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Endogenous Alkylglycerol Functions As a Mediator of Protein Kinase C Activity and Cell Proliferation

Fritz G. Buchanan
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ENDOGENOUS ALKYLGLYCEROL FUNCTIONS AS A MEDIATOR OF
PROTEIN KINASE C ACTIVITY AND CELL PROLIFERATION

A Dissertation
Presented to
the Faculty of the Department of Biochemistry and Molecular Biology
James H. Quillen College of Medicine
East Tennessee State University

In Partial Fulfilment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Science
with Emphasis in Biochemistry

by
F. Gregory Buchanan

May 1997
APPROVAL

This is to certify that the Graduate Committee of

F. Gregory Buchanan

met on the

Twenty-seventh day of March, 1997

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

Chair, Graduate Committee

Signed on behalf of the Graduate Council

Interim Dean, School of Graduate Studies
ABSTRACT

ENDOGENOUS ALKYLGLYCEROL FUNCTIONS AS A MEDIATOR OF

PROTEIN KINASE C ACTIVITY AND CELL PROLIFERATION

by

F. Gregory Buchanan

There is an increasing interest in the role of lipids as mediators in the regulation of many cellular activities including the control of cell proliferation. Synthetic ether linked glycerolipids such as 1-O-alkyl-sn-glycerol (alkylglycerol) have been shown to possess many biological properties. Alkylglycerol itself has been regarded only as a breakdown product of more complex ether linked lipids, typically platelet activating factor. Recently, we observed that the level of free alkylglycerol increased over 20 fold during the culture of MDCK (Madin-Darby canine kidney) cells to a contact inhibited growth state. A loss of protein kinase C (PKC) activity has also been associated with growth arrested cells. Furthermore, we have found that treatment of MDCK cells with 1-O-dodecyl-rac-glycerol inhibits the TPA induced release of arachidonic acid. To explore the possibility that alkylglycerol may serve a regulatory role in the control of cell proliferation or PKC activity, we examined the ability of alkylglycerol to influence PKC activity and subcellular distribution as well as the ability of alkylglycerol to effect cell proliferation.

MDCK cells grown to confluence show a loss of PKC activity associated with the membrane, as reported in fibroblasts. Preconfluent cultures of MDCK cells have a high level of PKC activity associated with the membrane. However, treatment of preconfluent cultures with alkylglycerol causes a reduction of PKC activity. A similar inhibition was observed with alkylglycerol when cells were treated with TPA, an activator of PKC. To confirm that alkylglycerol was exerting an effect directly on PKC, alkylglycerol was shown to inhibit PKC activity \textit{in vitro} in a dose dependent manner. Since PKC exists as a family of closely related isoymes, we have determined the effects of growth arrest and alkylglycerol treatment on PKC $\alpha$, $\epsilon$, and $\zeta$ (expressed in MDCK cells). The
active forms of PKC α and ε are lost early in the growth of MDCK cells during the endogenous accumulation of alkylglycerol and synthetic alkylglycerol inhibits the membrane form of PKC α and ε. However, alkylglycerol inhibits the TPA induced translocation of PKC α but not ε suggesting a differential inhibition among these isoforms. Neither TPA or alkylglycerol had any effects on the distribution of PKC ζ.

To examine the effect of alkylglycerol on cell proliferation, Swiss 3T3 cells were used. GLC analysis shows that 3T3 cells accumulate alkylglycerol in a similar manner as MDCK cells. Since this accumulation occurs just prior to cell growth arrest, the effects of alkylglycerol on preconfluent cells was observed. Preconfluent cultures of 3T3 cells were treated with alkylglycerol on day 1 of growth. After 8 days of culture, the treated group showed a slower growth rate and saturation density. Furthermore, after these cells were reseeded in the absence of alkylglycerol, the original growth rate and saturation density returned. Thus alkylglycerol induces a decrease in cell proliferation without causing any detrimental effects. Similarly, alkylglycerol was found to inhibit the induction of mitogenesis by TPA (a PKC dependent pathway) and these effects were shown not to be stereospecific.

To further investigate the effect of alkylglycerol on cell proliferation, the content of the monoglycerides in ras-transformed cells was analyzed. These cells have lost contact dependent growth arrest indicating a disruption of cell growth regulation. We observed a massive increase in the content of alkylglycerol during the culture of ras transformed cells. This increase is 3 fold higher than MDCK or 3T3 cells. This raises the possibility that alkylglycerol may be the end result of an increase number of cell-cell contacts.

We have observed an increase in the accumulation of alkylglycerol in normal and ras-transformed cells. This accumulation is accompanied by a decrease in PKC activity and alkylglycerol was shown to be a potent in vitro inhibitor of PKC. Similarly, alkylglycerol was shown to inhibit PKC α under stimulation by TPA. Alkylglycerol is an inhibitor of the TPA induced induction of mitogenesis and slows the growth rate of proliferating cultures of 3T3 cells. These results indicate that the endogenous ether-linked glycerolipid, alkylglycerol, is a regulator of cell proliferation through its inhibitory effects on protein kinase C.
DEDICATION

This is dedicated with love and appreciation to my wife, Melissa, and my daughter, Paige. Their love and understanding has made this endeavor possible. Also, this is dedicated to my parents, Jim and Marie. Their words of encouragement and support throughout my life have been a continual source of motivation. Thank you.
ACKNOWLEDGEMENTS

I would like to acknowledge the many people who have made the completion of this degree a possibility. First, I would like to thank the members of my committee, Drs. Jill Suttles, Scott Champney, Don Hoover, and John Kalbfleish. Taking the time from a normally busy schedule to serve on my committee and the advise they have given is greatly appreciated.

I would also like to thank my advisor Dr. Mitch Robinson. I know that I could not have chosen a better advisor or project to work with. As in any normal student-advisor relationship there are peaks and valleys. I feel blessed that the majority of time which I spent here was on high ground. I have enjoyed our working relationship and willingness to discuss other important topics besides lipid biochemistry, such as college sports. His advisement has prepared me for a successful future.

I would like to thank Karen and Ray for all the many things they do for graduate students. The openness in which they conduct themselves makes the initiation into a new environment much more enjoyable. They deserve a raise for all their work which often goes unnoticed.

Finally, I would like to thank several students and co-workers for their friendship and support. To Steven, thank you for listening and being a friend. I am not sure if telling you my problems or listening to yours was more helpful. To Charlie, thanks for the intellectual discussions and the occasional basketball game. To Bob and Jon, thanks for your advise and help while trying to learn cell signaling, also, hit ‘em straight. To all I wish you well and all the best be yours.
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CS</td>
<td>Calf Serum</td>
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<tr>
<td>DG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DPM</td>
<td>Disintegrations per minute</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(6-aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas liquid chromatography</td>
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<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<td>IP₃</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin Darby canine kidney</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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**PIP₂**  Phosphatidylinositol 4,5-bisphosphate

**PKC**  Protein kinase C

**PS**  Phosphatidylserine

**TLC**  Thin layer chromatography

**TPA**  12-O-tetradecanoylphorbol 13-acetate
CHAPTER 1

INTRODUCTION

An increasing number of cellular lipids are now known to participate in several aspects of cell regulation, including the control of cell proliferation and apoptosis. The sphingolipids have received much of the current interest in lipids as intracellular messengers. The sphingolipids include such mediators as ceramide, sphingosine, and sphingosine-1-phosphate. Research indicates that ceramide is involved in the regulation of cell proliferation, differentiation, and programmed cell death (Jayadev et al., 1995; Obeid et al., 1993; Sasaki et al., 1995; Riboni et al., 1995). Sphingosine and sphingosine-1-phosphate have been shown to inhibit cell proliferation and effect the growth and invasiveness of human breast neoplastic cells (Hannun and Linardic, 1993; Zhang et al., 1991; Spiegel et al., 1994). Other lipids such as lyso-phosphatidic acid and diacylglycerol have been found to act as potent mitogenic signals (Jalink et al., 1994; Fukami and Takenawa, 1992; Stoeck et al., 1992; Banfic et al., 1993). These compounds are a few of the many lipids which act as mediators of inter- and intra-cellular signaling.

Several of the bioactive lipids are glycerol linked compounds with an ether linkage at the sn-1 position. These ether linked lipids range in structure
from the complex (platelet activating factor) to the simplified (monoalkylglycerol). Regardless of complexity, the ether lipids possess many diverse biological activities. Platelet activating factor has been shown to stimulate mitogenesis and interleukin expression in fibroblasts, migration of endothelial cells, and platelet aggregation (Roth et al., 1996; Camussi et al., 1995; Snyder et al., 1989). Alkylacylglycerol stimulates protein kinase C activity (Ford et al., 1989; Cabot and Jaken, 1984) and other ether lipids show antineoplastic properties (Vagnetti et al., 1990; Ngwenya et al., 1992; Principe et al., 1992). The ether linked glycerolipids comprise a small but important group of diverse biologically active lipids.

Synthetic, ether linked alkylglycerols have been shown to possess many biological properties. 1-O-dodecylglycerol has been shown to induce neuronal differentiation (Ved et al., 1991), stimulation of antibody production in mice (Ngwenya and Foster, 1991), macrophage activation (Yamamoto et al., 1988), and antitumor activity (Ando et al., 1972). The structure of unsubstituted alkylglycerol is shown in Figure 1. The structure of alkylglycerol closely resembles that of the known activators of protein kinase C, 1,2-diacyl-sn-glycerol and 1-alkyl-2-acyl-sn-glycerol. Alkylglycerol forms the backbone of biologically active compounds such as platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycerophosphocholine). Alkylglycerol has been regarded primarily as
1,2-diacyl-sn-glycerol 1-alkyl-2-acyl-sn-glycerol
diglycerides

1-acyl-sn-glycerol 1-alkyl-sn-glycerol
monoglycerides

Figure 1. Chemical Structures of Monoglycerides and Diglycerides of Interest.
a product of the inflammatory degradation of complex ether lipids rather than a
constitutive component of normal cells (Yamamoto et al., 1988; Yamamoto and
Ngwenya, 1987). However, recently in our laboratory a substantial amount of
the free compound was detected in unstimulated cultured MDCK cells (Warne et
al., 1995). MDCK or Madin Darby canine kidney cells are an epithelial cell
line derived from a cocker spaniel kidney in 1958 by S. H. Madin and N. B.
Darby. Figure 2 shows the content of free monoglyceride during the culture
of MDCK cells from preconfluent proliferating cells to a confluent contact-
inhibited monolayer. During growth, the level of free alkylglycerol increases
over 20 fold and the level of the monoacylglycerides declines throughout growth
(Warne et al., 1995).

