we have tested the hypothesis that activators of PKC other than phorbol esters are capable of inhibiting the growth of A549 cells. Mezerein was an effective inhibitor of growth, even though a comparison of the respective IC_{50} values shows that TPA is 20 times more potent than mezerein. Bryostatins 1 and 2 also caused growth inhibition when incubated with A549 cells at concentrations within a narrow range, albeit less potently than mezerein or TPA. The inhibition of DNA synthesis caused by these PKC activators developed in 2 stages: within the first hour of incubation DNA synthesis was only slightly retarded, whereas the major inhibition emerged only after an incubation period of 6 hr. The result suggests that 2 different mechanisms may be responsible for the 2 phases of growth retardation. This hypothesis is currently being tested in our laboratory. Preliminary results suggest that the bryostatins at μM concentrations induce a small concentration-dependent translocation of PKC to cellular membranes, but bryostatin-induced down-regulation PKC is similar to that observed with TPA (unpublished results). Therefore, the biphasic effect of the bryostatins on growth does not appear to be correlated with concentration-dependent differences in their ability to activate PKC.

There are doubts concerning the importance of PKC activation for TPA-induced growth inhibition. We have recently shown that the diacylglycerols 1-oleoyl-2-acetylglycerol and 1,2-dioctanoylglycerol, synthetic analogues of the physiological ligands of PKC (Kishimoto *et al.*, 1980), cannot arrest the growth of A549 cells unless they are added to cellular incubates at concentrations in the 10^{-4} M range, at which they cause toxicity (Laughton *et al.*, 1988). In contrast, TPA and the PKC activators used in this study arrested cell growth at concentrations far below those at which they are cytotoxic. Furthermore, co-incubation of cells with TPA and the PKC inhibitor 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) did not alter the extent of the inhibition of DNA synthesis induced by TPA alone (data not shown). These results suggest that activation of PKC itself is not the only cause of the inhibition of A549 cell growth by TPA and its functional analogues. However, there are alternative explanations. Firstly, diacylglycerols may fail to mimic TPA due to the masking of any cytostatic influence by non-specific cytotoxicity and, secondly, both diacylglycerols and H-7 may be ineffective because they cannot penetrate A549 cells sufficiently to interact with their target(s). Nevertheless, the suggestion that activation of PKC on its own is perhaps not sufficient to trigger the

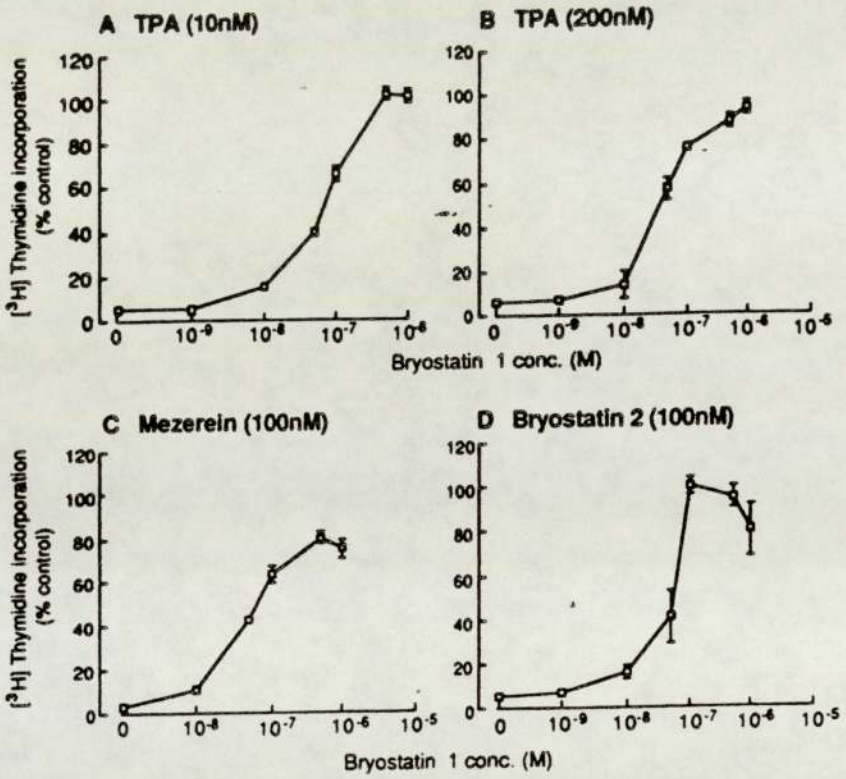

FIGURE 5 - Effect of bryostatin 1 on inhibition of DNA replication caused by TPA (10 nM) (*a*), TPA (200 nM) (*b*), mezerein (100 nM) (*c*) and bryostatin 2 (100 nM) (*d*). Values are the mean ± SD of 4 observations in one experiment representative of 3. The concentrations of bryostatin 1 required to retard the anti-replicative effect of compounds by 50% are: TPA (10 nM) 45 nM; TPA (200 nM) 35 nM; mezerein (100 nM) 63 nM; bryostatin 2 (100 nM) 56 nM.

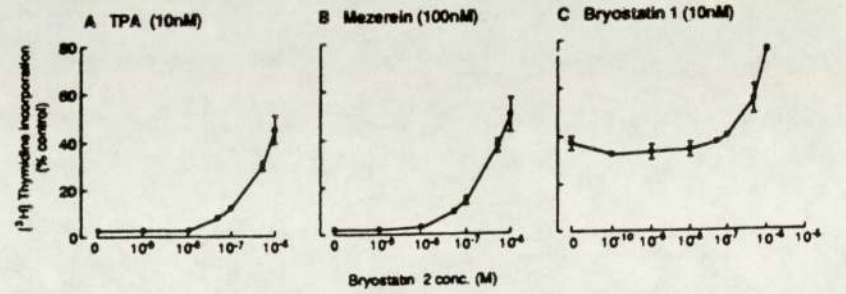

FIGURE 6 - Effect of bryostatin 2 on inhibition of DNA replication caused by TPA (10 nM) (*a*), mezerein (100 nM) (*b*) and bryostatin 1 (10 nM) (*c*). Values are the mean ± SD of 3–4 observations in one experiment representative of 3.

inhibition of A549 cell growth is compatible with conclusions drawn from studies in which the effects of TPA and diacylglycerols were compared in other cell types, for example the HL-60 promyelocytic leukaemia (Morin *et al.*, 1987; Kreutter *et al.*, 1985; Yamamoto *et al.*, 1985).

Studies on phorbol esters suggest a very close relationship between their chemical structure and their biological efficacy (Diamond *et al.*, 1980). Similarly, the biological activities of the bryostatins seem to be very sensitive to slight changes in the molecule. The structure of bryostatin 1 differs from that of bryostatin 2 by just one acetylated alcoholic hydroxy moiety (Pettit *et al.*, 1983). This slight structural difference caused the concentration-inhibitory response curve for bryostatin 1 to be shifted further to the left by a factor of one compared to that of bryostatin 2, even though a comparison of the extent of the inhibition of DNA synthesis at the optimal concentrations of either compound shows that bryostatin 2 was the more effective inhibitor.

