PROTEIN KINASE C ACTIVATION IN
HYPERGLYCEMIC BOVINE LENS
EPITHELIAL CELLS

THESIS

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Wen-Lin Fan, B.S.
Denton, Texas
December, 1993
Fan, Wen-Lin, Protein Kinase C Activation in Hyperglycemic Bovine Lens Epithelial Cells. Master of Science (Biochemistry), December, 1993, 76 pp., 6 tables, 12 illustrations, bibliography, 73 titles.

This study demonstrates the presence of protein kinase C activity in both cytosolic and membrane fractions of bovine lens epithelial cells in culture. Protein kinase C activity is similar in normal and hyperglycemic cells. Furthermore, the ability of the enzyme to translocate from the cytosol to the membrane following phorbol ester treatment is unimpeded by hyperglycemic conditions. Moreover, protein kinase C activation had no effect on myo-inositol uptake either in normal cells or in cells exposed to hyperglycemic conditions.
ACKNOWLEDGEMENTS

I would like to express my appreciation for the direction and support throughout the project by Dr. Patrick R. Cammarata. I sincerely appreciate the encouragement, fruitful discussion and support from Dr. Thomas Yorio. I would also like to thank Ms. Jinhua Yang and Hai-Qing Chen for the cooperation on tissue culture and performing [\(^3\)H]myo-inositol uptake experiments. Special thanks go to Dr. Robert T. Mallet and Ms. Su-Hua Xu, who read the manuscript and made many helpful suggestions.
# TABLE OF CONTENTS

**LIST OF TABLES**

| LIST OF ILLUSTRATIONS | vii |

**CHAPTER**

I. INTRODUCTION

- Diabetic Complications
- Bovine Lens Epithelial Cells: An *In Vitro* Model
- Lens Metabolism: the Polyol Pathway
- Hyperglycemia vs Hypergalactosemia
- Myo-inositol Depletion: Secondary Event?
- Protein Kinase C In The Lens
- The Properties of Protein Kinase C
- The purpose of the study

II. MATERIALS AND METHODS

- Cell culture
- Protein Kinase C Assay
- $[^3H]$-Myo-Inositol Uptake

III. RESULTS

- The Enzyme Is Phosphatidylerine Dependent
- The Enzyme Is Diacylglycerol Dependent
- The Interaction of Phosphatidylerine and 1-Oleoyl-2-acetylglycerol on Protein Kinase C Activity
- The Enzyme Is Calcium Dependent
Dependencies of Mixed Micelle Assay on other Factors
Partial Purification of Protein Kinase C
Comparison of Commercial Kit vs. Optimum Assay
Galactose and Glucose Effects on Protein Kinase C Activity
Phorbol Ester-Stimulated Translocation
Protein Kinase C Activators and Myo-Inositol Uptake
The Effect of Prolonged Exposure of Bovine Lens Epithelial Cells to Phorbol Myristic Acid

IV. DISCUSSION  57
BIBLIOGRAPHY  65
LIST OF TABLES

Table                                                                 page
1. Partial Purification of Protein Kinase C                              45
2. Comparison of GIBCO Kit to Optimum Assay                             46
3. The Effect of Hypergalactosemic and Hyperglycemic Condition On Protein Kinase C Activation 48
4. Protein Kinase C Activity in Galactose and Glucose Medium Compared to in Minimum Essential Media by Independent t-Test 49
5. The Effect of Hyperglycemic Conditions on Protein Kinase C Activity in the Presence and Absence of Phorbol Myristic Acid 51
6. Comparison of Protein Kinase C Activity in the Presence and Absence of Phorbol Myristic Acid by Independent t-Test 52
LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Protein Kinase C Partial Purifications</td>
<td>17</td>
</tr>
<tr>
<td>2.</td>
<td>Phosphatidylserine Dose Curve</td>
<td>21</td>
</tr>
<tr>
<td>3.</td>
<td>1-Oleoyl-2-acetylglycerol dose curve</td>
<td>24</td>
</tr>
<tr>
<td>4.</td>
<td>The Interaction of Phosphatidylserine and 1-Oleoyl-2-acetylglycerol on Protein Kinase C Activity</td>
<td>26</td>
</tr>
<tr>
<td>5.</td>
<td>Calcium Dose Curve</td>
<td>29</td>
</tr>
<tr>
<td>6.</td>
<td>Ac-Myelin Basic Protein Substrate Peptide Dose Curve</td>
<td>31</td>
</tr>
<tr>
<td>7.</td>
<td>Enzyme Dose Curve</td>
<td>34</td>
</tr>
<tr>
<td>8.</td>
<td>Time Dependent Protein Kinase C Activity</td>
<td>36</td>
</tr>
<tr>
<td>9.</td>
<td>Adenosine Triphosphate Dose Curve</td>
<td>38</td>
</tr>
<tr>
<td>10.</td>
<td>Magnesium Dose Curve</td>
<td>40</td>
</tr>
<tr>
<td>11.</td>
<td>Protein Kinase C Inhibitor Peptide PKC(19-36) Dose Curve</td>
<td>43</td>
</tr>
<tr>
<td>12.</td>
<td>Effect of Protein Kinase C activators on [³H]-Myo-inositol uptake</td>
<td>54</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

Diabetic Complications

Diabetes is a systemic disease characterized by hyperglycemia, hyperlipidemia, and hyperaminoacidemia. It is caused by a decrease in the secretion or activity of insulin (1). Diabetes mellitus affects approximately six percent of the U.S. population (2). It has been estimated that diabetes and its associated vascular complications are the fourth leading cause of death in the United States (2). Diabetes is the leading cause of blindness in individuals between 20 and 74 years of age in developed countries (3). Recent reports from the World Health Organization announced that diabetes in adults is now a world-wide disease (4). Insulin treatment saves lives but has failed to prevent the retinopathy, nephropathy and neuropathy of diabetes, and also has failed to reduce the aggravated risk of atherosclerosis (5). The impact of diabetes resides almost entirely in these complications of diabetes. One explanation for the development of the long-term complications of diabetes is the failure of current antidiabetic therapy to effectively normalize metabolism. It is necessary to delineate the metabolism in the diabetic condition in various tissues more precisely so that treatments can be
developed to increase the lifespan of diabetic patients and also improve their quality of life.

The ocular lens is seriously damaged by diabetes, developing a condition known as sugar cataract (6). Animal models such as rats and rabbits are used to study this complication (7, 8). The whole lens culture has also been used (9).

The lens is enveloped by a homogeneous, carbohydrate-rich capsule coating the outer surface of the epithelial cells. The subcapsular epithelium consists of a single layer of cuboidal epithelial cells present only on the anterior surface of the lens. Underneath this layer are the lens fibers. They are highly differentiated cells derived from cells of the subcapsular epithelium. They eventually lose their nuclei and other organelles and become greatly elongated. These cell types differ in their functional and metabolic characteristics. Therefore, it is necessary to isolate a single cell type in order to perform a detailed metabolic study.

Bovine Lens Epithelial Cells: An In Vitro Model

A unique model system has been developed by Dr. Cammarata et al., which is termed cultured Bovine Lens Epithelial Cells (BLECs)(10). Although this is not a cloned cell line and the metabolism may still vary somewhat from cell to cell, we are actually studying the average property of a large quantity of the same type of cells rather than
studying an individual animal or lens. This will reduce the variability of our results. More importantly, since the lens fibers are derived from the epithelium and they interact with the environment through the epithelial layer and its coating capsule, knowledge of the metabolism in the epithelial cells could help us elucidate the metabolic events which lead to sugar cataract formation. This system has proven to be a very successful approach in diabetic complication research. Several diabetic complications in BLECs have been identified in Dr. Cammarata's Lab (11, 10, 12, 13). Recent studies have revealed that the enzyme aldose reductase may play a key role in initiating these diabetic complications (14).