McNeely et al. (Mcneely et al., 1991) has observed that 1-O-
alcoholglycerol, derived from the hydrolysis products of the lipophosphoglycan
(LPG) of Leishmania donovani, inhibited isolated rat brain PKC in vitro. In this
study they observed that LPG itself inhibited PKC activity. To determine which
elements of the compound caused this effect they fragmented the LPG molecule
by mild acid or phosphatidylinositol-specific phospholipase C (PI-PLC). The
mild acid treatment generated phosphorylated disaccharides and
phosphosaccharidyl phosphatidylinositol. PI-PLC treatment generated free 1-O-
alcoholglycerol and a phosphoglycan compound (PG). These four products were
Figure 2. Content of Monoacylglycerol and Monoalkylglycerol During the Growth of MDCK Cells. MDCK cells were seeded at a density of 1x10⁶ cells/cm². Cell lipids were extracted and analyzed by HPLC to determine the content of acylglycerol (●--) and alkylglycerol (□--) relative to the content of cell cholesterol. Cell density (............) was determined by trypsinitizing parallel cultures. The results are from a single experiment and are representative of three separate experiments.
assayed for their ability to inhibit PKC activity \textit{in vitro}. The phosphorylated disaccharides showed no inhibition and the other fragments inhibited PKC activity in the following increasing order; PG < LPG < phosphosaccharidyl phosphatidylinositol < 1-O-alkylglycerol. The 1-O-alkylglycerol compound was comprised primarily of a saturated, unbranched C$_{24}$ hydrocarbon. This work indicated that alkylglycerol may function as a regulator of PKC activity.

Protein kinase C consists of a family of closely related isoforms that have been shown to participate in various signal transduction pathways (Majumdar et al., 1993; Hug and Sarre, 1993; Müller et al., 1995; Haller et al., 1994). The classical pathway for PKC activation involves the hydrolysis of phosphatidylinositol 4,5-bisphosphate (P$_{i}$P$_{5}$). This produces diacylglycerol and inositol 1,4,5-trisphosphate (IP$_{3}$). An increase in IP$_{3}$ causes an influx of calcium from internal stores. Thus PKC is dependent upon lipid and calcium for activation. PKC exists in two forms \textit{in vivo}. The cytosolic form has a closed conformation and is considered to be inactive. The membrane associated form is in an open conformation, which exposes the active site, and is considered to be active.

The isoforms of PKC have been grouped into three classes. The classical PKC isoforms $\alpha$, $\beta$I, $\beta$II, and $\gamma$ require diacylglycerol, Ca$^{2+}$, and phosphatidylserine for activation. The novel isoforms $\epsilon$, $\delta$, $\eta$, and $\theta$ lack the calcium binding C2 domain and are therefore Ca$^{2+}$ independent. The atypical
isoform $\zeta$ acts independently of both lipid and Ca$^{2+}$ (Nishizuka, 1988; Kikkawa et al., 1989; Dekker and Parker, 1994). Recent research has investigated the localization of the isoforms in NIH 3T3 fibroblasts and cardiac myocytes through immuno-fluorescence (Wan et al., 1996; Strulovici et al., 1991).

Without activation most isoforms are found diffuse throughout the cytoplasm. In general, upon TPA induced activation, PKC $\beta$II tends to associate with the cytoskeleton, PKC $\delta$ and $\epsilon$ associate with the perinuclear region, PKC $\gamma$, $\delta$, and $\eta$ translocate to the Golgi, and PKC $\alpha$ and $\beta$I with small quantities of the others translocate to the plasma membrane (Goodnight et al., 1995). However their distribution varies depending on the cell type and other conditions. PKC $\zeta$ does not translocate in the presence of TPA (Walsh et al., 1989; Assender et al., 1994; Disatnik et al., 1994). This divergence in activation and localization suggests that each isoform of the PKC family plays a unique role in various cell functions. PKC$\alpha$ has been shown to regulate proliferation in glioblastomas and other cell lines (Ahmed et al., 1994; Jorgensen et al., 1996), while PKC $\epsilon$ is involved in antiviral resistance (Pfeffer et al., 1991), hormone secretion (Akita et al., 1994), and cell attachment of HeLa cells to gelatin (Chun et al., 1996).

Overexpression of PKC $\epsilon$ leads to oncogenic transformation in rat epithelial cells (Perletti et al., 1996).

The classical endogenous activators of PKC are diacylglycerol and
calcium. Phosphatidylserine lowers the requirement for calcium. Phorbol esters such as 12-O-tetradecanoylphorbol 13-acetate (TPA) are potent exogenous activators of PKC in vivo and in vitro (Chen, 1993; Terzian and Rubin, 1993). Other endogenous lipids that may serve to regulate the activity of PKC include the sphingolipids such as sphingosine and sphingosine-1-phosphate. These sphingolipid compounds have an inhibitory effect on PKC activity.

Protein kinase C is considered to play an important role in the regulation of cell proliferation. Therefore, this isozyme family is a potential target for the regulation of growth in density dependent cells. The inhibition of cell proliferation at a high cell density is characteristic of normal cultured cells. This effect is absent in many tumor cell lines and is a distinguishing factor in these cells. Several factors may contribute to the negative control of cell proliferation such as the formation of cell to cell contacts, removal of growth factors, and the release of growth inhibitors. This gives rise to negative signals which in turn inhibit signal transducing pathways including ras G-proteins and PKC. In Swiss 3T3 fibroblasts that are growth arrested by the removal of serum, activation of PKC induces these cells to exit the G1 phase and enter the S phase of the cell cycle. Exposure of fibroblasts to TPA, an activator of PKC activity, induces the cells to proliferate although they are in a contact-inhibited growth state (Diomede et al., 1993). Conversely, the addition of PKC inhibitors suppresses cell cycle
progression (Miyamoto et al., 1993; Sasaguri et al., 1993; Akinaga et al., 1994; Hofmann et al., 1994) and block proliferation in response to mitogens (Chiarugi et al., 1990). Furthermore, during the early stages of growth in fibroblasts (Halsey et al., 1987), PKC has been identified in its active form and shifts toward an inactive state as the cells grow to confluence.

Protein kinase C possesses an important role in the regulation of cell cycle progression. Other enzyme families have also been implied to serve some regulatory function in cell growth. These include the phospholipases, G-coupled receptors, and small G-proteins such as ras. Recently, ras has received increased attention in order to discern its general function and its role in the regulation of cell proliferation. The ability to study the function of ras in vivo, has utilized ras transformed cells (Quilliam et al., 1990; Behrend and Chambers, 1994). NIH 3T3 ras transformed cells have been transfected with a stable insert which contains a constitutively active form of ras. Many biological observations have been made with these cells. These include the absence of contact dependent growth regulation, the localization of PKC α primarily in the cytosolic inactive form, and the inability of TPA to translocate PKC to the membrane (Haliotis et al., 1990; Borner et al., 1992; Mills et al., 1992; Weyman et al., 1988). These properties indicate that ras is a likely candidate as a mediator in the control of cell proliferation.
Current investigators have identified several lipids that may serve to regulate these important enzyme families. The research of our laboratory has focused on the ether linked glycerolipids, namely alkylglycerol. The initial observation which showed the accumulation of alkylglycerol during the growth of contact-inhibited cells has sparked interest in determining the biological activity of alkylglycerol. Our preliminary studies have examined the effect of alkylglycerol on the TPA induced release of arachidonic acid from MDCK cells. We have observed that this TPA mediated response is inhibited in the presence of alkylglycerol (Warne et al., 1995). These results indicate that alkylglycerol may exert an inhibitory effect on PKC activity in vivo.

The studies in this dissertation were designed to examine the biologic role of alkylglycerol in cultured cells. We examined if alkylglycerol affects the distribution of PKC during the culture of MDCK cells. Similarly, we determined if alkylglycerol acts as a negative regulator of protein kinase C activity in vivo and in vitro. The effect of alkylglycerol on the in vivo distribution of the PKC isoforms during the growth of MDCK cells was determined. Also, we examined the effect of alkylglycerol on the distribution of the PKC isoforms during exposure to phorbol ester and examine if this effect occurs in a dose dependent manner. Due to the accumulation of alkylglycerol during contact dependent growth arrest, the ability of alkylglycerol
to effect the induction of mitogenesis was examined in Swiss 3T3 fibroblasts. Similarly, the effect of alkylglycerol on the proliferation of 3T3 cells was determined. We tested any toxic properties of alkylglycerol to assure that the effects observed are not the result of cytotoxicity. The concentration of alkylglycerol during the culture of Swiss 3T3 fibroblasts was determined and compared to that of MDCK cells. Furthermore, the levels of alkylglycerol during the growth of ras-transformed NIH 3T3 cells was examined in order to compare the concentrations present in untransformed cells to ras-transformed cells. This study determines the role of endogenous alkylglycerol as a regulator of cell proliferation through effects on protein kinase C.
CHAPTER 2
MATERIALS AND METHODS

Materials

Madin Darby Canine Kidney Cells (MDCK) and Swiss 3T3 fibroblasts were purchased from American Type Culture Collection. NIH 3T3 ras-transformed fibroblasts (H-ras, K-ras, N-ras, and pZIP-empty vector control) were a kind gift from Dr. Adrienne Cox, Department of Pharmacology, University of North Carolina, Chapel Hill. Purified rat brain protein kinase C (PKC) was purchased from Calbiochem. Broad range biotinylated molecular weight markers, nitrocellulose membranes, and Bio-Rad’s Protein Assay Dye Reagent were purchased from Bio-Rad. Bicinchoninic Acid (BCA) Protein Assay Reagent was from Pierce. Enhanced Chemiluminescence (ECL) detection reagent and Streptavidin-HRP (horse radish peroxidase) were purchased from Amersham Life Science. Antibodies specific for PKC α (270-427), β (126-324), ε (1-175), and anti-mouse IgG-HRP were purchased from Transduction Laboratories. Antibodies specific for PKC δ, ζ, and anti-rabbit IgG-HRP and the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit were from Promega. Antibodies for PKC β and βI-βII were also purchased from Promega and Sigma, respectively. Protogel acrylamide solution was purchased through Midwest
Dulbecco's modified eagle's medium (DMEM), fetal calf serum (FCS), calf serum (CS), penicillin/streptomycin solution, Triton X-100, phosphatidylserine (PS), diacylglycerol (DG), adenosine 5'-triphosphate (ATP), bovine serum albumin (BSA), 12-O-tetradecanoyl 13-acetate (TPA), trypan blue dye, DEAE-Sepharose, Nonidet P-40, 1-O-dodecyl-rac-alkylglycerol, and dimethyl sulfoxide (DMSO) were purchased from Sigma. Trypsin solution was from Gibco Life Technologies. \([^3H]\)Thymidine and \([\gamma^{32P}]\)ATP were from DuPont NEN. Silica gel H Uniplates were purchased from Analtech. All other solvents (HPLC grade) and reagents were purchased from Fisher Scientific. 1-O-dodecyl-sn-glycerol (sn-1 and sn-3) was prepared by Robinson and Warne using the method described by Baumann and Mangold.