One of the most fascinating aspects of the biological activities of bryostatins is the dual nature of their influence on cells responsive to TPA. Bryostatins have been shown to mimic TPA in that, for example, they inhibit phorbol ester binding and cause mitogenesis in Swiss 3T3 cells (Smith *et al.*, 1985), stimulate human polymorphonuclear leukocytes (Berkow and Kraft, 1985) and induce ornithine decarboxylase while inhibiting cell–cell communication in keratinocytes (Sako *et al.*, 1987; Pasti *et al.*, 1988). In contrast, bryostatins block the differentiation induced by phorbol esters in HL-60 cells (Kraft *et al.*, 1986), human colon cancer cells (McBain *et al.*, 1988) and primary mouse epidermal cells (Sako *et al.*, 1987). They also restore the differentiation response in Friend erythroleukaemia cells in which drug-induced differentiation is suppressed by PDBu (Dell'Aquila *et al.*, 1987). In our work, the dual nature of the efficacy of the bryostatins has now been demonstrated clearly in A549 cells. Both bryostatin 1 and 2 inhibited DNA replication and growth in these cells, but at higher concentrations they blocked both their own inhibitory effect and the anti-replicative action of TPA and mezerein. In order to explain the fact that the bryostatins can function in a concentration-dependent manner to either stimulate or inhibit cell growth and differentiation, McBain *et al.* (1988) have recently proposed that these processes in a cell responsive to TPA may be regulated by the balance of 2 separate and opposing pathways which are controlled by the phosphorylating action of enzymes of the PKC family. One of these pathways is pro-replicative and the other anti-replicative. Within this conceptual framework, the phorbol esters or the bryostatins may achieve their divergent effects through the differential activation of individual C kinases responsible for phosphorylating components of the separate pathways. Alternatively, the agents may alter the substrate specificities of certain enzymes of the PKC family or may selectively facilitate their metabolic processing.

It seems likely that the bryostatins are agonistic with phorbol esters and mezerein *via* the same mechanisms as those operated by these tumour promoters. It is possible that they exert their antagonistic action *via* interaction with a second receptor, distinct from the high-affinity phorbol ester binding site of PKC (Blumberg, 1988). This hypothesis is supported by our observation that in A549 cells the antagonistic property of the bryostatins does not appear to be mediated *via* the phorbol ester receptor site. This conclusion is based on the finding that bryostatin 1 at 100 nM or at higher concentrations abolished the TPA-induced inhibition of DNA synthesis at either 10 or 200 nM TPA, which suggests that even at high concentrations TPA was incapable of competing with bryostatin 1. Undoubtedly, the bryostatins, like TPA, can also activate PKC in A549 cells, as recently shown by the bryostatin-induced translocation of cytosolic PKC to the particulate fraction and subsequent down-regulation of enzyme activity (data not shown). Whether the second target is part of the PKC enzyme system or is situated further downstream along the cascade of events which are involved in TPA-induced growth inhibition is currently not known.

In summary, our results suggest that some activators of PKC are capable of causing growth inhibition without cytotoxicity in certain neoplastic cells. Whether this growth-inhibitory potential can be exploited therapeutically remains to be investigated. However, the emergence of different targets through which the bryostatins exert their effects indicates that the investigation of the mode of action of agents which modulate the activity of PKC may provide new stimuli in the search for novel anti-cancer agents.

ACKNOWLEDGEMENTS

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$Na_2S_2O_3$. Relative peroxide value against vitamin E (POV) was calculated.

Inhibition of Lipid Peroxide Formation (m-LPO). This was investigated by a method similar to that described by Malvy et al.[22] (rat liver microsomes, ferrous sulfate/cysteine).

Effects on Hyperlipoperoxidemia and Hyperlipidemia (ALLOXAN). Male BALB/c mice were used at the age of 8 weeks. The animals were fasted for 18 h, after which 75 mg/kg of alloxan was administered intravenously. Each of the test compounds was administered orally at a dose of 300, 200, 100, 50, or 10 mg/kg body weight 30 min before and 24 and 30 h after administration of alloxan. Blood was collected from an incision in the cervical region 48 h after administration of alloxan. The collected amount of blood was 100 or 200 μL. Then whole blood was diluted 10 or 20 times with a saline solution and centrifuged (3000 rpm, 10 min) to determine lipid content.

LPO was measured by the TBA method.[6] CHOL and TG were measured according to the enzyme method. A Determiner TC (a registered trade mark of Kyowa Medix) kit was used to measure CHOL, and a Triglyceride Measuring Agent (GPO-*p*-chlorophenol color developing method) (Wako Pure Chemical Industries) kit was used for TG.

As a control, the procedure was repeated, except that no test compound was administered.

Effects on Hyperglycemia (KK-MICE). Male KK-mice were housed in individual cages at the age of 8 weeks. They were used for the experiment when their body weight was more than 40 g at the age of about 4–5 months.

Test compound was finely suspended in 0.5% (carboxymethyl)cellulose saline (vehicle). Each of the test compounds was administered orally at a dose of 150 or 50 mg/kg body weight 18 h before blood sampling. Blood was collected from the tail vein in a heparinized hematocrit tube and then centrifuged and plasma was separated to measure blood glucose. Plasma glucose level was determined by a glucose analyzer (Mitsubishi Kasei Co., Ltd, Model-101).

As a control, the same test was done simultaneously after administration of the vehicle.

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Studies on Bioactive Compounds. 13.[1] Synthesis and Lack of Growth-Inhibitory Properties of Cyclohexane-1,2,4-triol 1,2-Diesters, Which Resemble Ring C of the Phorbol Ester Molecule

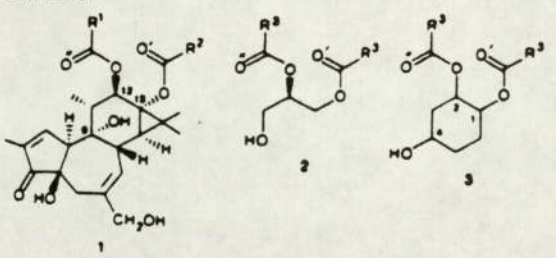
C. A. Laughton, I. L. Dale, and A. Gescher*

Cancer Research Campaign Experimental Chemotherapy Group, Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, U.K. *Received June 10, 1988*

It has been suggested that ring C of biologically active phorbol esters is an essential structural feature of the pharmocophore which confers activity on these compounds. In this study the hypothesis has been tested that compounds which resemble ring C of the phorbol ester molecule mimic the ability of phorbol esters to inhibit cell growth at nontoxic concentrations. All four diastereoisomers of (±)-1,2-di-*O*-octanoylcyclohexane-1,2,4-triol have been prepared from cyclohexen-4-ol and tested for growth-inhibitory and cytotoxic properties. The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate inhibited the growth of A549 human lung carcinoma cells by 50% at a concentration of 0.2 nM and exerted cytotoxicity at concentrations of >1 μM. Diacylglycerols are the physiological ligands and activators of protein kinase C, the receptor via which phorbol esters are thought to mediate their effects. The diacylglycerols 1-oleoyl-2-acetylglycerol and 1,2-dioctanoylglycerol and the cyclohexanetriol diesters inhibited the growth of A549 cells only at concentrations of 10^{-5} to 10^{-4} M, at which they were also cytotoxic. A computer-assisted analysis of the goodness of fit between the cyclohexanetriol diesters and ring C of the phorbol moiety revealed possible energetic grounds for conformational dissimilarities. The results suggest that activation of protein kinase C alone is probably not sufficient to reproduce phorbol ester induced growth arrest in A549 cells and that the cyclohexanetriol diesters may lack pivotal elements of the phorbol ester pharmacophore.