Lens Metabolism: the Polyol Pathway

The lens retains its shape and transparency by virtue of active metabolic processes. Nutrients and oxygen required for metabolism are taken up from the aqueous humor across the epithelium which maintains the ionic equilibrium within the lens (15). These cells derive energy from glucose metabolism, mainly through anaerobic glycolysis. Control of lens glycolysis resides in two enzymes, phosphofructokinase and hexokinase. Phosphofructokinase activity is regulated by ATP availability and hexokinase by the level of its product, glucose-6-phosphate (16). At physiological glucose levels, both enzymes are saturated. Raising the glucose concentration in blood or aqueous humor does not increase
glycolytic flux (17) and the intracellular glucose level will tend to rise. Excess glucose within the lens is metabolized by the hexose monophosphate shunt or by the polyol pathway which is made possible by the presence of aldose reductase in the lens epithelium (18).

Aldose reductase is the first and the rate-limiting enzyme of the polyol pathway. This reaction reduces hexoses to their corresponding polyols (sugar alcohol), for example, glucose to sorbitol, galactose to galactitol, with concomitant oxidation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The enzyme is widely distributed throughout the body and is found in those tissues susceptible to chronic diabetic complications. In the eye, aldose reductase has been localized to the capillary pericytes, pigment epithelium of the retina, and the cellular layers of the cornea and lens (19,20,21). Aldose reductase has a low affinity for glucose which, at physiological conditions, is preferentially phosphorylated by hexokinase to glucose-6-phosphate and directed into either the glycolytic or pentose phosphate pathways. Under high glucose concentrations, glucose is shifted into the polyol pathway and converted to sorbitol in those tissues which contain aldose reductase. Sorbitol is poorly diffusible and only slowly metabolized to fructose by the action of sorbitol dehydrogenase which utilizes nicotinamide-adenine dinucleotide as a co-factor. Therefore,
sorbitol accumulates in tissues containing aldose reductase. The accumulation of polyol could be responsible for the osmotic changes found in lens fiber cells. The hypothesis is that the accumulation of polyol causes osmotic stress and attracts water into the cell. The cells swell, and subsequent disturbances such as increased intracellular sodium concentration as well as myo-inositol and amino acid depletion are associated with it (22, 23).

**Hyperglycemia vs Hypergalactosemia**

Galactose is widely used in studying diabetic cataract formation. Galactose-fed systems, such as animals or cultured cells develop diabetic complications, such as cataract, more rapidly than glucose-fed systems (20). At least in cataract, this is the only difference because, morphologically and histologically, the hyperglycemic and hypergalactosemic cataracts appear to be identical (24). Thus, it is likely that the hyperglycemic and hypergalactosemic cataracts are caused by the same mechanisms.

Hypergalactosemic systems provide excellent models for studying the polyol effects on sugar cataract formation. Galactose is a better substrate for aldose reductase than glucose, and galactitol is not metabolized further by sorbitol dehydrogenase. In the galactose exposed lens, the level of galactitol accumulated was found to be higher than that of sorbitol found in the high-glucose exposed lens. The
retention of polyol was paralleled by an increase in lens hydration. In the galactose exposed system, studies showed that the point at which the curve plateaus indicated that the galactitol concentration was about 100 mmol/kg of lens water which was high enough to produce an osmotic consequence (25).

Although the polyol-osmotic hypothesis has considerable supporting evidence from animal and whole lens studies, and aldose reductase inhibition does prevent the onset of the diabetes complications in BLECs, it cannot explain the development of diabetic complications in BLECs and many other cells due to the fact that the polyol formed in those cells never reaches a level which can account for the osmotic stress. Other consequences of increased polyol pathway flux might also be important. One suggestion is that the altered redox state of pyridine nucleotides is critical, since pyruvate administration, which restores NAD⁺ levels, can prevent endothelial cell dysfunction (26). Another suggestion is that increased polyol pathway activity results in a decreased myo-inositol pool in the cells.

**Myo-inositol Depletion: Secondary Event?**

Myo-inositol is structurally related to glucose and is present in most animal and plant tissues. The concentration of myo-inositol in the cells is higher than in the extracellular fluid. It mostly comes from the diet but is
also synthesized in the cell from glucose-6-phosphate. It is actively transported across cell membranes (27, 28).

Under hyperglycemic conditions, glucose may compete with myo-inositol for uptake into cells, which accounts for myo-inositol depletion in diabetes (29, 30, 31). However, aldose reductase inhibitors have also been found to block the tissue depletion of myo-inositol, suggesting that there is a link between sorbitol accumulation and myo-inositol depletion (32). Actually, changes in myo-inositol in diabetes are limited to those tissues susceptible to long-term complications and in which the polyol pathway is active. The mechanism by which sorbitol affects the myo-inositol level remains uncertain but may involve diminished myo-inositol transporter function.

Tissue myo-inositol is an intermediate of phosphoinositide turnover, consequently the depletion of myo-inositol lowers the phosphoinositide metabolism and the availability of diacylglycerol and inositol 1,4,5 trisphosphate which are two important second messengers (33). These events lower the ability of protein kinase C to respond to external signals, as a result Na⁺-K⁺-ATPase activity and sodium dependent myo-inositol uptake decline and also the myo-inositol pool is depleted. The above scheme is well demonstrated in nerve (34, 35). However, in vascular tissues and in cell culture, hyperglycemia was associated with an increase in diacylglycerol level due to the increased
de novo synthesis of diacylglycerol from glucose and a corresponding increase in protein kinase C activation (36, 37, 38).

In nerve, myo-inositol depletion lowers protein kinase C activity and consequently decreases Na\(^+\)-K\(^+\)-ATPase activity. Diminished Na\(^+\)-K\(^+\)-ATPase is thought to impede Na\(^+\) extrusion from the nerve cell, the resultant high intracellular Na\(^+\) levels blocking nodal depolarization and slowing nerve conduction velocity. The activation of protein kinase C could be involved in abnormal growth and synthesis in the diabetic vasculature. What does protein kinase C do in lens cells? How is it related to polyol accumulation and myo-inositol depletion?

Protein Kinase C In The Lens

The knowledge of protein kinase C in the lens is very limited. The involvement of protein kinase C in the lens was first reported by Lampe and coworkers in the late 1980s (39, 40). MP26, a protein thought to form gap junctional channels in the lens, and other lens proteins were phosphorylated in isolated membrane by protein kinase C prepared from either bovine brain or lens. MP26 has also been phosphorylated by protein kinase A in vitro. In an in vivo type system, MP26 phosphorylation was detected in lens fiber cell fragments. In these experiments, calcium stimulated the phosphorylation of MP26 approximately fourfold and 12-O-tetradecanoylphorbol 13-acetate (TPA), a known protein kinase C activator,
increased the phosphorylation to sevenfold when calcium was present. MP26 was not a substrate for calcium/calmodulin-dependent protein kinase II, and Walsh inhibitor, which inhibits CAMP-dependent protein kinase, had no influence on the TPA-mediated increase in phosphorylation. These data suggest protein kinase C directly phosphorylates MP26.

Since multiple kinases are involved in MP26 phosphorylation, how these enzymes alter junctional activity in various cell types has been examined. Different groups have proposed that one kinase may increase and another decrease junctional activity. However in the lens, the issue has not been settled yet.

Besides the permeability of MP26 as a gap junction protein, the progressively limited proteolysis of MP26 has been used as an aging marker of mammalian lens. Acceleration of the limited proteolysis of lens fiber MP26 coincided with cataractogenesis of rats maintained on a 50% galactose diet. However, the precise relationship between the phosphorylation state of MP26 and the rate of its limited proteolysis has not been reported. Moreover, protein kinase C activity has not been reported in lens epithelium.

In other cell types, protein kinase C has been found to regulate membrane carrier proteins. For example, protein kinase C phosphorylation of the glucose transporter in vitro and in vivo has been reported in human erythrocytes (41). Induction of the translocation of the insulin-sensitive
glucose carrier (GLUT4) in fat cells by the phorbol ester, TPA, is clearly attributed to protein kinase C activation (42). Due to the structural similarity between glucose and myo-inositol, and the competitive nature of myo-inositol and glucose uptake in lens cells, a linkage between protein kinase C activity and myo-inositol transporter function has been proposed.