Cell Culture

MDCK and Swiss 3T3 cells were cultured in DMEM with 10% FCS, penicillin (100 units/ml), and streptomycin (100 \(\mu g/ml\)). NIH 3T3 ras-transformed cells were grown in DMEM with 10% CS, gentamicin (G418 200 mg/ml), penicillin (100 units/ml), and streptomycin (100 \(\mu g/ml\)). Cells were incubated at 37 °C in 5% CO\(_2\) and were passed every 3-5 days by trypsinization. Cells were stored frozen at -80 °C in Cell Freezing Medium containing 5% DMSO. Cell viability was determined by exclusion of trypan blue dye.
hemacytometer was used to determine cell number.

**Protein Determination**

Protein concentrations were determined by the Bradford method using the Bio-Rad assay kit with BSA as a standard. Protein determinations in samples which contain digitonin were obtained by using the BCA detection system.

**Isolation and Measurement of PKC Activity**

MDCK cells were homogenized in buffer A (2 mM EDTA, 0.5 mM EGTA, 50 mg/L phenylmethylsulfonylfluoride, 2 mM dithiothreitol, and 20 mM Tris HCl pH 7.5) containing 0.33 M sucrose and 25 μg/ml leupeptin. The cells were scraped and collected in buffer A and disrupted by sonication. The suspension was separated into a cytosolic and membrane fraction by ultracentrifugation (100,000 x g for 60 min). The pellet (particulate) fraction was suspended in buffer A containing 5 mg/ml Nonidet P-40. Both the supernatant (soluble) and the particulate fractions were applied to DEAE-Sepharose columns, washed with 3 column volumes of buffer A, and the activity was eluted from the columns by adding buffer A containing 150 mM NaCl. The eluant was stored at -80 °C. PKC activity in each fraction was measured by the transfer of phosphorus from \( [\gamma^{32}P]ATP \) to a synthetic peptide (pro-leu-ser-arg-
thr-leu-ser-val-ala-ala-lys-lys). The incubation mixture (120 \mu l) contained 0.2 
\mu g/\mu l peptide, 10 \mu M [\gamma^{32}P]ATP (1000 cpm/pmol), 10mM magnesium acetate,
0.5 mM CaCl$_2$, 20 mM Tris HCl pH 7.5, 8 \mu g/ml phosphatidyl-serine, and 0.8 
\mu g/ml diolein. Liposomes of phosphatidylserine and diolein were prepared by
sonication in 20 mM Tris. Incubations were at 30 °C for 5 min and were
initiated with 30 \mu l of column eluate. Reactions were stopped by addition of 1 
M ATP and the entire mixture was placed on ice. 100 \mu l of the reaction mixture
was spotted onto phosphocellulose P-81 paper which was washed 3 times in 75 
mM phosphoric acid. The amount of radioactivity that remained bound to the
paper was determined by scintillation counting. Protein kinase activity was
determined as the pmoles of phosphorus transferred to peptide per minute with
the activity in the absence of protein subtracted from each determination. PKC
activity was expressed as the difference in activity in the presence and absence of
calcium, diacylglycerol, and phosphatidylserine.

For studies on the effects of alkylglycerol on the activity of purified PKC,
phosphatidylserine and diacylglycerol were suspended in detergent micelles by
sonication in 0.03\% Triton X-100. Mixed micelles containing alkylglycerol
were prepared by suspending varying amounts 1-O-dodecyl-rac-glycerol in a
constant volume of 0.03\% Triton X-100. The final concentration of Triton X-
100 in the assay was 112 \mu g/ml. Incubations were initiated with the addition of
0.012 μg purified rat brain protein kinase C. All other conditions and concentrations were as described above.

**Determination of PKC isoform distribution**

Cytosolic and membrane-bound PKC were separated as described by Pelech et. al. with minor modifications. Briefly, after the appropriate culture time and treatment, cells were placed on ice and washed twice with cold PBS. The soluble extract was produced when the cells were incubated at 0 °C for 5 min with 0.5 mg/ml digitonin in buffer B (5 mM EGTA, 2 mM EDTA, 20 μM leupeptin, 200 μM PMSF, 100 mM NaF, and 20 mM Tris, pH 7.5). After removal of the supernatant, the cell remnants were solubilized with 1% Triton in buffer B at 0 °C for 30 min. A sample of each fraction was removed for protein determination using the BCA method.

SDS-polyacrylamide gel electrophoresis was preformed according to the procedure of Laemmli using 10% acrylamide (Mini-Protean II gel system from Bio-Rad). After electrophoresis, gels were equilibrated for 30 min in transfer buffer (40 mM glycine, 0.0375% SDS, 20% (v/v) methanol, and 50 mM Tris, pH 8.5-9.0). Proteins were transferred to nitrocellulose (0.45 μm pore size) for 30 min at 15 V using a semi-dry transfer apparatus (Trans-Blot SD from Bio-Rad). The membranes were blocked in wash buffer (100mM NaCl, 0.1%
Tween 20, and 10 mM Tris, pH 7.5) with 5% non-fat dry milk. All incubations were at 37 °C for 30 min. The primary antibodies were added in blocking buffer as above (α - 1:5,000, ε - 1:200, ζ - 1:400 dilution). The secondary antibodies were horseradish peroxidase conjugates (1:2,500 dilution). Proteins of interest were visualized using the enhanced chemiluminescence (ECL) system from Amersham Life Science. Densitometry analysis with the Millipore Whole Band Analyzer provided quantitation of the bands.

**Measurement of Monoglycerides**

Cells were collected by trypsinization and rinsed with phosphate buffered saline (PBS). The cells were centrifuged and resuspended in 1 ml PBS. 12:0 racemic monoalkylglycerol and 12:0 racemic monoacylglycerol (1 nM) were added as internal standards and the cellular lipids were extracted by the method of Bligh and Dyer. Neutral lipids were separated by thin layer chromatography (TLC) using a solvent system of hexane:diethyl ether:acetic acid (40:60:1). The area which corresponds to the combined monoacylglycerols and monoalkylglycerols was scraped and the lipids extracted. The monoglycerides were converted to their 1-radyl-2,3-O-isopropylidine derivatives by incubation in 0.01% perchloric acid in acetone for 60 min. The isopropylidine derivatives were analyzed by gas-liquid chromatography (GLC) using a SPB-5 capillary
column. The initial oven temperature was 160 °C and increased to 220 °C at 15 °C/min followed by and increase to 250 °C at 10 °C/min. The peaks were analyzed by the computer software program, Maxima and identified by comparison of their retention times with those of isopropylidene derivatives of standard monoglyceride species.

**Incorporation of [³H]Thymidine**

Cells were seeded at 2.5x10⁴ cells/cm² in 48-well tissue culture plates and grown to confluence in 3 days. The cells were made quiescent by incubation with serum-free DMEM for 24 h. Lipid and mitotic agent additions were made in serum-free DMEM containing 0.1% BSA. After a 12 h incubation, the cells were pulsed with 1 μCi of [³H]Thymidine for 6 h. Cells were rinsed with PBS and ice-cold trichloroacetic acid was added to each well with a final concentration of 10% (w/v). Cells were scraped and transferred to microfuge tubes. The wells were rinsed in 0.5 ml 10% trichloroacetic acid. After centrifugation for 2 min, the supernatant was removed by aspiration. The precipitate was dissolved in 0.5 ml 0.5 M NaOH for 15 min. After transfer to large scintillation vials, 10 ml of scintillation fluid was added and the radioactivity was assayed.

In experiments with proliferating cultures of 3T3 cells, monoglycerides
were added at a concentration of 10 μM for 12 hours. 0.5 μCi of [3H]thymidine was added for 4 hours. Cells were rinsed with PBS and solubilized with 100 μl of 3% Triton X-100. 10 μl of this suspension was measured by scintillation counting as above.

**Cytotoxicity Assay: Release of Lactate Dehydrogenase**

Cell treatments and incubations were performed as described for the [3H]thymidine incorporation assay. This assay was performed with the CytoTox96 Non-Radioactive Cytotoxicity Assay kit. A 10X lysis solution (9% v/v Triton X-100) was added 45 min prior to harvest to determine the maximum release of LDH. 50 μl of the supernatant was transferred to a 96 well plate. After the addition of 50 μl of the substrate mix, the plate was incubated for 30 min in the absence of light. 50 μl of a stop solution (1M acetic acid) was added and the absorbance at 490 nm was recorded with a microplate reader. Values are expressed as percent of the maximum release.