The multitude of recent studies on the mechanism by which tumor-promoting phorbol esters, of which 12-*O*-tetradecanoylphorbol 13-acetate (TPA, **1**; see Scheme I) is the most potent derivative, exert their pleiotropic effects in biological systems have left many intriguing questions unanswered. There is now little doubt about the contention that the ability of these compounds to bind to their receptor, the ubiquitous calcium and phospholipid-dependent enzyme protein kinase C (pkC), plays a pivotal role in the generation of their biological effects.[2,3] However it is not clear whether the diverse responses to TPA, such as, for example, induction or mitogenesis,[4] inhibition of growth,[5] and induction or inhibition of differentiation,[6] are

Scheme I



(1) For paper 12, see: Cunningham, B. D. M; Lowe, P. R.; Threadgill, M. D. *J. Chem. Soc., Perkin Trans. 2*, in press.
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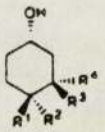
Scheme II



3a: $R^1 = R^4 = H$, $R^2 = R^3$ = octanoyloxy
b: $R^2 = R^3 = H$, $R^1 = R^4$ = octanoyloxy
c: $R^1 = R^3 = H$, $R^2 = R^4$ = octanoyloxy
d: $R^2 = R^4 = H$, $R^1 = R^3$ = octanoyloxy

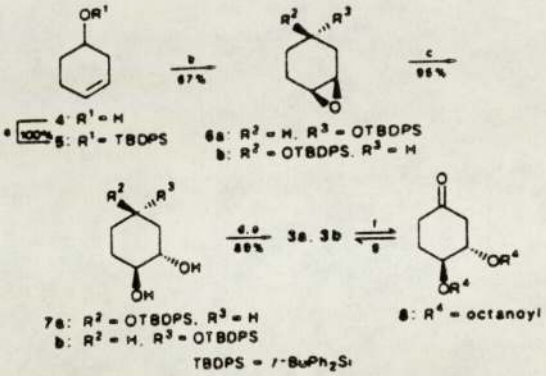
caused by the same or by different biochemical events. Extensive structure–tumor promotory activity studies have defined the importance of several structural features of the phorbol ester molecule for tumor promotion.[4] The ability of TPA to activate pkC has been suggested to be linked to the diester portion of the molecule which bears a structural resemblance to diacylglycerols (e.g. dioctanoylglycerol, DiC_8, 2), the physiological ligands of pkC.[5] However, it is unclear to what extent the structure–activity relationships valid for the tumor-promotory efficacy hold for other biological effects of phorbol esters.

We have been particularly interested in the ability of phorbol esters to cause growth inhibition at nontoxic concentrations.[7] This property suggests that the interactions between phorbol esters and some of their biochemical targets might be exploited for the therapeutic intervention with proliferative diseases. As part of a program of rational drug design we wished to determine relationships between chemical structure and growth-inhibitory activity associated with phorbol esters with the ultimate aim to exploit this property therapeutically. The structural simplicity of the diacylglycerols suggests that, firstly, only a few of the functional groups present in phorbol esters are involved in the binding interaction with the receptor protein and that, secondly, these functionalities lie close to each other. One candidate partial structure for consideration consists of the hydroxyl group at C-9 and the two acyloxy groupings at C-12 and C-13 which are disposed around ring C of the phorbol esters. On the basis of this hypothesis, we report here the synthesis of cyclohexane-1,2,4-triol 1,2-diesters, 3, as stripped-down versions of this ring. These compounds also mimic a diacylglycerol molecule upon which severe conformational restrictions have been imposed. The degree of similarity of the newly synthesized cyclohexanetriol esters to ring C of phorbol esters has been assessed by computer-assisted modeling. Furthermore the ability of the cyclohexanetriol derivatives to inhibit cell growth or to exert cytotoxicity has been tested with the A549 human lung carcinoma cell line, which is exquisitely sensitive to the growth-inhibitory potential of TPA.[7]

Chemistry

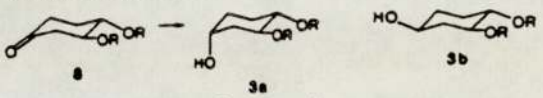
The stereochemistry of the oxygen substituents about the C ring of phorbols corresponds to that of the 1*R*,2*R*,4*S* isomer of cyclohexane-1,2,4-triol. The synthetic target chosen was the 1,2-dioctanoate derivative 3a in view of the superior pkC activating potency of 1,2-dioctanoylglycerol (DiC_8) as compared to other glycerol diesters.[8] However,

Scheme III[a]



[a](a) TBDMSCl, imidazole, DMF; (b) mcpba, CH_2Cl_2; (c) $Mo(CO)_6$, H_2O, dioxane; (d) octanoyl chloride, pyridine, $CHCl_3$; (e) TBAF, THF; (f) PCC, CH_2Cl_2; (g) $NaBH_4$, EtOH, or K-Selectride, THF.

Table I. Reduction of Ketone 8



8 → 3a + 3b

R = octanoyl

reducing agent	ratio 3a:3b[a]
$NaBH_4$	15:85
L-Selectride	92:8

[a]Determined by GLC.

all four diastereoisomers of this target (Scheme II) are of interest as a structure–activity relationship is being sought. All are potentially available from a suitably protected 4-cyclohexenol (4); the *tert*-butyldiphenylsilyl derivative 5 was chosen as it was hoped that the bulky protecting group would encourage diastereoselectivity in reactions at the double bond, despite its remoteness. The required cyclohexenol was synthesized in racemic form from the mixed cyclohexane-1,4-diols.[9] The chiral material is not available; however, it is known that, at least in the case of diacylglycerols, the presence of the unnatural isomer in assays does not affect the activity of the natural isomer;[10] therefore an enantiospecific synthesis was not attempted at this stage.

After silylation (Scheme III), epoxidation of 5 (mcpba) yielded the two epoxides 6a and 6b in a ratio of 11:5, respectively. The stereochemistry of the products, which were not separated, was determined from their ^1H NMR spectra and confirmed by the unambiguous synthesis of isomer 6b via the *tert*-butyl hydroperoxide/$Mo(CO)_6$ epoxidation of cyclohexenol 4.[11] No attempt was made to separate the epoxide isomers as it was expected that the hydrolysis of either would produce largely diol 7a. The preferred mode of trans-diaxial ring opening should be that which maintains the bulky silyloxy substituent in an equatorial conformation, i.e. attack at C-1 for isomer 6a and at C-2 for isomer 6b. Examination of the literature did, however, reveal a similar case where this appeared not

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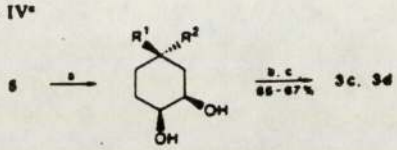
Scheme IV[a]



7c: R^1 = OTBDPS, R^2 = H (28%)
d: R^1 = H, R^2 = OTBDPS (36%)

[a] (a) OsO_4, *N*-methylmorpholine *N*-oxide, Me_2CO, H_2O; (b) octanoyl chloride, pyridine, $CHCl_3$; (c) TBAF, THF.