The Properties of Protein Kinase C

Protein Kinase C was first characterized in 1977 as a proteolytically activated protein kinase that was capable of phosphorylating histone (43, 44), and later found to be activated reversibly by association with membrane phospholipid in the presence of diacylglycerol and physiological concentrations of Ca\(^{2+}\) (45). Protein kinase C per se is inactive. Its activation depends on Ca\(^{2+}\) as well as phospholipid. Among various phospholipids, phosphatidylserine is absolutely required for the enzyme activation (46). This enzyme is highly specific for Ca\(^{2+}\): none of the other divalent cations tested is able to substitute for Ca\(^{2+}\), except Sr\(^{2+}\) which is only about 10% as potent as Ca\(^{2+}\) at comparable concentrations. Various diacylglycerols are able to activate the enzyme by dramatically increasing the affinity of this enzyme for Ca\(^{2+}\), provided they have 1,2-sn-configuration (47). PKC can also be activated by tumor promoting phorbol esters for which it is the cellular receptor (48). Phorbol esters are
valuable tools for studying the function of protein kinase C, because DAG, the natural physiological activator of PKC is rapidly metabolized and will activate PKC in a transitory fashion while phorbol esters are degraded very slowly and can activate PKC over a longer time (49).

To inhibit the PKC activity, theoretically, at least three classes of inhibitors may exist (50): first, compounds that antagonize the action of diacylglycerol; second, phospholipid-interacting compounds that prevent the activation of the enzyme; and third, compounds that inhibit the catalytically active center of the enzyme. No inhibitor that falls into these three entities is specific for protein kinase C. For instance, W-7 [N(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide], a phospholipid-interacting drug which inhibits PKC also inhibits calmodulin-dependent protein kinases such as myosin light chain kinase by competing with calmodulin (51); H-7[1-(5-isoquinolinesulfonyl)-2-methylpiperazine] profoundly inhibits PKC, however it inhibits protein kinase A as well (52).

A new class of protein kinase C inhibitor emerged in 1987 with the finding that the regulatory domain of PKC contains a "pseudosubstrate" region which is apparently involved in maintaining the enzyme in the inactive state. This made the development of a specific PKC inhibitor possible (53, 54). During the inactive state, the
"pseudosubstrate" region is bent over to occlude the substrate binding site of PKC. When PKC is activated, the conformational change of the PKC moves the "pseudosubstrate" region away from the substrate binding site, allowing the interaction of the substrate with the PKC substrate binding site. Antibodies against the pseudosubstrate sequence activate PKC in the absence of Ca\(^{2+}\) and diacylglycerol (55). Peptides containing this pseudosubstrate sequence are potent inhibitors because the conformational change of the PKC will not affect the binding of these peptides to the substrate binding site. The pseudosubstrate peptide PKC(19-36) has a \(K_i\) of 0.15 uM for PKC with virtually no inhibition of other protein kinases (54).

It has been reported that a synthetic peptide from myelin basic protein (MBP) can act as a specific substrate for PKC (56). Phosphorylation of Ac-MBP(4-14) by PKC and its specific inhibition by PKC(19-36) form the basis of the PKC assay in the present study.

The purpose of the study

This study aimed to answer two questions:

1) Is protein kinase C present in Bovine Lens Epithelial Cells (BLECs)?

2) What is the relationship between protein kinase C activity and myo-inositol uptake during hypergalactosemic conditions in cultured bovine lens epithelial cells?
CHAPTER II

MATERIALS AND METHODS

Cell culture

Bovine (Bos taurus) eyes obtained from a local slaughterhouse were brought on ice to the laboratory where the lenses were removed aseptically. After making incisions on each side of the equator, the anterior capsule of each lens, with its epithelium attached, was peeled away from the cortex and placed in a 60 mm petri dish in 5 ml of a growth medium composed of Eagle's minimal essential medium (MEM) supplemented with 10% calf serum, nonessential amino acids, 5 mg/L ascorbic acid, 20 mg/L gentamycin sulfate and basal medium Eagle vitamin solution. This growth medium contains approximately 10–15 μM myo-inositol, the primary contribution deriving from the vitamin solution. Cells were maintained in a water humidified atmosphere of 5% CO₂-95% air at 37 °C (10). Cell outgrowth after 2-3 weeks from the capsule to the petri dish was dispersed in Ca²⁺-Mg²⁺ free MEM containing 0.125% trypsin-0.05% EDTA and transferred to a 75-cm² culture flask. The cells originating from two to three capsules were placed in each culture flask with 25 ml of growth medium. Upon reaching confluence, the cells were again dispersed and subcultured in a split ratio of 1:10 in 25-cm² culture flasks containing 5 ml of growth medium.
Studies were performed with confluent monolayers in 25-cm$^2$ or 150-cm$^2$ culture flasks (representing second passage cells).

For the measurement of protein kinase C (PKC) activity the BLECs were divided into the following groups: 1) MEM—minimum essential medium containing 5.5 mM glucose; 2) Galactose—glucose-free physiological medium containing 40 mM galactose; and 3) Glucose—physiological medium containing 40 mM glucose. The cultures were preincubated for 20 hr prior to their harvesting for determination of PKC activity. For some experiments, 2 µM 4-β-phorbol 12-myristate 13-acetate (PMA) was administered 5 min prior to cell harvesting to determine its effect on PKC translocation from cytosol to membrane under various conditions.

Following the 20 hr exposure and additional treatments, the cells were washed with 2 X 10 ml basic media for each flask. Cells were briefly trypsinized, suspended in 20 ml of basic media or calcium-free phosphate buffer (D-PBS) containing 0.01% trypsin inhibitor, and centrifuged at 1200 rpm at 4 °C for 10 min in a Beckman table-top centrifuge. The supernatant was discarded, and the cells were washed with 20 ml D-PBS. The pelleted cells were homogenized in cold Buffer A with 15 strokes on ice in a precooled Dounce homogenizer. (Buffer A: Tris-HCl, 20 mM, pH=7.5; EDTA, 0.5 mM; EGTA, 0.5 mM; aprotinin and leupeptin, 25 µg/ml each; and 10 mM β-mercaptoethanol). A fraction of the homogenate
was taken out for PKC assay in order to monitor the purification process in the initial study. The majority of the homogenate was transferred and centrifuged at 100,000 x g at 4 °C for 30 min, yielding soluble (cytosolic) and particulate (membrane) fractions. The soluble fraction was designated as "crude cytosolic enzyme". The particulate fraction was rehomogenized in Buffer A containing 0.5 % Triton X-100, incubated on ice for 30 min, and centrifuged at 100,000 x g for 30 min at 4 °C. The supernatant, containing detergent-solubilized membrane-bound enzyme, was designated as "crude membrane enzyme". Partial purification of the enzyme was achieved by passing either the crude cytosolic enzyme or detergent-solubilized crude membrane enzyme through a DE52 column (bed volume, 0.4 ml) that was equilibrated with Buffer B (20 mM Tris-HCl, pH=7.5; 0.5 mM gwTA; 0.5 mM EGTA and 10 mM β-mercaptoethanol). The column was washed with 1.25 ml of Buffer B, the enzyme eluted with 1.25 ml of Buffer C (Buffer B containing 0.2 M NaCl) and 1.25 ml of 20 mM Tris-HCl was added to make a total volume of 2.5 ml. The eluate was then desalted through a Sephadex G-25 column that was equilibrated with 20 mM Tris-HCl. The enzyme was eluted with 4 ml of 20 mM Tris-HCl (pH=7.5). In the preliminary studies, all 4 ml were fractionated and tested for PKC activity. Knowing from these studies that only the first 3 ml have PKC activity, in the subsequent studies, only the first 3 ml were collected, pooled and
stored on ice 3 hr before assaying PKC activity. This procedure is illustrated in figure 1.