**Effect of Alkylglycerol on the Growth of Swiss 3T3 Fibroblasts**

Swiss 3T3 cells were seeded at 1.25x10⁴ cells/cm² in Falcon 24 multiwell plates and cultured overnight in DMEM with 10% FCS at 37 °C. The media was removed and the cells were rinsed with PBS. Untreated cells (control) were
cultured with DMEM with 4% FCS and treated cells were cultured in the presence of 10 μM 1-0-dodecyl-rac-glycerol in DMEM with 4% FCS. Daily cell counts were observed by using a hemacytometer. Cells were trypsinized and viable cells were counted by trypan blue exclusion. The media was removed and replenished on day 4 of the growth curve.
CHAPTER 3
RESULTS

The Distribution of Protein Kinase C in Proliferating and Confluent Cultures of MDCK Cells

In fibroblasts, protein kinase C has been found in an active, membrane associated state during the early log phase of cell growth. Also, PKC shifts toward its cytosolic, inactive state as the cells reach confluence and their growth has slowed (Halsey et al., 1987; Goin et al., 1993). We examined the distribution of PKC activity in MDCK cells to determine if a similar shift in the enzyme activity occurred as the cells form a contact-inhibited monolayer. The distribution of protein kinase C in MDCK cells was determined by measuring the lipid (phosphatidylserine and diacylglycerol) and calcium dependent phosphorylation of a synthetic peptide substrate in the cytosolic and membrane fractions from proliferating and confluent cultures of MDCK cells. As shown in Figure 3, the distribution of PKC activity undergoes a growth dependent change resembling that of fibroblasts. The amount of PKC activity associated with the membrane in confluent cells was found to decrease to a level that was only 14% of that in proliferating cells. Moreover, there is also an overall decrease in total PKC found in MDCK cells as they achieve confluence. The decrease in total
Figure 3. Effect of Cell Growth Status on the Subcellular Distribution of PKC Activity in MDCK Cells. MDCK cells were seeded at a density of 1x10^4 cells/cm^2 and cultured to a density of 8-9x10^4 cells/cm^2 (preconfluent, open bars) or 3-4x10^5 cells/cm^2 (confluent, shaded bars). Cells were rinsed and PKC activity in the soluble (upper panel) and particulate (lower panel) fractions were determined as described in "Experimental Procedures". Values are the mean ± S.E. of three determinations performed in triplicate (*, p < 0.01 significance of difference from preconfluent cells using Student's t test).
PKC present in these cells is consistent with published data which suggest that, in its active form, PKC is very susceptible to down-regulation by protease activity (Newton, 1995; Bell and Burns, 1991).

**The Effect of Alkylglycerol on the Distribution of Protein Kinase C in Proliferating MDCK Cells**

Our laboratory has observed that exposure of MDCK cells to alkylglycerol inhibits the phorbol ester induced release of arachidonic acid (Warne et al., 1995). This suggests that alkylglycerol can inhibit PKC in intact MDCK cells. We have also observed a growth-dependent accumulation of alkylglycerol that correlates with the decline in membrane associated PKC activity. Therefore, we examined the ability of alkylglycerol to inhibit PKC activation in preconfluent cultures of MDCK cells. Preconfluent cultures of MDCK cells were treated for 1 hour in the presence of 30 μM 1-O-dodecyl-rac-glycerol and the cytosolic and membrane fractions isolated. The presence of PKC was measured as the lipid and calcium dependent activity. As shown in Figure 4, the amount of PKC activity associated with the membrane decreased by greater than 40% in cells treated with alkylglycerol. Similarly, the PKC activity in the cytosolic fraction was reduced to 65% of the control activity. As noted in MDCK cells grown to confluence (Figure 3), there is a decrease in the
Figure 4. Effect of Alkylglycerol on PKC Activity in Preconfluent MDCK cells.

Preconfluent cultures of MDCK cells were incubated for one hour in the absence (control, open bars) or presence (alkylglycerol, shaded bars) of 30μM 1-O-dodecyl-sn-glycerol. Cells were rinsed and PKC activity in the soluble (upper panel) and particulate (lower panel) fractions were determined as described in "Experimental Procedures". Values are the mean ± S.E. of seven separate determinations performed in triplicate (*, p < 0.01 significance of difference from control cells using paired Student's t test).
total PKC activity in the alkylglycerol treated cells. Thus, the addition of alkylglycerol to preconfluent cultures of MDCK cells inhibits the amount of PKC activity found in the membrane fraction in a manner similar to high density growth.

**Effect of Alkylglycerol on the Phorbol Ester Induced Translocation of PKC in MDCK Cells**

The ability of phorbol esters (TPA) to induce the translocation of the lipid dependent isoforms of PKC has been well characterized (Chen, 1993; Terzian and Rubin, 1993). In view of the ability of alkylglycerol to inhibit the association of PKC with the membrane, we investigated the ability of alkylglycerol to inhibit the TPA induced translocation of PKC to the active, membrane-associated form. Preconfluent cultures of MDCK cells were treated with 30 μM 1-O-dodecylglycerol followed by a 15 min exposure to 100 nM TPA. Cytosolic and membrane fractions were prepared and assayed for the presence of PKC activity. As seen in Figure 5, the addition of TPA caused a 5 fold increase in the membrane form of PKC. Supplementation with alkylglycerol caused a slight decrease in the control levels of membrane PKC. Treatment with 30 μM alkylglycerol caused a 50% decrease of particulate PKC activity in cells treated with TPA. Therefore, in the presence of alkylglycerol...
Figure 5. Effect of Alkylglycerol on TPA Stimulated PKC Translocation in MDCK Cells. Preconfluent cultures of MDCK cells were incubated for one hour in the absence (control) or presence (alkylglycerol) of 30μM 1-O-dodecyl-sn-glycerol. Cells were subsequently rinsed and stimulated with 100nM TPA or DMSO for 15 m. PKC activity in the soluble and particulate fractions was determined as described in "Experimental Procedures". Values are the total PKC activity in the particulate fraction as a percent of the total recovered PKC activity (particulate + soluble) and are the means ± S.E. of four separate determinations performed in triplicate.
the endogenous and TPA induced levels of PKC activity in MDCK cells are reduced.

The *in vitro* Inhibition of Protein Kinase C by *rac*, *sn*-1, and *sn*-3 Isomers of Alkylglycerol

Our previous studies suggest that alkylglycerol is an *in vivo* inhibitor of PKC activity. To determine if alkylglycerol is inhibiting PKC directly, *in vitro* studies were performed using a mixed micelle assay system and purified rat brain PKC. As shown in Figure 6, alkylglycerol inhibits the activity of purified PKC in a dose dependent manner. At concentrations as low as 1 μM, alkylglycerol proved to be an effective inhibitor of PKC activity. At higher concentrations the activity of PKC was reduced to 40% of the control response. This data indicates that alkylglycerol is an inhibitor of PKC and the effects of alkylglycerol on intact cells is the result of its ability to inhibit PKC directly.

The studies described above used 1-O-dodecyl-*rac*-glycerol, a 50:50 racemic mix of the *sn*-1 and *sn*-3 isomers of alkylglycerol. To determine if the ability of alkylglycerol to inhibit PKC activity and translocation is stereospecific, the racemic isomers of alkylglycerol were analyzed in the *in vitro* system described above. The purified rat brain extract was assayed in the presence of 30 μM *sn*-1, *sn*-3, and *rac* dodecylglycerol. As shown in Figure 7, all isomers of alkylglycerol
Figure 6. Inhibition of Protein Kinase C by Alkylglycerol. Incubations contained phosphatidylserine (8 µg/ml), diolein (0.8 µg/ml) and varying amounts of alkylglycerol (1-O-dodecyl-rac-glycerol) in Triton X-100 micelles. The final concentration of Triton X-100 in the assay was 112 µg/ml. Incubations were initiated with the addition of 0.012 µg protein kinase C purified from rat brain. Values are the means ± S.E. of four separate determinations performed in triplicate.
Figure 7. Effect of Alkylglycerol Isomers on PKC Activity \textit{in vitro}. Incubations contained phosphatidylserine (8 µg/ml), diolein (0.8 µg/ml) and 10 µM alkylglycerol in Triton X-100 micelles. The final concentration of Triton X-100 in the assay was 112 µg/ml. Incubations were initiated with the addition of 0.012 µg protein kinase C purified from rat brain. Values are the means ± S.E. of one experiment performed in triplicate.
inhibited the activity of PKC in a similar fashion. These data indicate that the inhibitory effects of alkylglycerol on the \textit{in vitro} activity of PKC are not stereospecific.

\textbf{The Endogenous Distribution of the Protein Kinase C Isoforms During the Culture of MDCK Cells}

Protein kinase C consists of a family of at least eleven isoforms that have been classified as conventional (lipid and calcium dependent), novel (lipid but not calcium dependent), and atypical (lipid and calcium independent). Our studies involving the inhibition of PKC activity by alkylglycerol has measured these effects on the lipid and calcium dependent activity. To determine if the inhibition of PKC by alkylglycerol is isoform specific, western blotting of the individual isoforms was performed. Cytosolic and membrane fraction were obtained and the proteins were separated by SDS gel electrophoresis. After transfer to a nitrocellulose membrane, the samples were probed with PKC \(\alpha\), \(\epsilon\), and \(\zeta\) antibodies. MDCK cells have been shown to express these isoforms of PKC and each represents one of the three classes of PKC isoforms.

The initial experiments determined the endogenous distribution of the PKC isoforms during the growth of MDCK cells. Daily isolations were performed during the culture of MDCK cells and the results are shown in Figure 8. The
Figure 8. PKC Isoform Distribution During The Culture of MDCK Cells. Cells were seeded at a density of 1x10⁴ cells/cm². Cell counts were performed daily. Sample preparations were taken on days 2-11 as described under "Experimental Procedures". Bands were analyzed by densitometry. These results are representative of two separate experiments. Values shown represent the percent of total PKC localized to the membrane (● ■ ▲) and cell density (-).
membrane associated form of PKC ε was observed to decrease early in the growth of MDCK cells. The active form of this isoform was completely removed by day 4 of growth. Similarly, membrane associated PKC α declined early in the growth of MDCK cells. However, the active form increased on days 6 thru 8. This followed a media change on day 5 of growth. The membrane form of PKC ζ slightly declined then remained consistent throughout the growth of the cells. These experiments suggest that the presence of the lipid dependent isoforms of PKC (α and ε) decrease as the levels of endogenous alkylglycerol increase.

The Effect of Alkylglycerol on the Endogenous Distribution of the Protein Kinase C Isozymes

We have shown that alkylglycerol has an inhibitory effect on the endogenous activity of PKC (Figure 4). To determine if these effects are isoform specific, antibody detection of western blots were used. Proliferating MDCK cells were exposed to an increasing concentration of 1-O-dodecyl-rac-glycerol and the soluble and membrane extracts were probed with PKC antibodies. These results are shown in Figure 9. The increasing dose of alkylglycerol caused a displacement of PKC α from the membrane with a concentration as low as 1 µM. The presence of PKC α was reduced to less than
Figure 9. Effect of Alkylglycerol on the Endogenous Distribution of PKC Isoforms.