Table II. Growth-Inhibitory Properties and Cytotoxicities of TPA, Mezerein, Diacylglycerols, and Dioctanoylcyclohexanetriols

compd	growth inhibition: IC_{50},[a] μM	cytotoxicity: LC_{50},[b] μM
TPA	0.002 (0.0001, 0.0004)[c]	>1
OAG	70 (60, 81)	75 (67, 82)
DiC_8	44 (35, 53)	56 (42, 68)
3a	43 (27, 66)	36 (30, 41)
3b	58 (50, 69)	46 (43, 49)
3c	21 (17, 24)	36 (30, 41)
3d	49 (47, 53)	31 (9, 47)

[a] Concentration which caused half-maximal growth inhibition determined by cell counting. [b] Concentration which caused half-maximal LDH release. [c] Values were obtained by linear regression analysis of the linear portion of the concentration–response curve constructed with values measured at at least five concentrations in each of four separate experiments. Values in parentheses are the 90% confidence limits.

to have been the case.[12] Hydrolysis of the mixed epoxides yielded the two *trans*-diols 7a and 7b in a ratio of 9:2, respectively. The two isomers were inseparable by TLC, flash chromatography, or HPLC, and the stereochemistries were determined by ^1H NMR: the downfield shifts of protons involved in 1,3-diaxial interactions with oxygen functionalities proved particularly informative in this and other analyses.[13] Octanoylation of the mixed diols followed by deprotection yielded the two target isomers 3a and 3b, which were now readily separable by flash chromatography. Both isomers could be oxidized to give the same ketone 8. The results of the reduction of 8 with sodium borohydride and K-Selectride (Aldrich) provide further support for the original stereochemical analysis of the epoxide opening reaction (Table I).[14]

The second pair of target isomers were approached (Scheme IV) via the cis-hydroxylation of 5, which gave diols 7c and 7d in a ratio of 7:6 as established by HPLC. In this case the diols could be separated by careful flash chromatography. The relative stereochemistries of the products were again deduced by ^1H NMR. Dioctanoylation of 7c followed by desilylation gave target isomer 3c. Similar treatment of 7d led to the final target isomer 3d.

Biological Properties

The growth-inhibitory and the cytotoxic potentials of the cyclohexanetriol diesters were investigated in the human A549 lung carcinoma cell line and compared with those of TPA and the diacylglycerols OAG and DiC_8. The effect of the compounds on cell growth was assessed by cell counting (Table II) and by measurement of DNA synthesis after exposure to the test compounds (results not shown). Both methods furnished virtually identical IC_{50} values. Cytotoxicity was quantified by assaying the leakage of lactate dehydrogenase (LDH) from cells into the culture medium. Table II shows that, in accordance with a previous report,[7] TPA inhibited cell growth at concentrations below 1 nM, whereas cytotoxicity was only evident at micromolar concentrations. Neither the diacylglycerols nor any of the cyclohexanetriol esters inhibited cell growth at noncytotoxic concentrations. They also failed to elicit the change in cell morphology (result not shown) which accompanied phorbol ester induced growth inhibition.[7]

Studies involving the repeated addition of DiC_8 (100 μM) to cultures revealed that its cytotoxicity was dependent on cell density. At densities of 2×10^5 or 1×10^6 cells/mL LDH leakage was 56 ± 4% and 54 ± 14%, respectively (n = 4), of total enzyme release achieved by detergent, whereas at cellular concentrations of 0.5×10^6 or 9×10^5 cells/mL LDH release was decreased to 16 ± 18% and 12 ± 8%, respectively (n = 4).

The cytotoxicity of the cyclohexane triol diesters showed little variation.

Table III. Energetic Penalties Associated with the Fitting of the Cyclohexanetriol Diacetates to the Low-Energy Conformation of Phorbol Diacetate

compd	energy, kcal: unrestrained	fitted	difference
3a	58.7	63.4	4.7
3b	57.9	112.4	54.5
3c	68.6	79.8	11.2
3d	67.1	107.1	40.0

Computer-Assisted Modeling

In a preliminary assessment of the degree of resemblance between the cyclohexanetriol diesters and ring C of the phorbol ester molecule, the spatial relationship between the relevant oxygen atoms was investigated. Attempts were made to superimpose the O-1, O-2, O-4 oxygens of the cyclohexane-1,2,4-triol diesters over the O-13, O-12, and O-9 oxygens, respectively, of the phorbol ester molecule. Both chair conformations of each isomer were examined, and the goodness of fit, defined as the minimum mean separation between corresponding oxygen atoms in the two molecules, was determined (results not shown). Derivatives 3a and 3d exhibited the best fit, with a minimum mean separation between correlated atoms of 0.36 Å. In view of the biological results, a more detailed computer-aided conformational analysis of the diol diester portion of both the cyclohexanetriol diesters and TPA was undertaken. Analysis of the phorbol ester revealed a single low-energy conformation of the diester moiety. The atoms that are hypothesized as playing a key role in the hydrogen bonding of the molecule to the enzyme are the carbonyl oxygens O′ and O″ and the hydroxyl hydrogen OH-9 (see Scheme I). Each cyclohexanetriol diester isomer was minimized subject to the restraints that the separations between the corresponding atoms, O′, O″, and OH-4, lay within 0.1 Å of the values for the phorbol ester. Table III lists, for each conformation, the difference in energy between the conformation thus found and the unconstrained minimum-energy conformation. The conformational analysis of the phorbol ester revealed a particularly short distance (3.00 Å) between O′ and OH-9, and it seems possible that the 13-acetyl and C9-hydroxyl groups interact via hydrogen bonding. It is the difficulty that the cyclohexanetriol diesters have in adopting a conformation with a correspondingly short O′ to OH-4 separation that mainly accounts for the large energy differences between the constrained and unconstrained conformational minima that

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are observed, and particularly when O' and OH-4 are attached to opposite faces of the cyclohexane ring (isomers 3b and 3d).

Discussion

Recently two models have been advanced[15,16] which explain in terms of common structural features the resemblance in biological properties between TPA and other tumor promoters and pkC activators. Both models attempt to establish the relationship in space between various functional groups in these compounds. The guiding hypothesis in the chemical synthesis conducted in this project is based on the original proposal[3] that the vicinal diester moiety is a molecular feature crucial for the activity of both phorbol esters and diacylglycerols, and that the pharmacophore common to TPA and other activators of pkC contains two hydrogen bond acceptors (the diester carbonyl oxygens), one hydrogen bond donor (an alcoholic hydroxyl) and a hydrophobic region, suitably disposed.[8] The cyclohexane part of the TPA molecule (ring C) was chosen as the basic structural skeleton, and the four novel stereoisomeric 1,2,4-cyclohexanetriol diesters 3a–d were synthesized.

When tested in cultures of A549 carcinoma cells, the newly synthesized compounds were found to lack the growth-inhibitory properties of TPA at nontoxic concentrations. This result suggests that either the structural similarity with phorbol esters achieved in these compounds is inconsequential for activity or, alternatively, that the hypothesis which guided the design of the cyclohexanetriol diesters does not take important elements of the phorbol ester pharmacophore into account.