**Protein Kinase C Assay**

The PKC assay is based on the mixed micelle assay of Hannun et al. (57). PKC activity was assayed by measuring the incorporation of $^{32}$P from $[\gamma^{32}$P]ATP into the substrate peptide Ac-MBP(4-14) (Gibco BRL, Gaithersburg, MD). The reaction was carried out at 30 °C in an incubation volume of 50 μl for 5 min. The assay mixture contained the following unless otherwise indicated: ATP, 200 μM; MgCl$_2$, 2 mM; Ca(OAc)$_2$, 200μM; Ac-MBP(4-14) substrate, 25 μM; phosphatidylserine, 16 mol%; 1-oleoyl-2-acetylglycerol (OAG), 8 mol%; enzyme, 5-15 μg/tube; Tris-HCl, 20 mM, pH=7.5 with or without 5 μM PKC(19-36) pseudosubstrate inhibitor peptide (Gibco BRL). About 500,000 cpm of $[\gamma^{32}$P]ATP was added to each test tube. The reaction was started by the addition of ATP and stopped by spotting 25 ul of assay mixture onto phosphocellulose paper (p81, 2x2 cm). Free $^{32}$P-ATP and $^{32}$P-labeled substrate were separated by washing the paper twice with 0.85 % phosphoric acid for 5 min, followed by another two washes with distilled water. The radioactivity retained on the phosphocellulose paper after washing was determined by counting the paper in 10 ml of scintillation fluid (Ecolite, ICN pharmaceuticals, Costa Mesa, CA). PKC activity is determined as the difference
Figure 1: Protein Kinase C Partial Purifications

Cells (preincubated for 20 hrs in 5.5mM glucose or 40mM galactose)
Trypsin
Suspend in the presence of 0.01% trypsin inhibitor
Centrifuge and resuspend in PBS
Centrifuge and homogenize at 4°C
In Buffer A (no Triton X-100)
Centrifuge at 100,000 x g for 30 min at 4°C

Pellet
Homogenize at 4°C
In Buffer A (with Triton X-100)
Store on ice for 30 min
Centrifuge at 100,000 x g for 30 min at 4°C

Supernatant (crude cytosol)
DE-52
Wash with Buffer B
Elute with Buffer C

Cytosol Fraction
Sephadex G-25
20 mM Tris, pH7.5

Cytosol Fraction
Pool fractions 1-3
Store on ice 3 hrs.

PKC assay

Pellet
Supernatant (crude membrane)
DE-52
Wash with Buffer B
Elute with Buffer C

Membrane Fraction
Sephadex G-25
20 mM Tris, pH7.5

Membrane Fraction
Pool fractions 1-3
Store on ice 3 hrs.

PKC assay

Buffer A: 20 mM Tris pH7.5, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml each aprotinin and leupeptin, 10 mM β-mercaptoethanol ± 0.5% Triton X-100

Buffer B: 20 mM Tris pH7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol

Buffer C: 20 mM Tris pH7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.2 M NaCl
between the activities in the absence and presence of the PKC inhibitor. In order to directly compare the ratio of PKC activity in the membrane to that in the cytosol, PKC activity should be normalized to the total protein. Thus a unit of PKC activity was defined as the incorporation of 1 umol of phosphate into the substrate per minute per mg total protein. However, to monitor the purification process, PKC activity was normalized to the mass of substrate protein.

\[^{3}\text{H}\]-Myo-Inositol Uptake

Myo-inositol uptake was determined as follows: The cultures were maintained overnight (20 hr) with and without 40 mM galactose before the addition of myo-\[^{3}\text{H}\]inositol to the serum-supplemented media. The accumulation of myo-\[^{3}\text{H}\]inositol was achieved by incubating the cultured cells in the presence of 0.1 uCi/ml myo-\[^{3}\text{H}\]inositol (94 Ci/mmol; Amersham, Arlington Heights, IL) for up to 10 hr. After isotope incubation, the medium was removed, and the culture flasks were rinsed three times with ice-cold Ca\(^{2+}\)-added phosphate-buffered saline (137 mM NaCl, 8 mM dibasic sodium phosphate, 0.7 mM calcium chloride, pH 7.2) and drained overnight at 4 °C. Five milliliters of 2 % sodium carbonate in 0.1 N sodium hydroxide was added to each flask and left overnight at room temperature to ensure cell lysis. Replicate 1.0 ml aliquots were taken for liquid-scintillation counting (Packard TriCarb 4640, Laguna Hills, CA). Triplicate 25 ul aliquots were taken for protein
determination by the method of Bradford et al. (58) with bovine serum albumin (Sigma, St. Louis, MO) as a standard. Accumulation of $[^3]$H]myo-inositol was expressed as counts per minute (cpm) per milligram of protein in individual culture flasks.
CHAPTER III

RESULTS

Since protein kinase C activity has not been demonstrated in Bovine Lens Epithelial Cell culture before, caution should be taken in concluding the existence of the enzyme in this particular cell. In other words, we have to identify the properties of protein kinase C in this cell type.

The Enzyme Is Phosphatidylserine Dependent

G-25 pooled cytosol and membrane fractions were incubated at 30 °C for 5 min in a reaction mixture of 50 ul. The reaction mixture is the same as described in chapter II except that 50 uM Ac-MBP and 20 uM ATP were used and no PKC inhibitor was applied. Various amounts of phosphatidylserine (PS) were added to the reaction mixture as indicated in Figure 2. Similar dependencies were shown for cytosol enzyme and membrane enzyme. When PS concentrations were lower than 10 mol % (1 mol % = 43 umol), virtually no enzyme activities were detected. The enzyme activities quickly increased when PS concentration became greater than 10 mol % and plateaued at about 15 mol %. The three repeats of this experiment were in excellent agreement with the representative experiment shown in Figure 2. We conclude from this experiment that
Figure 2. Phosphatidylserine Dose Curve. Protein Kinase C activity was determined on the G-25 pooled cytosol (8.9 ug) and membrane (3.4 ug) fractions incubated at 30 °C for 5 min in 10 mM Tris-HCl, pH=7.5 containing 8 mol % 1-oleoyl-2-acetylglycerol (OAG), 50 μM Ac-Myelin Basic Protein (Ac-MBP), 200 μM calcium acetate (CaOAc), 2 mM MgCl₂, 20 μM ATP[Na₂] with trace [γ-³²P]ATP (specific activity, 385 cpm/pmol) and phosphatidylserine (PS) as indicated in a total reaction volume of 50 ul. 1 mol % is equivalent to 43 μM. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
phosphatidylserine is absolutely required for activating the enzyme, and at least 15 mol % PS in the reaction mixture is required to reach maximum activation.

The Enzyme Is Diacylglycerol Dependent

G-25 pooled cytosol and membrane fractions were incubated at 30 °C for 5 min in 10 Mm Tris-Hcl, Ph=7.5 containing 15 mol % PS, 50 uM Ac-MBP, 200 uM CaOAc, 2mM MgCl₂, 20 uM \([\mathrm{Na}_2]\)ATP with trace \(\gamma^3\mathrm{P}\)ATP and OAG (total volume 50 ul) as indicated in Figure 3. Three determinations were performed. One was excluded because of the attempt to prepare very small amount of lipid (20 ul each), causing a big variation. The other two experiments agree with each other. One of the representative experiments is depicted in Figure 3. When no OAG was added, the enzyme had no activity. However, a very small amount of OAG (as little as 4 mol %) dramatically increased the enzyme activity which plateaued at low OAG concentrations.

The Interaction of Phosphotidylserine and 1-Oleoyl-2-Acetylglycerol on Protein Kinase C Activity

To study the combined effect of PS and OAG on PKC activity and to find the best ratio of PS and OAG in the reaction mixture, six different lipid mixtures were prepared from the combination of PS (14, 15, 16 mol %) and OAG (6, 8 mol %). The other components of the reaction mixture were
Figure 3. 1-Oleoyl-2-acetylglycerol dose curve. Protein kinase C activity was determined on the G-25 pooled cytosol (9.3 ug) and membrane (3.4 ug) fractions incubated at 30 °C for 5 min in 10 mM Tris-HCl, pH=7.5 containing 15 mol % PS, 50 uM Ac-MBP, 200 uM CaOAc, 2 mM MgCl₂, 20 uM ATP[Na₂] with trace [γ-³²P]ATP (specific activity, 518 cpm/pmol) and OAG as indicated in a total reaction volume of 50 ul. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
Figure 4. The Interaction of Phosphatidylserine and 1-Oleoyl-2-Acetylglycerol on Protein Kinase C Activity.
Protein kinase C activity was determined on the G-25 pooled cytosol (9.16 ug) and membrane (3.56 ug) fraction incubated at 30 °C for 5 min in 10 mM Tris-HCl, pH=7.5 containing 50 uM Ac-MBP, 200 uM CaOAc, 2 mM MgCl₂, 20 uM ATP[Na₂] with trace [γ-³²P]ATP (specific activity, 518 cpm/pmol) and PS and OAG as indicated in a total reaction volume of 50 ul. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
The Interaction of Phosphatidylserine and 1-Oleoyl-2-Acetylglycerol on Protein Kinase C Activity

![Graphs showing the interaction between phosphatidylserine (PS) and oleoylacylglycerol (OAG) on protein kinase C activity in cytosol and membrane fractions.](image-url)
the same as above. Two experiments were performed. Both supported the same conclusions as shown in Figure 4. It appeared that the combination of 16 mol % PS and 8 mol % OAG is the best one for maximum activation of this enzyme.