MDCK cells were seeded at a density of 5x10^4 cells/cm^2 and cultured for 2 days. Cells were incubated in the presence of 30 μM 1-O-dodecyl-rac-glycerol in DMEM-0.1% BSA or DMEM-0.1% BSA for 75 min. Cell fractions were obtained as described under "Experimental Procedures". A, western blot analysis. These results are representative of two separate experiments. 5 μg of protein was added to each lane. Gel stain confirmed equal loading of samples (data not shown). Molecular weight standards confirmed sizes; α - 78kDa, ε - 88kDa, and ζ - 75kDa. B, densitometry analysis of bands. Values shown represent the percent of total PKC localized to the membrane.
5% of the total PKC at 30 μM. A similar effect was observed with PKC ε. The increasing dose of alkylglycerol caused a decrease in the membrane form of PKC ε. However, the decrease was not greater than 40% of the membrane associated PKC at the higher concentrations. The addition of alkylglycerol had no effect on the distribution of PKC ζ at any concentration. These data indicate that alkylglycerol displaces PKC α and ε from the membrane in a dose dependent manner.

The Effect of Alkylglycerol on the Phorbol Ester Induced Translocation of PKC α, ε, and ζ

Our data demonstrates that alkylglycerol inhibits the TPA induced translocation of PKC in MDCK cells. We sought to determine if this inhibition is isoform specific. The treatment of cells with phorbol esters, such as TPA, results in the translocation of PKC from the cytosol to the membrane fraction (Chen, 1993; Terzian and Rubin, 1993). Through western blotting, the distribution of individual PKC isoforms in response to TPA was observed. Unstimulated PKC α was found primarily in a cytosolic form (Figure 10A). Upon stimulation with TPA for 5 min we observed a shift to the active membrane associated form. Particulate levels tripled from 31% to 98% in the presence of TPA (Figure 10B). The
Figure 10. Effect of alkylglycerol on the TPA induced translocation of PKC α.
Figure 10 (continued). MDCK cells were seeded at a density of 5x10^4 cells/cm² and cultured for 3 days. Cells were incubated in the presence of 30 μM 1-O-dodecyl-rac-glycerol or vehicle for 75 min. Alkylglycerol additions were made in the presence of 0.1% BSA. Cells were then incubated with 100 nM TPA or DMSO for 5 min. Cell fractions were obtained as described under "Experimental Procedures". A, western blot analysis. 10 μg of protein was added to each lane. Gel stain confirmed equal loading of samples (data not shown). Molecular weight standards confirmed size; α - 78kDa. B, densitometry analysis of bands are displayed as the mean of six separate experiments ± SEM (*, p < 0.001; **, p < 0.005). Values are normalized so that the total in the particulate and soluble = 100%.

endogenous distribution of PKC ε, in contrast to PKC α, was more associated with the membrane as seen in our earlier studies (Figure 11). PKC ε did show a substantial variation in the initial distribution. The membrane associated form varied from 50% to 90%, which is highly dependent upon the growth status of the cells (Figure 8). Regardless of the initial distribution, treatment with TPA caused a 100% shift to the particulate fraction (Figure 11). In untreated cells PKC ζ was primarily associated with the cytosolic fraction and TPA, as reported (Cardone et al., 1994), had no stimulatory effect on this isoform (Figure 12). Therefore, TPA translocates the lipid dependent isoforms α and ε from the cytosol to the membrane while having no effect on the distribution of the atypical isoform ζ.
Once TPA induced translocation of PKC had been established in our system, the effects of alkylglycerol on this response was determined. MDCK cells were pre-treated for 60 min in the presence of 1-O-dodecyl-rac-glycerol or vehicle, followed by incubation with 100nM TPA or dimethyl sulfoxide. In the presence of alkylglycerol, the TPA induced response on particulate PKC α was inhibited by 35% (Figure 10B). Alkylglycerol reduced the amount of endogenous membrane bound PKC α as seen before (Figure 9). However, supplementation with alkylglycerol had no effect on the TPA induced translocation of PKC ε (Figure 11). As expected, treatment of cells with alkylglycerol had no effect on the subcellular distribution of PKC ζ (Figure 12).

Monoglyceride Composition During the Culture of Swiss 3T3 Fibroblasts

Swiss 3T3 fibroblasts have been used extensively to study cell growth and the induction of mitogenesis (Takuwa et al., 1988; Desai et al., 1993; Rozengurt et al., 1981). In order to investigate the effects of alkylglycerol on the induction of mitogenesis, we employed this well characterized system. Our initial studies examined the endogenous levels of the monoglycerides. Swiss 3T3 cells were seeded at a density of 1x10⁴ cells/cm² and cultured for 8 days. On days 2 thru 8 the cells were counted and harvested by trypsinization. Lipids were extracted and the monoglycerides isolated by TLC. Monoglycerides were converted to
Figure 11. Effect of Alkylglycerol on the TPA Induced Translocation of PKC ε.
Figure 11 (continued). MDCK cells were seeded at a density of 5x10⁴ cells/cm² and cultured for 3 days. Cells were incubated in the presence of 30 μM 1-O-dodecyl-rac-glycerol or vehicle for 75 min. Alkylglycerol additions were made in the presence of 0.1% BSA. Cells were then incubated with 100 nM TPA or DMSO for 5 min. Cell fractions were obtained as described under "Experimental Procedures". A, western blot analysis. 10 μg of protein was added to each lane. Gel stain confirmed equal loading of samples (data not shown). Molecular weight standards confirmed size; ε - 88kDa. B, densitometry analysis of bands are displayed as the mean of six separate experiments ± SEM. Values are normalized so that the total in the particulate and soluble = 100%.
Figure 12. Effect of Alkylglycerol on the TPA Induced Translocation of PKC ζ.
Figure 12 (continued). MDCK cells were seeded at a density of 5x10⁴ cells/cm² and cultured for 3 days. Cells were incubated in the presence of 30 µM 1-O-dodecyl-rac-glycerol or vehicle for 75 min. Alkylglycerol additions were made in the presence of 0.1% BSA. Cells were then incubated with 100 nM TPA or DMSO for 5 min. Cell fractions were obtained as described under "Experimental Procedures". A, western blot analysis. 10 µg protein was added to each lane. Gel stain confirmed equal loading of samples (data not shown). Molecular weight standards confirmed size; ζ - 75kDa. B, densitometry analysis of bands. Values are normalized so that the total in the particulate and soluble = 100%.

1,2-O-isopropylidene derivatives and separated into molecular species and quantified by GLC. As shown in Figure 13, the cells grew in a typical log phase growth that peaked at 13x10⁴ cells/cm². The levels of the monoacylglycerides did not change throughout the growth of these cells. However, the levels of monoalkylglycerol increased as the cells were cultured to a contact-inhibited monolayer. The monoalkylglycerols increased over 8 fold as the cells grew to confluence. The highest alkylglycerol concentration occurred just prior to the inhibition of growth. The endogenous levels of alkylglycerol decreased during the later stages of culture.

Table 1 shows the individual species distribution of the alkylglycerols during the growth of Swiss 3T3 cells. The species of alkylglycerol include
Figure 13. Content of Monoacylglycerol and Monoalkylglycerol During the Growth of Swiss 3T3 Fibroblasts. 3T3 cells were seeded at a density of 1x10^4 cells/cm^2. Cell lipids were extracted and monoglycerides were isolated by TLC. Isopropylidine derivatives were made and quantified by GLC analysis. The results are from two experiments performed in triplicate.
TABLE 1

ALKYLGLYCEROL SPECIES COMPOSITION OF SWISS 3T3 FIBROBLASTS

<table>
<thead>
<tr>
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<th>cell growth status</th>
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<td></td>
<td>preconfluent</td>
<td>confluent</td>
<td>postconfluent</td>
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<tr>
<td>pmol/10^6 cells</td>
<td></td>
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<tr>
<td>hexadecylglycerol</td>
<td>22.7 ± 5.0 (49%)</td>
<td>184.5 ± 18.6 (44%)</td>
<td>75.7 ± 17.1 (51%)</td>
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<tr>
<td>octadecylglycerol</td>
<td>9.5 ± 2.1 (21%)</td>
<td>72.2 ± 10.8 (17%)</td>
<td>20.0 ± 7.4 (13%)</td>
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<tr>
<td>octadecenylglycerol</td>
<td>13.8 ± 2.6 (30%)</td>
<td>161.1 ± 26.0 (39%)</td>
<td>53.2 ± 11.1 (36%)</td>
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<tr>
<td>total alkylglycerol</td>
<td>46.0 ± 9.2</td>
<td>417.8 ± 53.0</td>
<td>148.9 ± 35.5</td>
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3T3 cells were seeded at a density of 1x10^5 cells/cm^2. Cell lipids were extracted and monoglycerides were isolated by TLC. Isopropylidene derivatives were prepared and then quantified by GLC analysis. The results are from two experiments performed in triplicate.

hexadecylglycerol (16 carbon form of AG with no double bonds),
octadecylglycerol (18 carbon form of AG with no double bonds), and
octadecenylglycerol (18 carbon form of AG with 1 double bond). Although the total concentration of alkylglycerol changes throughout the culture of these cells, the distribution remains relatively constant. The percent of 16:0 AG remains at approximately 50%. The percent of 18:0 declines slightly from 21% to 13%
while the level of 18:1 AG slightly increases to 36%. Therefore, the increase of alkylglycerol occurs as the result of an increase in the entire population of alkylglycerols, not a single species.

The Effect of Alkylglycerol on the Proliferation of Swiss 3T3 Fibroblasts

We have observed that the level of alkylglycerol increases during the culture of MDCK and Swiss 3T3 cells. Furthermore, this increase precedes a decrease in cell growth. We therefore examined the effect of exogenous alkylglycerol on the growth of Swiss 3T3 cells. 3T3 cells were seeded at a density of 1.25x10⁴ cells/cm² and cultured for 24 hours. The cells were changed to media that contained 4 % FCS and 10 μM alkylglycerol. These cells were then cultured for 8 days. On each day viable cells were counted by trypan blue exclusion. The results are shown in Figure 14. The control cells showed a typical log phase growth and grew to a saturation density of 1.1x10⁵ cells/cm². Cells cultured in the presence of alkylglycerol showed a slower growth rate and grew to a lower maximum cell density of 7x10⁴ cells/cm². These results show that alkylglycerol decreases the rate of cell proliferation and saturation density of Swiss 3T3 fibroblasts. Therefore, this suggest that endogenous alkylglycerol contributes to growth arrest at a critical density.