The latter alternative is a distinct possibility as shown by the analysis of the goodness of fit in which the orientation and position of the carbonyl groups were taken into consideration. This analysis revealed that considerable energetic penalties could result from the cyclohexanetriol diesters adopting conformations resembling those of phorbol esters, which could explain the lack of biological similarity. The cyclohexanetrol diesters share the lack of growth-inhibitory properties in A549 cells with the diacylglycerols OAG and DiC_8, which were used in this study as representatives of the physiological ligands and activators of pkC. It could be argued that the concentrations of diacylglycerols to which the cells were exposed in these experiments were too low to elicit a growth-inhibitory response, as diacylglycerols are known to undergo rapid metabolism in cells.[17,18] However this explanation seems unlikely as the diacylglycerols were added at 6 hourly intervals at concentrations in the 10^{-4} M range. At such concentrations diacylglycerols have been reported to stimulate pkC.[8,19] Furthermore in a preliminary experiment the ability of the diacylglycerol kinase inhibitor R 59022[20] to influence the effect of DiC_8 on A549 cell growth was studied. Inhibition of this enzyme should increase the biochemical stability of diacylglycerols. The presence of the inhibitor at concentrations as high as 45 μM did not render nontoxic but pkC-activating amounts of DiC_8 growth inhibitory. These results suggest that activation of pkC alone may not be sufficient to trigger growth arrest in A549 cells.

The concentrations at which diacylglycerols were cytotoxic against these cells were of an order of magnitude, which is tolerated by HL-60 myeloid leukemia cells.[17,21-23] The reason for this discrepancy in sensitivity might be associated with the relationship between cytotoxicity and cell density which was observed here. HL-60 cells are maintained in suspension cultures in which cell densities are obtained which far surpass those reached in cultures in cells grown, like those of the A549 line, as monolayers.

The cytotoxicities determined for the cyclohexanetriol diesters were similar to those of DiC_8 and OAG. It seems likely that the cytotoxicity caused by these compounds is the consequence of nonspecific cellular damage rather than receptor-mediated processes. That the cytotoxicity of these molecules is influenced by their lipophilicities is borne out by the finding that of two 1,4-cyclohexanediol monoesters synthesized for comparative purposes the tetradecanoyl derivative was significantly more cytotoxic than the octanoyl ester (result not shown). We are currently investigating if the cyclohexanetriol diesters are capable of binding to the phorbol ester receptor or of activating pkC. Preliminary results show that the cyclohexanetriol diesters at concentrations in the 10^{-6} M range compete with phorbol dibutyrate for phorbol ester binding sites on purified pkC. The affinity of the cyclohexanetriol diesters to the phorbol ester receptor appears to be 1 order of magnitude lower than that for dioctanoylglycerol (results not shown). Therefore the failure of the cyclohexane derivatives to arrest A549 cell growth at nontoxic concentrations may well be linked to their inability to activate pkC.

Experimental Section

Chemistry. Melting points were determined in open capillaries with an Electrothermal apparatus and are uncorrected. ^{1}H NMR spectra were recorded on Varian EM360A, Bruker WH300, and WH400 spectrometers. ^{13}C NMR spectra were recorded on the latter two instruments. IR spectra were recorded on a Perkin-Elmer 1310 spectrophotometer. Mass spectra were recorded on a VG Micromass 12 instrument. Elemental analyses were performed by Butterworth Laboratories Ltd., Middlesex (U.K.) and Elemental Microanalysis, Okehampton (U.K.). TLC was performed on fluorescent silica gel coated aluminum-backed plates (Merck Art. 5554); spots were visualized with UV light or by spraying with dodecamolybdophosphoric acid. Flash chromatography was performed with May and Baker Sorbisil C-60 flash silica and dry column chromatography[24] with Merck silica gel 60H (Art. 7736). HPLC was performed on a Waters instrument fitted with a Merck LiChrosorb Si 60 column using a solvent system of 5% methanol in chloroform and GLC on a Pye Unicam instrument fitted with a 2.5-m glass column packed with 3% SP2100 DOH on Supelcoport operating at 200 °C. Ether and THF were freshly distilled from sodium; ethyl acetate and hexanes (both HPLC grade) were used as received. Dichloromethane and chloroform were dried just prior to use by passage through a column of active basic alumina.

Dioctanoylglycerol (DiC_8). DiC_8 was prepared in racemic form according to standard literature procedures.[25,26]

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4-[(*tert*-Butyldiphenylsilyl)oxy]cyclohexene (5). *tert*-Butyldiphenylsilyl chloride (4.31 g, 16 mmol) was added to a solution under nitrogen of 4-cyclohexenol (4)[8] (1.4 g, 14 mmol) and imidazole (2.14 g, 31 mmol) in DMF (25 mL). After 24 h the solution was poured into water (200 mL) and extracted with 3:1 hexanes–ether (200 mL). After drying (Na_2SO_4) and evaporation of the solvent, dry column chromatography[26] (eluant 0–12% ether in hexanes) led to the isolation of **5** (4.83 g, ~100%) as an oil: NMR ($CDCl_3$) δ 1–2 (6 H, m), 1.05 (9 H, s, Me_3C), 3.6 (1 H, m, H-4), 5.5 (2 H, m, H-1, H-2), 7.3–7.7 (10 H, m, Ph_2Si); MS (EI, 70 eV), *m/z* 279 (M^+ – *t*-Bu). Anal. ($C_{22}H_{30}OSi$) C, H.

3-[(*tert*-Butyldiphenylsilyl)oxy]-7-oxabicyclo[4.1.0]heptanes 6a and 6a. To a solution of **5** (2.0 g, 5.9 mmol) in dichloromethane (40 mL) was added in portions 85% *m*-chloroperbenzoic acid (1.54 g, 7.6 mmol). After stirring for 18 h, the solution was washed with 1 M Na_2CO_3 and brine and dried (Na_2SO_4). After evaporation of the solvent, flash chromatography (20% ether in hexanes) led to the recovery of starting material **5** (170 mg, 8.5%) and isolation of the mixed epoxides **6a** and **6b** as an oil (1.34 g, 67% based on recovered starting material): NMR ($CDCl_3$) δ 1.03 and 1.05 (9 H, 2 s, ratio 5:11, Me_3C), 1.3–2.2 (6 H, m), 2.95 (~0.7 H, m, H-1, H-6, minor isomer), 3.1–3.2 (~1.4 H, m, H-1, H-6, major isomer), 3.55 (~0.3 H, m, H-3, minor isomer), 3.85 (~0.7 H, m, H-3, major isomer), 7.3–7.65 (10 H, m, Ph_2Si); MS (EI, 70 eV), *m/e* 295 (M^+ – *t*-Bu). Anal. ($C_{22}H_{30}O_2Si$) C, H.

4-*O*-(*tert*-Butyldiphenylsilyl)cyclohexane-1,2,4-triols 7a and 7b. A solution of the mixed epoxides **6a** and **6b** (1.28 g, 3.6 mmol) and $Mo(CO)_6$ (~5 mg, 0.02 mmol) in dioxane (12 mL) and water (1.5 mL) was heated at 95 °C for 24 h.[27] After evaporation of the solvents, the residue was taken up in ether, washed with brine, and dried (Na_2SO_4). HPLC analysis revealed a single peak (retention time 5.8 min). After evaporation of the solvent, flash chromatography (eluant 33% hexanes in ethyl acetate) gave the monoprotected triols **7a** and **7b** as an oil (1.27 g, 95%): NMR ($CDCl_3$) δ 1.03 and 1.05 (9 H, 2 s, ratio 2:9 Me_3C), 1.2–2.0 (6 H, m), 3.35 (~0.7 H, ddd, J = 11.5, 9, 4.5 Hz, H-1, major isomer), 3.6–3.7 (~0.6 H, m, H-1, H-2, minor isomer), 3.85 (~0.3 H, m, H-4, minor isomer), 3.95 (~0.7 H, ddd, J = 11.5, 9, 4.5 Hz, H-2, major isomer), 4.1 (~0.7 H, m, H-4, major isomer), 7.3–7.65 (10 H, m, Ph_2Si).