The Enzyme Is Calcium Dependent

Both cytosol and membrane G-25 fractions were incubated in a standard reaction mixture as described in chapter II except 20 uM ATP[Na₂] was added, and no inhibitor was used. Calcium acetate concentrations varied from 0 to 800 uM. The result is shown in Figure 5. Apparently, the enzyme activation was heavily dependent upon the calcium ion in the reaction mixture. However, since OAG was present in the mixture, as little as 50 uM of calcium in the mixture would be able to near-maximally activate the enzyme. It was also shown that the relationship between calcium concentration and the enzyme activity was independent of the amount of enzyme added in the test tube.

The above studies not only confirmed the existence of PKC in BLECs, but also created the basis of the PKC assay system. To establish an optimized assay system for PKC, further studies to determine the dependencies of the mgwed micelle assay on other factors were carried ogw as reported below.

Dependencies of Mixed Micelle Assay on other Factors

Pooled G-25 cytosol and membrane fractions were tested for PKC activity by using the method described in chapter II
Figure 5. Calcium Dose Curve. Protein kinase C activity was determined on the G-25 pooled cytosol (6.46 ug and 12.92 ug) and membrane (2.28 ug and 4.56 ug) fractions incubated at 30 °C for 5 min in 10 mM Tris-HCl, pH=7.5 containing 16 mol % PS, 8 mol % of OAG, 25 uM Ac-MBP, 2 mM MgCl$_2$, 20 uM ATP[Na$_2$] with trace [$\gamma$-$^{32}$P]-ATP (specific activity, 523 cpm/pmol) and CaOAc as indicated in a total reaction volume of 50 ul. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
Calcium Dose Curve

**CYTOSOL FRACTION**

![Graph of calcium dose curve for the cytosol fraction.](image)
- **Cytosol:**
  - ○ 6.46 ug protein
  - ● 12.92 ug protein

**MEMBRANE FRACTION**

![Graph of calcium dose curve for the membrane fraction.](image)
- **Membrane:**
  - ○ 2.28 ug protein
  - ● 4.56 ug protein
Figure 6. Ac-Myelin Basic Protein Substrate Peptide Dose Curve. Protein kinase C activity was determined on the G-25 pooled cytosol (11.3 ug) and membrane (4.5 ug) fractions incubated at 30 °C for 5 min in 10 mM Tris-HCl, pH=7.5 containing 16 mol % PS, 8 mol % of OAG, 2 mM MgCl₂, 200 uM CaOAc, 20 uM ATP[Na₂] with trace [γ⁻³²P]ATP (specific activity, 557 cpm/pmol) and Ac-MBP as indicated in a total reaction volume of 50 ul. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
Ac–Myelin Basic Protein Substrate Peptide Dose Curve

![Graphs showing the relationship between AC-MBP concentration and pmol P0₄/min/mg protein for cytosol and membrane fractions.](image-url)
with minor modifications: 20 uM instead of 200 uM ATP[Na_2] was added and no PKC inhibitor was involved in the assay. Various amounts of Ac-MBP were added to the reaction mixture as indicated in Figure 6. The results showed that either 25uM or 50 uM Ac-MBP could be used in the assay. Because of the high cost of this substrate peptide, 25 uM was routinely used in the subsequent studies.

Various amounts of enzyme extract were added to the assay mixture. Figure 7 shows that the PKC specific activity was independent of the amount of PKC protein added within a wide range of protein concentrations (for cytosol, from 5 ug to 20 ug; for membrane, from 1 ug to 9 ug). It also shows no difference between using 25 uM Ac-MBP and using 50 uM Ac-MBP.

Protein kinase C specific activity is expressed as pmol PO_4/min/mg protein. Valid determinations of enzyme activity require that the reaction be linear with respect to time. Time-dependent protein kinase C activity was assessed by using the reaction mixture described in chapter II. Again 20 uM ATP instead of 200 uM was used in this study. Figure 8 shows that under this condition, 5 min would be the limit of linearity. Accordingly, the reaction time throughout the entire study was set at 5 min.

The optimum concentration of ATP in the reaction mixture was also tested. As expected, PKC specific activity increases with the increased ATP concentration (Figure 9).
Figure 7. Enzyme Dose Curve. Protein kinase C activity was determined on the G-25 pooled cytosol and membrane fractions as indicated incubated at 30 °C for 5 min in 10 mM Tris-HCl, pH=7.5 containing 16 mol % PS, 8 mol % of OAG, 2 mM MgCl₂, 200 µM CaOAc, 20 µM ATP[Na₂] with trace [γ-³²P]ATP (specific activity, 539 cpm/pmol) in a total reaction volume of 50 ul. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
Enzyme Dose Curve

For Ac-MBP:
- 25 uM
- 50 uM

Graphs show the pmol P04/min/mg protein as a function of protein-cyto (ug) and protein-memb (ug).
Figure 8. Time Dependent Protein Kinase C Activity. Protein kinase C activity was determined on the G-25 pooled cytosol (15.82 ug) and membrane (5.20 ug) fractions incubated at 30 °C for periods indicated with 10 mM Tris-HCl, pH=7.5 containing 16 mol % PS, 8 mol % of OAG, 25 uM Ac-MBP, 2 mM MgCl$_2$, 200 uM CaOAc, 20 uM ATP[Na$_2$] with trace [7-$^{32}$P]ATP (specific activity, 460 cpm/pmol) in a total reaction volume of 50 ul. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
Figure 9. Adenosine Triphosphate Dose Curve. Protein kinase C activity was determined on the G-25 pooled cytosol (12.4 ug) fraction incubated at 30 °C for periods indicated with 10 mM Tris-HCl, pH=7.5 containing 16 mol % PS, 8 mol % of OAG, 25 μM Ac-MBP, 2 mM MgCl$_2$, 200 μM CaOAc, and ATP[Na$_2$] as indicated. [γ-$^{32}$P]ATP (460,000cpm/test tube) was added in a total reaction volume of 50 ul. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
Adenosine Triphosphate Dose Curve

![Graph showing Adenosine Triphosphate Dose Curve with ATP (uM) on the x-axis and pmol PO₄/min/mg protein on the y-axis.]
Figure 10. Magnesium Dose Curve. Protein kinase C activity was determined on the G-25 pooled cytosol (6.03 ug and 12.06 ug) fractions incubated at 30 °C for periods indicated with 10 mM Tris-HCl, pH=7.5 containing 16 mol % PS, 8 mol % of OAG, 25 uM Ac-MBP, 200 uM CaOAc, 200 uM ATP[Na₂] with trace [γ-³²P]ATP (specific activity, 116 cpm/pmol) and MgCl₂ as indicated in a total reaction volume of 50 ul. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
Megnesium Dose Curve

![Graph showing the effect of magnesium chloride (MgCl₂) on protein phosphatase activity. The x-axis represents MgCl₂ (mM) concentration ranging from 0.0 to 20.0, and the y-axis represents pmol P₀₄/min/mg protein. Two curves are depicted: one for cyto 6.03 ug and another for cyto 12.06 ug. The curves show an initial increase in activity followed by a decrease as the MgCl₂ concentration increases.]
The optimum ATP concentration would be 200 uM while other factors were fixed.