We determined if the inhibitory effects of alkylglycerol on the cell
Figure 14. Effect of Alkylglycerol on the Proliferation of Swiss 3T3 Fibroblasts. Cells were seeded at $1.25 \times 10^4$ cells/cm$^2$ in DMEM with 10% FCS. After 24 hours (day 1) the media was changed to DMEM containing 4% FCS with (■) or without (○) 10 μM alkylglycerol. The media was changed on day 4. Viable cells were counted each day by trypan blue exclusion. Values are the mean ± S.E. of two determinations performed in triplicate.
proliferation and saturation density of 3T3 cells is reversible. The growth of cells that were cultured to saturation density in the presence of alkylglycerol were subcultured and observed after the removal of alkylglycerol. The growth of these cells was followed for another 8 days in the presence of fetal calf serum in DMEM. As shown in Figure 15, the cells which were treated earlier with alkylglycerol and then subcultured in the absence of alkylglycerol, return to a normal growth rate and saturation density. This suggests that growth inhibition observed during the addition of exogenous alkylglycerol is reversible.

**The Induction of Mitogenesis in Quiescent Swiss 3T3 Fibroblasts with Insulin, TPA, and Serum**

Previous studies examined the effect of alkylglycerol on proliferating 3T3 cells. Swiss 3T3 cells have been used extensively as a model system to explore the signaling pathways leading to the induction of cell proliferation. We investigated the effects of alkylglycerol on the induction of mitogenesis in quiescent fibroblasts to determine if the effects were limited to PKC-mediated growth activation. 3T3 cells were cultured for 3 days and made quiescent by the removal of serum for 24 hours. Mitogens were added followed by the addition of [³H]thymidine 12 hours later. Following a 6 hour incubation, the cells were scraped and all TCA precipitable material was counted by scintillation.
Figure 15. Proliferation of Swiss 3T3 Fibroblasts after Alkylglycerol Treatment. Cells were grown for 8 days in the presence (○) or absence (●) of 10 μM alkylglycerol. The cells were trypsinized and seeded at 1.25x10⁴ cells/cm² in DMEM with 4% FCS. The media was changed on day 4. Viable cells were counted each day by trypan blue exclusion. Values are the mean ± S.E. of one determination performed in triplicate.
measurement. As Figure 16 shows, control cells in the absence of any mitogens had little increase of [3H]thymidine incorporation. The mitogenic effects of insulin and TPA were similar (approximately a 20 fold increase over control). The largest increase in [3H]thymidine incorporation was observed with the addition of 10% serum. Therefore, in the presence of insulin, TPA, and serum we observed an increase in the induction of DNA synthesis in Swiss 3T3 fibroblasts.

The Effect of Alkylglycerol on the Incorporation of [3H]Thymidine in Quiescent Swiss 3T3 Fibroblasts

Alkylglycerol has been shown to inhibit the activity and translocation of PKC. The addition of alkylglycerol also induces a slower growth rate and decreased saturation density. We therefore determined if the addition of exogenous alkylglycerol would inhibit the mitogenic response of insulin, TPA, and serum. 3T3 cells were treated with increasing concentrations of alkylglycerol 1 hour prior to stimulation with mitogens. The results are shown in Figure 17. Alkylglycerol caused a dose dependent decrease in the induction of TPA-stimulated mitogenesis. In contrast, we observed an increase in the incorporation of [3H]thymidine. At concentrations up to 10 μM, alkylglycerol had no effect on mitogenesis initiated with serum. These results indicate that the
Figure 16. Effect of Mitogens on the Induction of DNA Synthesis in Swiss 3T3 Fibroblasts. Quiescent cultures were treated with 100 nM TPA, 10% serum, or insulin for 12 hours. 0.5 μCi [³H]thymidine was added and the cells incubated for 6 hours. All TCA precipitable material was quantified by scintillation counting. Values are the mean ± S.E. of three experiments performed in triplicate.
addition of exogenous alkylglycerol inhibits the TPA (PKC-dependent) induced mitogenic response. However, alkylglycerol caused an increase in the mitogenic response to insulin (PKC-independent).

We then determined if these effects were stereospecific. The previous experiment was repeated with 1-O-dodecyl-\textit{sn}-glycerol and 3-O-dodecyl-\textit{sn}-glycerol. As shown in Figures 18 and 19, 1-O-dodecyl-\textit{sn}-glycerol and 3-O-dodecyl-\textit{sn}-glycerol inhibited the mitogenic response to TPA while increasing the response to insulin. Thus, the effects of alkylglycerol on the induction of mitogenesis are not stereospecific.

\textbf{The Effect of Alkylglycerol isomers and Acylglycerol on the Incorporation of [3H]Thymidine in preconfluent Swiss 3T3 Fibroblasts}

We have observed that alkylglycerol can inhibit the induction of TPA-stimulated mitogenesis in 3T3 cells and inhibit proliferation in actively dividing cells. As shown in Figure 20, we compared the ability of the \textit{sn}-1, \textit{sn}-3, and racemic isomers of alkylglycerol to inhibit the incorporation of [\textsuperscript{3}H]thymidine into preconfluent 3T3 cells. As we observed in the mitogenesis experiments, stereoisomers of alkylglycerol inhibit the incorporation of [\textsuperscript{3}H]thymidine in proliferating cultures of 3T3 cells equally. However, monoacylglycerol had no effect on the [\textsuperscript{3}H]thymidine incorporation in these cells. These results confirm
Figure 17. Effect of Alkylglycerol on the Induction of Mitogenesis in Swiss 3T3 Fibroblasts. Quiescent cultures were treated with increasing concentrations of alkylglycerol (1-0-dodecyl-rac-glycerol) for 60 min. Cells were rinsed and cultured with 100 nM TPA, 10% serum, or insulin for 12 hours. 0.5 μCi $[^3]$H]thymidine was added and the cells incubated for 6 hours. All TCA precipitable material was quantified by scintillation counting.
Figure 18. Effect of 1-0-dodecyl-sn-glycerol on the Induction of Mitogenesis in Swiss 3T3 Fibroblasts. Quiescent cultures were treated with increasing concentrations of 1-0-dodecyl-sn-glycerol for 60 min. Cells were rinsed and cultured with 100 nM TPA, 10% serum, or insulin for 12 hours. 0.5 μCi [3H]thymidine was added and the cells incubated for 6 hours. All TCA precipitable material was quantified by scintillation counting. Values are the mean ± S.E. of one experiment performed in triplicate.
Figure 19. Effect of 3-0-dodecyl-sn-glycerol on the Induction of Mitogenesis in Swiss 3T3 Fibroblasts. Quiescent cultures were treated with increasing concentrations of 3-0-dodecyl-sn-glycerol for 60 min. Cells were rinsed and cultured with 100 nM TPA, 10% serum, or insulin for 12 hours. 0.5 \( \mu \text{Ci} \) [\( ^3\text{H} \)] thymidine was added and the cells incubated for 6 hours. All TCA precipitable material was quantified by scintillation counting. Values are the mean ± S.E. of one experiment performed in triplicate.
that the inhibitory effects of alkylglycerol are not stereospecific. The inability of the monoacylglycerol analog to inhibit mitogenesis demonstrates that ether-linkage is required for the biological activity of the compound.

The Cytotoxicity of Alkylglycerol in Swiss 3T3 Fibroblasts

We have identified several biological effects of exogenous alkylglycerol. To assure that these results were not due to cytotoxic effects, we measured the release of lactate dehydrogenase into the media of cells treated with alkylglycerol. These experiments were performed using conditions described for the mitogenesis assays. As shown in Figure 21, the addition of up to 30 \( \mu \text{M} \) alkylglycerol does not cause an increase in the release of LDH. Also alkylglycerol had no effect on LDH release from cells that had been stimulated by TPA or insulin.

Monoglyceride content of H-ras Transformed NIH 3T3 Fibroblasts at Various Cell Densities

Our results indicate that alkylglycerol may play a possible role in the regulation of cell proliferation. To further investigate this role, we have examined ras-transformed cell lines. Ras-transformed cells possess many unique characteristics such as the loss of contact-dependent growth arrest. We
Figure 20. Effect of Alkylglycerol and Acylglycerol on the Proliferation of Swiss 3T3 Fibroblasts. Cell cultures were treated with 10 μM alkylglycerol or acylglycerol for 60 min. 0.5 μCi [³H]thymidine was added and the cells were incubated for 4 hours. Cells were rinsed and solubilized with 3% Triton X-100. An extract was quantified by scintillation counting. Values are mean ± S.E. of one experiment performed in quadruplicate.
Figure 21. Cytotoxicity Effects of Alkylglycerol on Swiss 3T3 Fibroblasts. Quiescent cultures were treated with 100 nM TPA, 10% serum, or insulin for 12 hours. A sample of the medium was assayed for the presence of LDH. Values are the mean ± S.E. of three experiments performed in triplicate.
measured the level of alkylglycerol in NIH 3T3 fibroblasts that had been stably transfected with oncogenic H-, K-, and N-ras genes using the pZIP expression vector. These cells display characteristics of oncogenic transformed cells including anchorage independent growth and the lack of contact inhibition of growth. NIH 3T3 cells transfected with the empty expression vector served as controls. We determined the monoglyceride content during the culture of H-ras cells (Figure 22). H-ras cells were collected at various stages of growth and the level of monoglycerides were determined as their isopropylidene derivatives by GLC. H-ras cells show a dramatic increase in the level of alkylglycerol at a high density. The monoacylglycerol content remained relatively unchanged throughout the culture of H-ras cells. As shown in Table 2, the composition of the alkylglycerol species remained similar during the culture of H-ras transformed cells. Hexadecylglycerol remained at 48% in preconfluent and confluent cells. Similarly, octadecylglycerol and octadecenylglycerol remained at 13% and 49% respectively. These results demonstrate a massive accumulation of alkylglycerol during the culture of H-ras transformed cells.