(±)-(1*R,2*R**,4*S**)-1,2-Di-*O*-octanoylcyclohexane-1,2,4-triol (3a) and the (±)-1*R**,2*R**,4*R** Isomer 3b.** Octanoyl chloride (1.67 g, 10 mmol) was added dropwise to a solution of the mixed monoprotected triols **7a** and **7b** (1.27 g, 3 mmol) in dry chloroform (30 mL) containing pyridine (1.08 g, 14 mmol). After 48 h the solution was washed with 1 M H_2SO_4, 1 M Na_2CO_3, and brine and dried (Na_2SO_4). After evaporation of the solvent, the residue was taken up in dry THF (20 mL) and tetrabutylammonium fluoride solution (1 M in THF; 6 mL, 6 mmol) was added. After 42 h the solvent was removed under reduced pressure, the residue partitioned between ether and water, and the organic layer dried (Na_2SO_4). After evaporation of the solvent, dry column chromatography[26] (hexane–ethyl acetate gradient) led to the isolation of the mixed diesters **3a** and **3b** (1.17 g, 89%). Flash chromatography (eluant 33% hexane in ether) led to the isolation of pure **3a** (770 mg, 58%) as a waxy solid and pure **3b** (140 mg, 11%) as an oil, in addition to mixed fractions (130 mg, 10%). Isomer **3a**: mp 36–38 °C (hexanes); NMR ($CDCl_3$) δ 0.85 (6 H, t, J = 7 Hz, 2 *Me*), 1.2–1.3 (16 H, m), 1.5–2.1 (11 H, m), 2.25 (4 H, m), 4.05 (1 H, m, H-4), 4.8 (1 H, ddd, J = 7.8, 7.8, 5.0 Hz, H-1), 5.15 (1 H, ddd, J = 7.8, 7.8, 4.2 Hz, H-2); MS (EI, 70 eV), *m/z* 367 (M^+ – OH). Anal. ($C_{22}H_{40}O_5$) C, H.

Isomer **3b**: NMR ($CDCl_3$) δ 0.85 (6 H, t, J = 7 Hz, 2 *Me*), 1.2–1.3 (16 H, m), 1.3–2.4 (m, 15 H), 3.8 (1 H, m, H-4), 4.85 (2 H, m, H-1, H-2); MS (EI, 70 eV), *m/z* 367 (M^+ – OH). Anal. ($C_{22}H_{40}O_5$) C, H.

Oxidation/Reduction of Alcohols 3a and 3b. To a mixture of alcohols **3a** and **3b** (ratio 6:1; 200 mg, 0.52 mmol) in dichloromethane (10 mL) were added first powdered 4-Å molecular sieve (300 mg) and then pyridinium chlorochromate (280 mg, 1.3 mmol). After 30 min the solution was diluted with ether (15 mL) and filtered through a pad of silica topped with Celite. Evaporation of the solvents afforded (±)-(3*R**,4*R**)-3,4-bis(octanoyloxy)cyclohexanone (**8**), which was used immediately in the reduction procedures: NMR ($CDCl_3$) δ 0.85 (6 H, t, J = 6.5 Hz), 1.2–1.3 (16 H, m), 1.5–1.7 (4 H, m), 2.05 (1 H, m), 2.1–2.6 (8 H, m), 2.8 (1 H, ddd, J = 15.3, 4.6, 1.1 Hz), 5.1 (1 H, ddd, J = 5.5, 5.5, 3.4 Hz), 5.25 (1 H, ddd, J = 5, 5, 5 Hz). Reductions were performed at room temperature with 3 equiv of reducing agent in ethanol ($NaBH_4$) or THF (K-Selectride). When the reactions were complete as judged by TLC, they were quenched with saturated NH_4Cl and extracted with ether. The ether layers were then analyzed by GLC (retention times: 73 min (**3b**), 77 min (**3a**)).

4-*O*-(*tert*-Butyldiphenylsilyl)cyclohexane-1,2,4-triols 7c and 7d. A solution of alkene **5** (780 mg, 2.32 mmol) in acetone (16 mL) was added to a solution of *N*-methylmorpholine *N*-oxide monohydrate (530 mg, 3.94 mmol) and OsO_4 (1 crystal) in 50% aqueous acetone (12 mL). After 18 h the acetone was removed by evaporation under reduced pressure and the residue partitioned between water and ether. HPLC analysis revealed two peaks in a ratio of 6:7 in order to elution (retention times: 4.1 and 5.0 min). After evaporation of the solvent, flash chromatography (eluant 5% MeOH in $CHCl_3$) led to the isolation of first diol isomer **7c** (240 mg, 28%) and then mixed fractions (150 mg, 18%) and finally isomer **7d** (310 mg, 36%), all as oils. Isomer **7c**: NMR ($CDCl_3$) δ 1.08 (s, 9 H, Me_3C), 1.25 (1 H, m), 1.5–1.65 (3 H, m), 1.95 (1 H, m), 2.2 (1 H, m), 2.45 (1 H, d, J = 9 Hz), 3.45 (1 H, m, H-4), 3.8 (1 H, br, *OH*), 3.85 (1 H, m, H-2), 4.0 (1 H, m, H-1), 7.3–7.7 (10 H, m, Ph_2Si); MS (EI, 70 eV), *m/e* 313 (M^+ – *t*-Bu). Anal. ($C_{22}H_{30}O_3Si$) C, H.

Isomer **7d**: NMR ($CDCl_3$) δ 1.05 (9 H, s, Me_3C), 1.4 (1 H, m), 1.6–2.0 (7 H, m), 3.85 (1 H, m, H-1), 4.1 (2 H, m, H-2, H-4), 7.3–7.7 (10 H, m, Ph_2Si); MS (EI, 70 eV), *m/e* 313 (M^+ – *t*-Bu). Anal. ($C_{22}H_{30}O_3Si$) C, H.

(±)-(1*R,2*S**,4*S**)-1,2-Di-*O*-octanoylcyclohexane-1,2,4-triol (3c).** To a stirred solution of diol **7c** (230 mg, 0.62 mmol) in dry $CHCl_3$ (4 mL) were added first pyridine (196 mg, 0.20 mL, 2.42 mmol) and then octanoyl chloride (305 mg, 0.32 mL, 1.86 mmol). After 8 h the solution was poured into water and extracted with hexanes–ether (3:1). After drying (Na_2SO_4), evaporation of the solvent, and flash chromatography (eluant 10% ether in hexanes), the protected diester was immediately taken up in dry THF (3 mL) and tetrabutylammonium fluoride solution (1 M in THF; 0.9 mL, 0.9 mmol) was added. After 18 h the solvent was evaporated; flash chromatography of the residue led to the isolation of diester **3c** (160 mg, 67%) as an oil: NMR ($CDCl_3$) δ 0.8 (6 H, t, J = 7 Hz, 2 Me), 1.0–2.4 (31 H, m), 3.7 (1 H, m, H-4), 4.8–5.2 (2 H, m, H-1, H-2); MS (EI, 70 eV), *m/z* 367 (M^+ – OH). Anal. ($C_{22}H_{40}O_5$) C, H.