The effect of magnesium cation on PKC activity was also tested. Figure 10 shows the lens PKC was dependant on the presence of magnesium with 2 mM MgCl₂ giving optimum activity.

The efficacy of PKC(19-36) in inhibiting PKC activity was tested. Even at the lowest concentration tested, 2.5 uM, PKC(19-36) produced a maximum inhibition (Figure 11). To ensure the maximum inhibition of PKC in the later studies, 5 uM PKC(19-36) was routinely applied.

Partial Purification of Protein Kinase C

PKC specific activities in various stages of purification were tested. The results are listed in Table 1. There was a 30-fold increase in the specific activity of the cytosolic enzyme of fraction 2, and a greater than 50-fold enrichment in the activity of the membrane-bound form of fraction 1 as compared to the activities in the original homogenate. Fraction 4 of both cytosol and membrane G-25 eluate contained no PKC activity. In subsequent experiments the first three fractions of the G-25 column were pooled before the enzyme activity was determined. This table also shows that cells growing under physiological conditions have a greater proportion of PKC activity in the cytosol than in the membrane.
Figure 11. Protein Kinase C Inhibitor Peptide PKC (19-36) Dose Curve. Protein kinase C activity was determined on the G-25 pooled cytosol (12.06 ug) and membrane (4.28 ug) fractions incubated at 30 °C for periods indicated with 10 mM Tris-HCl, pH=7.5 containing 16 mol % PS, 8 mol % of OAG, 25 uM Ac-MBP, 200 uM CaOAc, 2 mM MgCl₂, 200 uM ATP[Na₂] with trace [γ-³²P]ATP (specific activity, 121 cpm/pmol) and PKC (19-36) as indicated in a total reaction volume of 50 ul. The results shown are the mean ± SEM of duplicate determinations from a representative individual experiment.
Protein Kinase C Inhibitor Peptide PKC(19–36) Dose Curve
### Table 1: Partial Purification of Protein Kinase C

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (pmol/min)</th>
<th>Specific Activity (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.90</td>
<td>12.34</td>
<td>887</td>
<td>72</td>
</tr>
<tr>
<td>Crude cytosol</td>
<td>1.70</td>
<td>6.33</td>
<td>2198</td>
<td>347</td>
</tr>
<tr>
<td>DE52 cytosol</td>
<td>1.25</td>
<td>2.73</td>
<td>741</td>
<td>271</td>
</tr>
<tr>
<td>G-25 cytosol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 1</td>
<td>0.50</td>
<td>0.22</td>
<td>183</td>
<td>830</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>0.50</td>
<td>0.54</td>
<td>1185</td>
<td>2194</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>2.00</td>
<td>1.80</td>
<td>379</td>
<td>210</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>1.00</td>
<td>0.02</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>homogenate</td>
<td>2.00</td>
<td>8.02</td>
<td>64</td>
<td>8</td>
</tr>
<tr>
<td>Crude membrane</td>
<td>1.83</td>
<td>4.14</td>
<td>67</td>
<td>16</td>
</tr>
<tr>
<td>DE52 membrane</td>
<td>1.25</td>
<td>1.03</td>
<td>60</td>
<td>58</td>
</tr>
<tr>
<td>G-25 membrane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 1</td>
<td>0.50</td>
<td>0.01</td>
<td>5</td>
<td>445</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>0.50</td>
<td>0.11</td>
<td>23</td>
<td>210</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>2.00</td>
<td>0.58</td>
<td>75</td>
<td>129</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>1.00</td>
<td>0.01</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. = Not Detected
Table 2. Comparison of GIBCO Kit to Optimum Assay

<table>
<thead>
<tr>
<th></th>
<th>GIBCO Kits</th>
<th></th>
<th>Established</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEM</td>
<td>GAL</td>
<td>MEM</td>
<td>GAL</td>
</tr>
<tr>
<td>n</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Membrane</td>
<td>23±3</td>
<td>32±2</td>
<td>98±16</td>
<td>85±19</td>
</tr>
<tr>
<td>Cytosol</td>
<td>190±11</td>
<td>249±33</td>
<td>727±142</td>
<td>978±205</td>
</tr>
<tr>
<td>Total</td>
<td>213±14</td>
<td>281±34</td>
<td>825±156</td>
<td>1063±222</td>
</tr>
<tr>
<td>% Membrane</td>
<td>10.9±0.8</td>
<td>11.5±1.2</td>
<td>13.5±1.8</td>
<td>8.3±0.9</td>
</tr>
</tbody>
</table>

The results are given as the mean ± SEM.

unit= pmol P0₄/min/mg total protein

MEM = Minimal Essential Medium (5.5 mM glucose)

GAL = 40 mM galactose

The cell cultures were incubated for 20 hr in either MEM or GAL prior to harvesting the cells for the PKC assay.
Comparison of Commercial Kit vs. Optimum Assay

The assay developed in this study is similar to the commercially available kit from Gibco BRL. The profiles of enzyme activity for both the kit and the optimum assay developed in this study were compared. Table 2 shows that the optimum assay significantly enhances the apparent PKC activity compared to the commercial kit, while the percentage of the membrane activity remains the same.

Galactose and Glucose Effects on Protein Kinase C Activity

PKC activity was measured under three conditions: 20 hr preincubation in 1) MEM--physiological medium containing 5.5 mM glucose; 2) Galactose--glucose-free physiological medium containing 40 mM galactose; and 3) Glucose--physiological medium containing 40 mM glucose. Both cytosolic and membrane-bound fractions were measured. Since the specific activity was normalized to total protein, the total PKC activity can be obtained by summing the membrane and cytosolic PKC activities. Hence the percentage of PKC activity residing in the membrane fraction, an important index of the enzyme activation, could be obtained. Table 3 compares the membrane, cytosol and total PKC activities in the cells (BLECs) under three incubation conditions. The PKC specific activity was expressed as pmol PO₄ transferred per min per mg total protein. The percentage of PKC activity in membrane was also compared. Two-tailed independent t-tests indicate no significant effects of galactose and glucose on
<table>
<thead>
<tr>
<th>Membrane</th>
<th>Cytosol</th>
<th>Total</th>
<th>% Membrane</th>
<th>size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>98±16</td>
<td>727±142</td>
<td>825±156</td>
<td>13.5±1.8</td>
</tr>
<tr>
<td>GAL</td>
<td>85±19</td>
<td>978±205</td>
<td>1063±222</td>
<td>8.3±0.9</td>
</tr>
<tr>
<td>GLU</td>
<td>86±42</td>
<td>637±321</td>
<td>723±364</td>
<td>12.4±0.7</td>
</tr>
</tbody>
</table>

The results are given as the mean ± SEM.

unit = pmol PO₄ /min/mg total protein

MEM = Minimum Essential Medium (5.5 mM glucose)

GAL = 40 mM galactose

GLU = 40 mM glucose

The cell cultures were incubated for 20 hr in MEM, GAL or GLU medium prior to harvesting the cells for the PKC assay.
Table 4. Protein Kinase C Activity in Galactose and Glucose Medium Compared to in Minimum Essential Media by Independent t-Test

<table>
<thead>
<tr>
<th></th>
<th>MEM</th>
<th></th>
<th></th>
<th>GAL</th>
<th></th>
<th></th>
<th>GLU</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>t value</td>
<td>P value</td>
<td></td>
<td>t value</td>
<td>P value</td>
<td></td>
<td>t value</td>
<td>P value</td>
</tr>
<tr>
<td>Membrane</td>
<td>---</td>
<td>0.52</td>
<td>0.61</td>
<td>NS</td>
<td>0.36</td>
<td>0.73</td>
<td>NS</td>
<td>0.38</td>
<td>0.72</td>
</tr>
<tr>
<td>Cytosol</td>
<td>---</td>
<td>-1.04</td>
<td>0.32</td>
<td>NS</td>
<td>0.30</td>
<td>0.77</td>
<td>NS</td>
<td>0.30</td>
<td>0.76</td>
</tr>
<tr>
<td>Total</td>
<td>---</td>
<td>-0.90</td>
<td>0.39</td>
<td>NS</td>
<td>0.31</td>
<td>0.76</td>
<td>NS</td>
<td>0.31</td>
<td>0.76</td>
</tr>
<tr>
<td>% Membrane</td>
<td>---</td>
<td>2.17</td>
<td>0.53</td>
<td>NS</td>
<td>0.38</td>
<td>0.72</td>
<td>NS</td>
<td>0.38</td>
<td>0.72</td>
</tr>
</tbody>
</table>

The sample sizes in each group are MEM, 8; GAL, 5; GLU, 3.