Monoglyceride Content in Confluent Cultures of MDCK, Swiss 3T3, and Ras-Transformed Cell Lines

We have observed an accumulation of alkylglycerol in several cell types
Figure 22. Monoglyceride Content of H-ras NIH 3T3 cells. Cell lipids were extracted and monoglycerides were isolated by TLC. Isopropylidine derivatives were made and quantified by GLC analysis. Values are the mean ± S.E. from three experiments performed in triplicate.
### TABLE 2

**CONTENT OF ALKYLGLYCEROL SPECIES IN H-RAS NIH 3T3 CELLS**

<table>
<thead>
<tr>
<th>Cell Growth Status</th>
<th>Preconfluent pmol/10⁶ cells</th>
<th>Confluent pmol/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecylglycerol</td>
<td>142.30 ± 39.08 (49%)</td>
<td>939.18 ± 69.63 (47%)</td>
</tr>
<tr>
<td>Octadecylglycerol</td>
<td>37.17 ± 14.43 (13%)</td>
<td>277.49 ± 14.24 (13%)</td>
</tr>
<tr>
<td>Octadecenylglycerol</td>
<td>108.10 ± 47.95 (38%)</td>
<td>805.62 ± 37.77 (40%)</td>
</tr>
<tr>
<td>Total Alkylglycerol</td>
<td>287.6 ± 101.3</td>
<td>1996.0 ± 99.85</td>
</tr>
</tbody>
</table>

Cell lipids were extracted and monoglycerides were isolated by TLC. Isopropylidene derivatives were prepared and then quantified by GLC analysis. Values are the mean ± S.E. from three experiments performed in triplicate.

When grown to confluence. A comparison of alkylglycerol content in high density cell types is shown in Figure 23. MDCK and 3T3 cells show a similar concentration of alkylglycerol at this stage of growth. Similarly the empty vector pZIP NIH 3T3 cells have a comparable increase. However, the ras transformed lines, H- and K-ras, show a massive accumulation of alkylglycerol
at a high density. In comparison the MDCK, 3T3, and pZIP cells exhibited a 20 fold increase over low density cells. The ras transformed cells show almost a 100 fold increase over low density cells. These data indicate that there may be some correlation between alkylglycerol accumulation and contact dependent growth regulation.

Gas-Liquid Chromatography Analysis of pZIP and H-ras Transformed NIH 3T3 Cells

We have performed many GLC separations with various cell types. Figure 24 is an example of a GLC chromatogram comparing late preconfluent pZIP and H-ras 3T3 cells. This figure shows the individual monoalkyl- and monoacyl-glycerol species contained within these cells. The two internal standards appear first in the chromatogram. These were added as standards and were used to quantify the other peaks. These peaks were identified by comparison of retention times to a standard monoglyceride mix. As shown in the figure, the 16:0, 18:1, and 18:0 alkylglycerol peaks were greatly elevated in the H-ras cells. The monoglyceride peaks were relatively the same once the internal standards have been compensated for.
Figure 23. Alkylglycerol Content of High Density MDCK, 3T3, p-ZIP, K-ras, and H-ras NIH 3T3 cells. Cell densities at the time of extraction were approximately $1 \times 10^5$ cells/cm$^2$. Cell lipids were extracted and monoglycerides were isolated by TLC. Isopropylidene derivatives were made and quantified by GLC analysis. Values are the mean ± S.E. from three experiments performed in triplicate.
Figure 24. GLC Chromatogram of p-ZIP and H-Ras Cells. Cell densities at the time of extraction were approximately $1 \times 10^6$ cells/cm$^2$. Cell lipids were extracted and monoglycerides were isolated by TLC. Isopropylidine derivatives were made and quantified by GLC analysis.
CHAPTER 4
DISCUSSION

Until recently, free alkylglycerol in cultured cells was thought to be the breakdown product of more complex ether linked glycerolipids such as platelet activating factor. Our results have shown that unsubstituted alkylglycerol accumulated as MDCK cells are cultured to a contact inhibited epithelial monolayer. Similar results were observed during the culture of Swiss 3T3 fibroblasts. The peak accumulation of alkylglycerol occurred as the rate of cell proliferation slowed in both cell lines. This increase in alkylglycerol content during the late log phase of growth may serve to slow cell proliferation and to induce contact inhibited growth arrest. The accumulation of alkylglycerol during growth inhibition appears to be regulated in a very specific manner since the levels of monoacylglycerol and diacylglycerol have been shown to decline during the growth of these cells. The increase in alkylglycerol could arise from de novo intermediates through the dephosphorylation of alkylglycerolphosphate or deacylation of alkylacylglycerol (Wykle and Snyder, 1970). An interesting possibility is the production of alkylglycerol by the deacylation of alkylacylglycerol. Since alkylacylglycerol has been shown to be an activator of
PKC, this process may serve to produce an inhibitor of PKC from an activating molecule.

We have observed that the majority of alkylglycerol species in MDCK and Swiss 3T3 cells are the 16:0, 18:0, and 18:1 isomers. The relative distribution of these isomers remains consistent throughout the accumulation of these compounds during cell culture. Taken together with the observation that there is no significant decrease in the phospholipid pool, this implies that the increase of alkylglycerol in these cells arises from de novo synthesis and not from the breakdown of complex ether lipids. The synthetic alkylglycerol that was used for the in vivo treatment of cell cultures is the 12 carbon form of alkylglycerol. The natural occurring 16 and 18 carbon species of alkylglycerol are very hydrophobic and are not easily incorporated into cultured cells. However, the 12 carbon, 1-O-dodecyl-rac-glycerol species is much less hydrophobic and is readily incorporated into cells.

Protein kinase C consists of an enzyme family with a diverse expression in many cell types. PKC contributes to a multitude of signaling pathways including the control of cell proliferation. PKC is found in vivo in two general locations depending upon its state of activation. The inactive form is localized to the cytosol in a closed configuration. In the presence of the activators diacylglycerol, phosphatidylinerine, and calcium, PKC translocates to the
membrane. When diacylglycerol binds to the C1 domain of PKC at the membrane, the pseudosubstrate is removed from the active site thus activating the enzyme. We have shown during the culture of MDCK cells that the level of membrane associated PKC declines. This inactivation occurs in other cells, including fibroblasts, during serum deprivation or growth to confluence (Miloszewska et al., 1986; Halsey et al., 1987; Whatley et al., 1993; Überall et al., 1991). It is interesting to take note that the decrease in PKC activity during the culture of MDCK cells occurs as the accumulation of alkylglycerol increases.

In our previous studies, we observed that alkylglycerol inhibited the TPA induced release of arachidonic acid in MDCK cells. The use of phorbol esters such as 12-0-tetradecanoyl phorbol 13-acetate (TPA) has been shown to stimulate the translocation of PKC from the cytosol to the membrane fraction. In the presence of alkylglycerol we observed a 50% decrease in the ability of TPA to increase PKC activity. Also, the total PKC activity in the alkylglycerol treated group was lower than that of the untreated. This effect is consistent with published data that the level of total PKC present declines in the presence of inhibitors or extended exposure of TPA (Huang et al., 1989; Olivier and Parker, 1992). This decrease in the active form of PKC is the result of its downregulation. In the active state, the exposed hinge region, which opens the active site, is proteolytically labile. Cleavage in the hinge region dissociates...
PKC from the membrane.

Our results demonstrate that alkylglycerol is an *in vivo* inhibitor of PKC activity. This implies that alkylglycerol exerts this effect on PKC either directly or indirectly. We investigated the ability of alkylglycerol to inhibit PKC activity *in vitro*. Isolated rat brain PKC, the natural activators, and alkylglycerol were used in a mixed micelle system to determine if this effect occurs through a direct interaction of alkylglycerol and PKC. Alkylglycerol was found to be a very potent *in vitro* inhibitor of PKC activity at concentrations as low as 1 μM. This is a relevant physiologic concentration in MDCK cells. The *in vitro* experiments revealed that alkylglycerol could inhibit PKC activity by 50% at an equal molar ratio of the activator diacylglycerol. This suggests that the accumulation of alkylglycerol can endogenously regulate the activity of protein kinase C.

Through the use of the *sn*-1 and *sn*-3 stereoisomers of alkylglycerol, we determined if the inhibitory effects of alkylglycerol on PKC are stereospecific. 1-O-dodecyl-*sn*-glycerol, 3-O-dodecyl-*sn*-glycerol, and dodecyl-*rac*-glycerol equally inhibited PKC activity *in vitro*. Therefore the inhibitory effects of alkylglycerol on PKC activity *in vitro* are not stereospecific. This observation is unusual in consideration of other lipid-enzyme interactions. Usually the interaction of glycerolipids with proteins are stereospecific. The proposed binding of alkylglycerol to PKC occurs at the diacylglycerol binding site or C1.
domain. This domain is comprised of a cysteine rich region in a β-sheet formation. This forms a hydrophobic groove with a hydrophilic core. In the normal activation of PKC, diacylglycerol binds to the hydrophobic groove and conceals the hydrophilic core. This allows the enzyme to associate with the membrane and opens the active site by inducing a conformational change which removes the pseudosubstrate form the active site. Diacylglycerol fits into this domain in a stereospecific manner. Alkylglycerol may bind to this C1 domain of PKC in a similar fashion to diacylglycerol. However, alkylglycerol does not induce the conformational change in the pseudosubstrate position. Therefore, alkylglycerol blocks the activation of PKC by diacylglycerol via occupation of the C1 binding domain. This effect is not stereospecific due to the structure of alkylglycerol. The only difference between the $sn$-1 and $sn$-3 isomers of alkylglycerol is the position of a hydroxy group. This is vastly different from diacylglycerol in which an entire fatty acid chain is repositioned. Therefore, the inhibitory effects of alkylglycerol are not stereospecific and are proposed to act through the occupation of the C1 domain.

Since protein kinase C consists of a family of isozymes and alkylglycerol was shown to inhibit total PKC activity, we have examined the effect of alkylglycerol on the isoforms of PKC in MDCK cells. Under the conditions used, the $\alpha$, $\epsilon$, and $\zeta$ isoforms of PKC were detected in MDCK cells. While other reports
suggest the presence of PKC β and δ (Godson et al., 1990; Cardone et al., 1994), these isoforms were detected only in minute quantities in our system. The membrane levels of PKC α and ε were found to decrease during log phase, while the levels of PKC ζ slightly decreased then remained constant. The levels of PKC ε dropped immediately from the membrane after day two of culture. As previously shown (Martin and Michaelis, 1989; Yamamura et al., 1996), this suggests that PKC ε plays a role in the attachment of MDCK cells. However, PKC α was found to associate with the membrane until day five. The loss of PKC α from the membrane coincides with the end of log phase growth, suggesting its role in cell proliferation (Ahmed et al., 1994; Jorgensen et al., 1996). Interestingly, the level of membrane associated PKC α slightly increased on days 6-8. This increase directly followed a media change on day 5. The addition of fresh media, including 10% serum (a potent mitogen), appeared to cause an increase in the membrane form of PKC α alone. While many factors may control the function and distribution of the individual PKC isoforms, these results suggest, as others have shown, that the control of cell proliferation is mediated primarily through PKC α.