(±)-(1*R,2*S**,4*R**)-1,2-Di-*O*-octanoylcyclohexane-1,2,4-triol (3d).** Treatment of diol **7d** (310 mg, 0.84 mmol), essentially as described for isomer **7c**, led to the isolation of diester **3d** (210 mg, 65%) as an oil: NMR ($CDCl_3$) δ 0.85 (6 H, t, J = 6.5 Hz, 2 Me), 1.2–1.3 (16 H, m), 1.4–2.1 (11 H, m), 2.3 (4 H, m), 4.1 (1 H, m, H-4), 5.0 (1 H, m, H-1), 5.25 (1 H, m, H-2); MS (EI, 70 eV), *m/z* 367 (M^+ – OH). Anal. ($C_{22}H_{40}O_5$) C, H.

Biochemistry. TPA, OAG, NADH, and sodium pyruvate were purchased from Sigma Chemical Co. Tissue culture reagents were purchased from Gibco Ltd. TPA and the diacylglycerols were stored at −20 °C. Stock solutions were prepared in dimethyl sulfoxide (in the case of DiC_8, OAG, and the racemic cyclohexanetriol diesters immediately prior to use). The final concentration of dimethyl sulfoxide in the culture medium did not exceed 0.5%. Dimethyl sulfoxide was added to control cultures and this concentration did not affect cell growth.

Cell Culture and Measurement of Cell Growth. Human-derived A549 lung carcinoma cells were obtained from the American Type Culture Collection and routinely cultured as described previously.[7] In the experiments in which the effect of compounds on cell growth was assessed cells ((2–5) × 10^4/well) were seeded in six-well multidishes (35-mm diameter, Nunclon U.K.) and incubated with 3 mL of Nutrient Hams F12 medium supplemented with 10% fetal calf serum and different concentrations of the test compound in a humidified incubator gassed with 95% air/5% CO_2. Cells and drugs were incubated for 4–5 days after which control cultures reached confluence. Medium and test compounds were replaced every 2 days. DiC_8 or OAG was added

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three times daily at 6 hourly intervals for 3 days. At the end of the incubation period, cells were counted with either a hemocytometer or a Coulter Counter, Model ZM, after detachment of cells from the dish by short incubation with a solution of trypsin (0.1%) in versene. The IC_{50} values shown in Table II are the concentrations of test compounds which inhibited cell growth by 50%.

Cytotoxicity Assay. Cells (1×10^6/well) were incubated for 24 h with the test compound and with medium supplemented with only 1% fetal calf serum, as larger serum concentrations interfered with the LDH assay. Diacylglycerols were added 3 times at 6 hourly intervals. The supernatant was removed and briefly centrifuged at 1000 rpm at the end of the incubation or when it was replaced with fresh medium and agent. The supernatant was kept on ice until assayed. The activity of LDH in the cell supernatant was measured spectrophotometrically as described by Leathwood and Plummer[28] with a Beckman DU-7 spectrophotometer. The amount of maximally releasable LDH was measured in the supernatant of control cells lysed by 1% Triton X-100 immediately before the assay. The LC_{50} values shown in Table II are the concentrations of test compounds which caused 50% of the maximal LDH leakage.

Computer-Assisted Modeling. Molecular modeling was performed on a DEC8650 processor using the CHEM-X graphics package developed and distributed by Chemical Design Ltd., Oxford, U.K. The coordinates for phorbol were obtained from the published crystal structure;[29] the acetyl group coordinates and those for cyclohexane were obtained from the CHEM-X fragments database. Structural modification and conformational analysis was performed by using the supplied routines. Briefly, the energy of each molecule as a function of rotations about each carbon–oxygen single bond of the diester moiety was calculated and minima in this (4-dimensional) conformational space were located. Each was then subjected to a full molecular mechanics minimization, subject to interatom distance restraints if required, in order to determine the lowest energy conformation. For the cyclohexanetriol diesters, both chair conformations of the ring were considered separately as start points.

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Registry No. 3a, 117918-47-5; 3b, 117918-48-6; 3c, 117918-52-2; 3d, 117918-53-3; 4, 72137-22-5; 5, 117918-43-1; 6a, 117918-44-2; 6b, 118013-64-2; 7a, 117918-45-3; 7b, 117918-46-4; 7c, 117918-50-0; 7d, 117918-51-1; 8, 117918-49-7; $CH_3(CH_2)_6COCl$, 111-64-8; phorbol, 17673-25-5.

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those of Hultmark *et al* [2]: PB increases DPNA metabolism 9 times, when calculated per mg protein, and 3 times, when calculated per nmol P-450. But in mice, protein-related increase after PB is only 2 times (C57BL/6J) and 3 times (NMRI), and there is no P-450-related increase at all. That means, although PB did augment the total amount of cytochrome P-450, no isoenzyme(s) specific for DPNA-demethylation have been formed. Or: as there is no increase of DPNA-specific activity, DPNA is not a specific substrate for PB in mice.

TCPOBOP even decreases DPNA specific activity of cytochrome P-450 (Table 1b) in guinea-pigs. PCN decreases it in mice and rats. BNF decreases it in C57BL/6J/6J- but not in NMRI-mice, and seems to decrease it in rats and guinea-pigs also.

From these results may be drawn the following conclusions. (1) In mice, DPNA is not the prominent marker of PB induction as it is in rats. (2) Although TCPOBOP has been described as PB-like inducer [4] its effect on DPNA metabolism is clearly different from that of PB in C57BL/6J mice and rats. (3) DPNA may be called an atypical substrate, because, in C57BL/6J mice, DPNA-specific activity per cytochrome P-450 is decreased by as different inducers as TCPOBOP ("PB-like"), PCN and BNF ("3-methylcholanthrene-like").

‡ To whom reprint requests should be addressed.

*Institut für Pharmakologie und Toxikologie der Philipps-Universität
and
†Fachbereich Chemie der Philipps-Universität
Lahnberge
3550 Marburg
Federal Republic of Germany

FRIEDRICH HEUBEL*†‡
ERNST GERSTNER†
HELGA GROTJAHN*

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Effect of two inducers of cellular differentiation on the glutathione status of human HL-60 promyelocytic leukaemia and A549 lung carcinoma cells

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Human HL-60 promyelocytic leukaemia cells, undergo differentiation to mature granulocytes when they are incubated with polar solvents such as dimethylsulfoxide (DMSO), dimethylformamide (DMF) or *N*-methylformamide (NMF) at concentrations in the 10^{-1} M range [1]. Exposure to nM concentrations of the tumour promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) promotes maturation to a macrophagic monocytic phenotype [2]. In other cell lines TPA causes growth inhibition apparently without inducing differentiation [3]. This has been observed in, for example, A549 lung carcinoma [4] and A431 epidermoid carcinoma cells [5].

It has been suggested that the induction of differentiation of poorly differentiated tumours by compounds which lack cytotoxic properties may be worthy of investigation as a therapeutic strategy [6, 7]. The biochemical events which trigger chemically-induced cell differentiation *in vitro* are not known. It has been shown recently that DMF and NMF deplete glutathione stores in DLD-1 clone A human colon carcinoma cells without causing toxicity [8, 9]. This effect was observed at concentrations of the solvents which induced the expression of a more benign phenotype. The authors suggested that glutathione may play a role in regulating the growth of these cells. This suggestion has been investigated further in the work described here. In particular, the hypothesis has been tested that glutathione depletion is involved in the mechanism by which agents such as NMF or TPA cause HL-60 cells to differentiate. Glutathione levels have been measured in HL-60 cells before and after chemically-induced maturation. For comparison the effect of NMF and TPA on the glutathione status of A549 cells has been investigated and interpreted in the light of the ability of these compounds to interfere with the growth of A549 cells.