MEM = Minimum Essential Medium (5.5 mM glucose)

GAL = 40 mM galactose

GLU = 40 mM glucose

Independent two-tailed t-test was applied to compare GAL to MEM, GLU to MEM.

NS = not significant at 0.05 level.
the membrane-bound, cytosolic, or total PKC activities and the proportion of the PKC activity in the membrane at the level of significance of 0.05 (Table 4).

Phorbol Ester-Stimulated Translocation

Phorbol 12 myristate 13-acetate (PMA) is a known activator of PKC in tissue or cell preparations. Following the activation, the enzyme translocates from the cytosol to the plasma membrane (59). The BLECs, preincubated overnight under various conditions as described in Chapter II, were treated with 2 μM PMA for 5 min before harvest to determine the activation of PKC by PMA. Following this treatment, there was a more than six-fold increase in the membrane-bound enzyme in cells either maintained in physiological media or treated overnight with 40 mM galactose or 40 mM glucose (Table 5). However, the two-tailed independent t-test reveals that there was no significant difference (0.05 level) in the total PKC activity in the BLECs before and after PMA treatment in all three incubation media (Table 6). This result clearly indicates that PKC translocates from cytosol to membrane after PMA stimulation in BLECs as in other systems in the physiological, hypergalactosemic, and hyperglycemic conditions. This phenomenon provides a very useful tool for studying the regulation of certain biological functions by PKC.
Table 5. The Effect of Hyperglycemic Conditions on Protein Kinase C Activity in the Presence and Absence of Phorbol Myristic Acid

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Cytosol</th>
<th>Total</th>
<th>% Membrane</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>98±16</td>
<td>727±142</td>
<td>825±156</td>
<td>13.5±1.8</td>
</tr>
<tr>
<td>M/P</td>
<td>780±210</td>
<td>273±77</td>
<td>1053±280</td>
<td>74.4±3.0</td>
</tr>
<tr>
<td>G</td>
<td>85±19</td>
<td>978±205</td>
<td>1063±222</td>
<td>8.3±0.9</td>
</tr>
<tr>
<td>G/P</td>
<td>538±178</td>
<td>384±162</td>
<td>922±329</td>
<td>64.5±6.2</td>
</tr>
<tr>
<td>L</td>
<td>86±42</td>
<td>637±321</td>
<td>723±364</td>
<td>12.4±0.7</td>
</tr>
<tr>
<td>L/P</td>
<td>1029±36</td>
<td>381±2</td>
<td>1410±38</td>
<td>73.0±0.6</td>
</tr>
</tbody>
</table>

Data are mean ± SEM.
unit = pmol PO₄ ⁄min⁄mg total protein
M = Minimal Essential Medium (MEM, 5.5 mM glucose)
M/P = MEM + 5 min 2 uM PMA treatment
G = 40 mM galactose
G/P = 40 mM galactose + 5 min 2 uM PMA treatment
L = 40 mM glucose
L/P = 40 mM glucose + 5 min 2 uM PMA treatment

The cells were incubated for 20 hr in either MEM or GAL or GLU prior to PMA treatment and harvest for PKC assay.
Table 6. Comparison of Protein Kinase C Activity in the Presence and Absence of Phorbol Myristic Acid by Independent Two-Tailed t-Test

<table>
<thead>
<tr>
<th>Membrane</th>
<th>cytosol t</th>
<th>cytosol p</th>
<th>total t</th>
<th>total p</th>
<th>% membrane t</th>
<th>% membrane p</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>M/P</td>
<td>-3.47</td>
<td>0.004 S</td>
<td>2.70</td>
<td>0.018</td>
<td>-0.74</td>
<td>0.48 NS</td>
</tr>
<tr>
<td>G</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>G/P</td>
<td>-2.52</td>
<td>0.036 S</td>
<td>2.27</td>
<td>0.053 NS</td>
<td>0.36</td>
<td>0.73 NS</td>
</tr>
<tr>
<td>L</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>L/P</td>
<td>-15.5</td>
<td>0.001 S</td>
<td>0.62</td>
<td>0.581 NS</td>
<td>-1.46</td>
<td>0.24 NS</td>
</tr>
</tbody>
</table>

M = Minimal Essential Medium (MEM, 5.5 mM glucose)
M/P = MEM + 5 min 2 uM PMA treatment
G = 40 mM galactose
G/P = 40 mM galactose + 5 min 2 uM PMA treatment
L = 40 mM glucose
L/P = 40 mM glucose + 5 min 2 uM PMA treatment
t = t value
p = p value
S: significant (p < 0.05); NS: not significant

The cells were incubated for 20 hr in MEM, GAL or GLU prior to PMA treatment and harvesting the cells for the PKC assay.
Protein Kinase C Activators and Myo-Inositol Uptake

Induction of the translocation of the insulin-sensitive glucose carrier (GLUT4) in fat cells by the phorbol ester TPA is clearly attributed to protein kinase C activation (42). Due to the structural similarity between glucose and myo-inositol, and the competitive nature of myo-inositol and glucose uptake in lens cells, a linkage between protein kinase C activity and myo-inositol transporter function has been proposed. In BLECs, PMA increases the PKC activity in the cells by stimulating the translocation of PKC from cytosol to the membrane. The effects of activating PKC with phorbol esters on myo-inositol uptake were examined to determine if PKC plays any role in regulating myo-inositol transport in BLECs. Figure 12 shows that there was no significant change in \([^3H]\)myo-inositol uptake in cells maintained in physiological media as compared to cells exposed to PMA for 5 min. Although it was not tested in BLECs that mezerein (a non-phorbol activator of PKC) and SC-10 stimulate PKC translocation, they are all known PKC activators. The effects of activating PKC with mezerein and SC-10 on myo-inositol uptake were also examined, but no significant changes were found (Figure 12). Cells incubated in 40 mM galactose demonstrated a significant reduction in \([^3H]\)myo-inositol uptake, however, the PKC activators,
Figure 12. Effect of Protein Kinase C activators on $[^3H]$-Myo-inositol uptake. Confluent monolayers of bovine lens epithelial cells (25 cm$^2$ flasks) were maintained in galactose-free physiological medium (MEM, 5.5 mM glucose) or 40 mM galactose (Gal) for 20 hr. At the end of 20 hr, either phorbol 12-myristate 13-acetate (PMA, 2uM), N-heptyl-5-Cl-NAPH-thalene-1-sulfonamide (SC-10, 2 uM) or Mezerein (Mez, 2 uM) was added in fresh medium for 5 min prior to rinsing the cells, followed by the addition of $[^3H]$-myo-inositol (0.2 uCi/ml) and the incubation continued. Triplicate cultures were collected at the designated times and the data expressed as cpm/mg protein. The results represent the mean ± SEM.
phorbol or non-phorbol alike, failed to normalize this attenuated myo-inositol transport (Figure 12).

The Effect of Prolonged Exposure of Bovine Lens Epithelial Cells to Phorbol Myristic Acid

It has been reported that the prolonged exposure of cells to phorbol ester would cause a reduction of PKC activity (60). Although the excess PMA was washed out after 5 min exposure, the intercalated PMA on the membrane may have a prolonged effect. The PKC activity in BLECs collected 8 hours (2 hours longer than the above experiments as shown in Figure 12) after 5 min PMA treatment was compared to that collected immediately after 5 min exposure. The results were not conclusive. Two experiments showed a remarkable decrease in the percentage of PKC activity associated with the membrane fraction after 8 hour PMA treatment: from 81.1% to 45.1% and from 62.9% to 9.5% respectively. But another experiment showed no decrease: the percentages of PKC activity in the membranes were 75.8% and 82.2% respectively. In all three experiments, however, the total PKC activities in the cells were not changed significantly.
CHAPTER IV

DISCUSSION

Protein kinase C plays a key role in regulating cellular functions in many cell types (61,62,63). Its appearance in whole lens has been reported (39). In lens epithelium, however, PKC activity had not been reported prior to study.