Alkylglycerol shows inhibitory effects on the endogenous distribution of the PKC isoforms. With increasing concentrations of 1-0-dodecyl-rac-glycerol added to MDCK cultures, a rapid reduction of PKC α in the membrane fraction
was observed. Similarly, the membrane-associated form of PKC ε was reduced. However, this required over a 20 fold increase in the concentration of alkylglycerol, although there is a higher percentage of PKC ε localized to the membrane initially. As previously reported (Clark and Murray, 1995; Cardone et al., 1994), PKC α and ε, but not ζ, were translocated to the particulate in response to TPA. Pre-incubation with the cell permeable alkylglycerol, 1-0-dodecyl-rac-glycerol, inhibited the TPA-induced translocation of the α isoform but not ε. As expected neither TPA nor alkylglycerol had any effect on the initial translocation or final distribution of PKC ζ. These studies suggest that the effects of alkylglycerol on PKC activity are primarily the result of the inhibition of PKC α.

Both PKC α and ε contain highly conserved lipid binding domains and thus require diacylglycerol for activation. The main structural difference between these isoforms is the lack of a C2 calcium binding region in PKC ε. If alkylglycerol is exerting its effect through the lipid binding domain, one would assume that it would affect each of these isoforms in similar ways. Slater et al. have shown that PKC α contains two lipid binding domains, thus posing a possible mechanism for the variance of inhibition observed. Regardless, alkylglycerol is a more potent inhibitor of PKC α than PKC ε, especially under stimulation with TPA. These results indicate that the effects of alkylglycerol on
PKC activity are isoform specific. Taken together with the observation that the distribution of PKC α at the membrane decreased as the content of alkylglycerol increased in cultured MDCK cells, this evidence supports the hypothesis that the increase in alkylglycerol during the culture of MDCK cells regulates cell proliferation through its effects on PKC α.

We used Swiss 3T3 fibroblasts in order to directly investigate the possible regulatory role of alkylglycerol in cell proliferation. Studies on the induction and regulation of mitogenesis are well characterized in this cell system. We quantified the levels of free alkylglycerol in these cells and observed a similar accumulation and species distribution as that in MDCK cells. Therefore, MDCK and 3T3 cells, which both exhibit contact dependent growth arrest, also show similar increases in alkylglycerol content during growth to confluence.

In order to observe the effect of alkylglycerol on the rate of proliferation, we treated proliferating cultures of Swiss 3T3 fibroblasts with exogenous alkylglycerol. In the presence of 4% serum, alkylglycerol induced 3T3 cells to grow at a slower rate and achieve a lower saturation density than control cells. Furthermore, if these treated cultures are reseeded both in 4% serum and in the absence of alkylglycerol, both cultures maintain a normal growth curve. This evidence supports the ability of alkylglycerol to regulate the control of cell proliferation without causing any long term effects. The ability of alkylglycerol
to slow the rate of cell proliferation coincides with the initial observation of endogenous alkylglycerol accumulation. During the early stages of growth with low levels of endogenous alkylglycerol, there is a high rate of proliferation. However, as the level of endogenous alkylglycerol increases, there is a decline in the rate of cell growth.

Although alkylglycerol slows the rate of cell proliferation in growing cultures, the effect of alkylglycerol on the induction of mitogenesis has further enlightened the mechanism by which this may be occurring. The addition of alkylglycerol to quiescent 3T3 cells prior to the induction of mitogenesis caused a decrease in the mitogenic response to TPA. The induction of mitogenesis by TPA occurs directly through a PKC dependent pathway. Our earlier observation that alkylglycerol is an inhibitor of the TPA induced activity of PKC, agrees with the mitogenesis results. The addition of alkylglycerol caused an increase in the mitogenic response to insulin. This may be a result of the reduced phosphorylation of the insulin receptor. The insulin receptor is naturally phosphorylated by PKC. This causes a downregulation of the receptor and therefore a loss of function. As we have shown in the presence of alkylglycerol, the activity of PKC declines. This inhibits the downregulation of the insulin receptor and allows for an increase in the insulin response.

The effects of alkylglycerol on the induction of mitogenesis by TPA and
insulin were shown to be independent of the alkylglycerol isomers. The \textit{sn}-1, \textit{sn}-3, and racemic mix of alkylglycerol all produced similar effects on mitogenesis. This observation was also noted in the effect of alkylglycerol isomers on the \textit{in vitro} activity of PKC and the effect of alkylglycerol on proliferating cultures of 3T3 cells. The use of monoacylglycerol in these experiments had no effect on the proliferation of 3T3 cells and had no effect on the \textit{in vitro} activity of PKC (data not shown). Therefore, the ether linkage in alkylglycerol is important and distinguishable from the acyl linkage of acylglycerol. Furthermore, the additions of alkylglycerol were found not to be cytotoxic at concentrations up to 30 \(\mu\text{M}\). Therefore, the inhibitory effects of alkylglycerol are not stereospecific or cytotoxic.

In order to further investigate the effects of alkylglycerol on the regulation of cell proliferation, we have utilized ras transformed NIH 3T3 fibroblasts. Ras transformed cells have been used in the study of signal transduction and the general function of ras. Several properties of these cells have been identified. These include the absence of contact dependent growth arrest, PKC \(\alpha\) primarily localized in the inactive cytosolic form, and exposure to TPA does not induce a rapid translocation of the PKC. Although these cells have lost contact dependent growth arrest, they remain adherent to the culture flasks. At high densities the cells form large spherical structures composed of many cells. These structures
remain attached to the monolayer and may serve to increase surface area. Our data indicate that alkylglycerol serves a role in the regulation of contact dependent growth arrest, the inhibition of the endogenous activity of PKC α, and the inhibition of the TPA induced translocation of PKC α. We, therefore, investigated the endogenous concentrations of alkylglycerol in these ras transformed cells.

The concentration of free endogenous alkylglycerol in H-ras transformed cells increased in proportion to cell number as observed in non-transformed cells. However, the higher concentration of alkylglycerol was 4 to 5 fold higher in comparison to the empty vector pZIP control cells and 2 to 3 fold higher than MDCK and 3T3 cells. This is a massive accumulation of free alkylglycerol in the ras transformed cells. Although the concentration of alkylglycerol is much higher in these cells, the species distribution is very similar to that of 3T3 fibroblasts and remains consistent throughout their growth. This accumulation and possible function are of current and future investigation in our laboratory. This observation supports the hypothesis that alkylglycerol serves a function in the regulation of cell proliferation. A possible pathway for this regulation is described in Figure 25. In non-transformed cells, a mitogenic signal results in the production of diacylglycerol. This is the known endogenous activator of PKC. Once PKC is activated, it transmits a signal through a ras mediated
Figure 25. Flow Diagram of Proposed Pathway for Alkylglycerol, PKC, and Ras Mediated Cell Proliferation.
pathway. This results in an increase in DNA synthesis and eventually cell proliferation. As the cells grow in number, more cell to cell contacts are established and an increase in the concentration of alkylglycerol is observed. This increase in alkylglycerol serves to inhibit PKC activity thus terminating the mitogenic signal. This turns off the ras mediated pathway, DNA synthesis, and cell proliferation. However, the ras transformed cells function in a different manner. The ras transformed cells are transfected with a constitutively active form of ras. In these cells, regardless of a mitogenic signal, there is a ras mediated signal to increase DNA synthesis, which in turn increases cell proliferation. This is accompanied by an increase of cell to cell contacts and an increase in alkylglycerol concentration (as we have observed). However, the negative signal of alkylglycerol on PKC is mute due to the constitutively active form of ras. This in turn gives rise to the increase in cell proliferation and the additional accumulation of alkylglycerol. Eventually the cells lose contact dependent growth arrest and accumulate large quantities of alkylglycerol which are defining characteristics in these cells. The exact reason for the accumulation of alkylglycerol is still not clearly understood. The increase in the endogenous levels of alkylglycerol coincides with an increase in the cell density and therefore, the number of cell-cell contacts. The generation of free alkylglycerol could therefore arise from a membrane surface adhesion molecule, other receptor interactions, or
from an interaction with the integrin components. There has been an increasing interest in the role of lipids in signal transduction and as second messengers. Similar compounds to alkylglycerol have been investigated such as sphingosine. Sphingosine has been suggested to be the endogenous inhibitor of PKC. However, the concentration of endogenous sphingosine is 20 fold lower than that of alkylglycerol and alkylglycerol is a more potent inhibitor of PKC activity (Igarashi et al., 1989; Merrill, Jr. et al., 1989). Similarly, the alkyl-methoxy compounds have been used as antineoplastic agent until several side effects arose (Morimoto et al., 1991; Zhou et al., 1992). Unsubstituted alkylglycerol appears to be a better candidate considering the negative effects on cell proliferation and absence of cytotoxicity.

In summary, our results indicate that free alkylglycerol, a potent inhibitor of PKC activity, accumulates in MDCK, 3T3, and ras transformed cells. The increase of endogenous alkylglycerol coincides with a reduced level of membrane-associated PKC activity. The addition of synthetic alkylglycerol inhibits the TPA induced translocation of PKC and this effect is specific for PKC α. The addition of alkylglycerol to 3T3 fibroblasts inhibits the mitogenic response of TPA, which occurs through a PKC dependent pathway. Also, these effects are not stereospecific or cytotoxic. The levels of alkylglycerol in ras transformed cells are much higher than non-transformed cells at similar densities. Several properties of
ras cells have been identified, including the loss of contact dependent growth regulation and PKC remaining mostly cytosolic even under TPA stimulation. The inhibitory effects of alkylglycerol on these processes support the hypothesis that alkylglycerol serves to regulate cell proliferation through its effects on protein kinase C activity.


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