Materials and methods

Materials. TPA was purchased from Sigma Chemical Co. (U.K.), NMF and DMSO from Aldrich Chemical Co. (U.K.); HL-60 cells were obtained from Dr G. Brown, Birmingham University (U.K.) and A549 cells from the American Type Culture Collection (U.S.A.). Cell culture media were purchased from Gibco (U.K.).

Cell culture. HL-60 cells were grown in RPMI 1640 medium with 10% foetal calf serum, A549 cells in Nutrient Hams F12 medium with 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (10 μg/ml). HL-60 cells were routinely maintained in logarithmic phase growth between 2×10^4 and $1 \times 10^6 \times ml^{-1}$ by biweekly subculture. A549 cells were subcultured every 5–7 days and the medium was renewed every 2–3 days. All cells were maintained in an incubator at 37° with 5% CO_2. Whereas HL-60 cells grow in suspension, A549 cells adhere to the surface of the culture flask and had to be detached with trypsin (0.1%). Incubations were initiated with either $0.5–1 \times 10^5$ HL-60 cell $\times ml^{-1}$ or 0.5×10^5 A549 cells/flask and included either NMF 180 mM, DMSO 180 mM or TPA 5×10^{-9} M. These concentrations were found to induce maximum differentiation in HL-60 cells. Cells were counted with a haemocytometer or a Coulter Counter; cell viability was assessed by their ability to exclude trypan blue.

Assays for differentiation and glutathione. Functional differentiation of HL-60 cells to macrophagic cell was measured by staining cells for the presence of non-specific esterases [10]; differentiation to granulocytes was assessed by nitro blue tetrazolium reduction [11]. Total glutathione levels (GSH + GSSG) and levels of oxidised glutathione (GSSG) were determined according to Griffith [12].

Results and discussion

Incubation with TPA at nM concentrations causes differentiation in HL-60 cells [2] and growth inhibition in A549 cells [4]. Figure 1 shows that exposure of these cells to 5 nM TPA for 96 hr did not lead to a change in intracellular glutathione levels. In these experiments TPA induced 80–

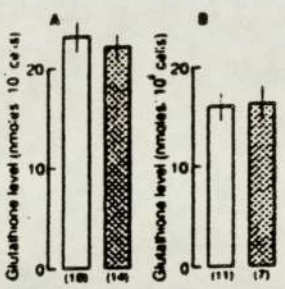

Fig. 1. Glutathione levels in HL-60 cells (A) or A549 cells (B) exposed to TPA (5×10^{-9} M) for 4 days. Values are the mean ± SE, number of determinations in brackets.

85% of the HL-60 cells to differentiate as measured by the presence of non-specific esterases. The ratio of GSSG to GSH concentrations, which was between 0.1 and 0.2 in control cells, was not affected by TPA. Incubation with TPA for periods of above 5 days rendered A549 cells refractory towards the growth inhibitory action of TPA [4] and also under these conditions, glutathione levels did not deviate from those in control cells (results not shown).

Incubation of HL-60 cells for 96 hr with 180 mM NMF induced 80–85% of the cells to adopt granulocyte-like properties as assessed by superoxide formation. This cellular maturation was not accompanied by a change in glutathione levels (Fig. 2). A similar result was obtained when HL-60 cells were exposed to 180 mM DMSO (result not shown). These results are in accordance with the recent finding [13] that glutathione levels in HL-60 cells after 72 hr in a medium containing 60 mM DMF, were unchanged from control levels even though after incubation with DMF for 7

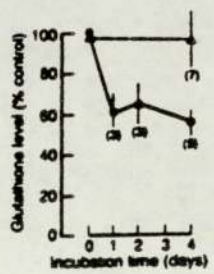

Fig. 2. Glutathione levels in HL-60 cells (▲) or A549 cells (●) exposed to NMF (180 mM). Values are the mean ± SE, number of experiments in brackets. The levels in A549 cells were significantly reduced from control levels ($P < 0.05$, paired t-test).

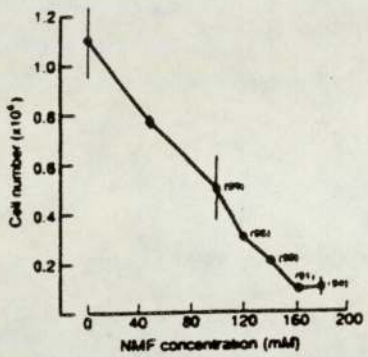

Fig. 3. Influence of NMF on the growth of A549 cells. Cells (0.5×10^5/flask) were seeded and counted after 4 days. Numbers in brackets are % of cells which excluded trypan blue. Points are the mean of two or the mean ± SE of three experiments.

days glutathione levels were reduced by 35%. In contrast, exposure of DLD-1 clone A colon carcinoma cells to 170 mM NMF [8] or of murine TLX5 lymphoma cells to 106 mM NMF for 96 hr [14] depleted intracellular glutathione stores by almost 90%. At these concentrations, NMF inhibited cell growth. In the case of the TLX5 cells, 106 mM NMF was cytostatic, but this does not appear to be as a consequence of a change of state of maturation of the cellular phenotype [14]. Likewise, A549 cells stopped growing when incubated with NMF (180 mM) for 96 hr (Fig. 3). This may well also be a cytostatic rather than a cytotoxic effect of NMF as exposure of confluent A549 cells to NMF for 2 days resulted in the detachment of only 1.5% of cells from the plastic surface, compared to 0.3% in incubations omitting NMF. The cytostatic effect of NMF was accompanied by a 45% fall in intracellular glutathione concentrations (Fig. 2). Even though this value constitutes a significant glutathione depletion it does not resemble the dramatic emptying of glutathione stores which NMF caused in DLD colon carcinoma [8] or TLX5 lymphoma cells [14].

When the evidence is summarised to relate changes in intracellular glutathione status to the mechanism by which compounds such as NMF cause induction of differentiation or growth inhibition, a complex picture emerges (Table 1): NMF inhibits growth of the four cell lines which have been studied under comparable conditions. This growth arrest appears to be the result of the induction of differentiation in HL-60 leukaemia cells and of maturation in DLD-1 colon carcinoma cells. There is no evidence that the state of differentiation is altered by NMF in the other cell lines. A dramatic depletion of glutathione pools which accompanies growth arrest has only been observed in DLD-1 and TLX5 cells. Growth inhibition caused by TPA does not appear to

Table 1. Effect of NMF on growth and glutathione levels of four tumour cell lines *in vitro*

Cell type	Growth inhibition	Induction of differentiation or maturation	Glutathione depletion
DLD-1 colon carcinoma [8]	+	+	+
HL-60 leukaemia	+	+	−
A549 lung carcinoma	+	?	(+)
TLX5 lymphoma [14]	+	−	+

+, effect; (+), weak effect; −, no effect.

affect glutathione levels. The results presented in this paper show that there is no clear mechanistic link between chemically-induced growth arrest and changes in glutathione status. Therefore caution should be exercised when attempts are made to extrapolate findings concerning the relationship between mechanisms of cell growth modulation and glutathione status from one cell type to others.

MRC Mechanisms of Drug Resistance Research Group and MRC Experimental Chemotherapy Research Group
Pharmaceutical Sciences Institute
Aston University, Birmingham B4 7ET, U.K.

CHARLES M. LAZENBY
ANDREAS GESCHER*
IAN L. DALE

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* Author to whom correspondence should be sent.