Protein kinase C per se is normally inactive. Its activation requires Ca^{2+} and phospholipid. However, when diacylglycerol is present, the affinity of PKC for Ca^{2+} is increased. This allows the enzyme to be activated at physiological Ca^{2+} concentrations. In living cells, diacylglycerol is produced in membranes from inositol phospholipids in a signal-dependent fashion. It has been shown that a wide variety of hormones, neurotransmitters, and many other biologically active substances provoke the breakdown of phosphatidylinositol-4,5-bisphosphate (PIP_2), and immediately produce 1,2-diacylglycerol and inositol-1,4,5-trisphosphate (IP_3) (64). 1,2-diacylglycerol is one of the PKC activators. IP_3 mobilizes the Ca^{2+} in the cell, which produces physiological responses and also it could take part in PKC activation. The PIP_2 is regenerated by inositol phospholipid turnover, subsequently diacylglycerol converts to CDP-diacylglycerol and IP_3 further degrades to myo-
inositol, these two intermediates reassemble to form phosphatidylinositol (PI), which is converted to PIP and PIP$_2$ by phosphorylation (65).

Theoretically, any change in an intermediates' pool size may alter the rate of its turnover. Since inositol phospholipid turnover occurs in lens tissue (66) as well as in lens epithelial cell culture, and the hyperglycemic condition attenuates the active uptake of myo-inositol (67), the alteration of PKC activity in lens cells during hyperglycemia could play a role in regulating or perturbing lens epithelial function.

Protein kinase C shares many characteristics with other kinases. Ca$^{2+}$, which is required for PKC activation, is also required for activation of calmodulin-dependent protein kinase. H-7, which is commercially available as a protein kinase inhibitor, also inhibits protein kinase A. Since protein kinase C has not been reported to be present in lens epithelium, a careful assessment is required.

In this study, phospholipid, calcium and diacylglycerol dependency of the enzyme eluted from a G-25 column was measured in both the cytosol and membrane portions from bovine lens epithelial cells in culture using a mixed micelle assay and a selective PKC substrate and PKC pseudosubstrate inhibitor. The results duplicated all the known properties of this enzyme, and provided convincing
evidence that PKC activity is indeed present in bovine lens epithelial cells.

As the dependency of this enzyme on various factors and substrates was determined, an optimum assay for PKC was also developed. This assay was compared to a commercially available kit from Gibco BRL. It revealed that the optimum assay yields higher specific activities than the Gibco Kit, but the qualitative responses were similar (Table 2). These results suggest that the establishment of optimum conditions for each tissue is important, especially when low PKC activity is expected.

Of special interest, protein kinase C was found to be strikingly magnesium-dependent (Figure 10). A likely explanation for this finding is that Mg\textsubscript{2}ATP rather than unchelated ATP alone serves as the true substrate for protein kinase C. An increased magnesium concentration in the reaction mixture up to 2.5 mM will convert more ATP molecules to the active substrate Mg\textsubscript{2}ATP. However, once all the ATPs are chelated, the excess magnesium may block or hinder the access of Mg\textsubscript{2}ATP to the active site of the enzyme, causing a decrease in enzyme activity.

Given the definitive finding that protein kinase C exists in bovine lens epithelial cells and having established an optimum assay for this enzyme, protein kinase C activity of BLECs under normal and hyperglycemic conditions was determined.
Under the normal physiological conditions, approximately 10% of the total enzyme activity was recovered in the membrane fraction of bovine lens epithelial cells in culture (Table 3). The cellular distributions of PKC in cells incubated in 40 mM glucose or galactose were similar to those in physiological media (Table 3), suggesting that the hyperglycemic condition does not alter its cytoplasmic to membrane distribution. The percentage of PKC activity recovered in the membrane fraction was small in all three incubation conditions (about 10%). At least part of these activities could be attributed to mass action during the partial purification procedure. This means that under normal conditions, most of the protein kinase C in the cells remains inactive or at least not constitutively active. Hyperglycemic conditions do not alter the cellular PKC distribution which indicates 20 hr exposure of bovine lens epithelial cells to hyperglycemia does not produce any endogenous activator that will permanently bind PKC to the membrane.

Neither 40 mM glucose nor 40 mM galactose significantly altered the total PKC activity significantly in the cells (Table 3,4). It is emphasized that the assay was conducted under the optimized condition which may not resemble any true intracellular environment under normal or hyperglycemic conditions. There are two alternative explanations for the absence of glucose or galactose effects on PKC activity: a)
the average mass of PKC per cell and the activity of each individual PKC do not change under hyperglycemic conditions, or b) the average mass of total PKC per cell does change due to increased rate of enzyme synthesis or decreased rate of enzyme degradation while the individual enzyme activities change in opposite directions. The latter is unlikely and difficult to prove. The present studies cannot definitively prove or disprove the two different possible consequences of hyperglycemic conditions on PKC activation.

It is safe to assume that the average PKC mass per cell remains unaltered after one day of hyperglycemic exposure and the efficacy of enzyme activation under the same standard conditions remains the same. It is important to determine whether the ability of the enzyme to translocate from cytosol to membrane and become active during endogenous stimulation remains constant. Tables 5 and 6 showed a remarkable increase in PKC activity recovered in membrane fractions, and no significant differences in total PKC activity after a 5 min 2 uM PMA treatment in all three incubation conditions.

Independent t-tests were also conducted to compare PKC activity in MEM with PMA treatment (MP) to that in 40 mM glucose and in 40 mM galactose with PMA treatment (GP and LP respectively). When comparing MP to GP, p values for membrane, cytosol, total, and % membrane PKC activities were 0.56, 0.27, 0.24, 0.43, respectively. When comparing MP to
LP, p values of 0.33, 0.88, 0.27, 0.45 were obtained respectively. Thus, no differences were found between MP and the two high-sugar conditions. This means that one day exposure to hyperglycemic conditions does not change the ability of PKC to be translocated following stimulation. This suggests that the metabolic damage produced by the short-term high ambient glucose or galactose in BLECs cannot be attributed to the inability of PKC to translocate.

It has been demonstrated that lens epithelial cells in culture display a reduced capability to take up myo-inositol in the presence of elevated galactose (13) or glucose (68, 69). This hyperglycemia-induced reduction in myo-inositol uptake has also been described in other tissues (70, 71, 72). A depletion of myo-inositol from cells could lead to a deficit in myo-inositol-containing phospholipids, which could severely attenuate diacylglycerol and inositol trisphosphate release. Alterations in diacylglycerol formation would ultimately lead to a decrease in PKC activation and a reduction in activated PKC substrates. In this study, the activation of PKC by phorbol esters had no effect on the uptake of myo-inositol in the presence or absence of hyperglycemic conditions. This is similar to what has been reported for phorbol effects on myo-inositol uptake in rat aorta (73), suggesting that PKC may not play a role in regulating the myo-inositol transporter.
It may be argued that the downregulation of PKC contributed to this result because even after washing out the excess PMA after a 5 min exposure, the intercalated PMA on the membrane may have a prolonged effect. PKC activity in BLECs collected 8 hours (2 hours longer than the above experiments as shown in figure 12) after a 5 min PMA treatment was compared to that collected immediately after a 5 min exposure. The results suggest that 8 hours might be the critical point in terms of maintaining or decreasing membrane PKC activity. Since 6 hours was the longest incubation period in myo-inositol uptake experiments, the above result (Figure 12) was not due to a reduction of membrane PKC activity.

In conclusion, this study demonstrates the presence of PKC activity in both cytosolic and membrane fractions of bovine lens epithelial cells in culture. The PKC activity is similar in normal and hyperglycemic cells. Furthermore, the ability of the enzyme to translocate from the cytosol to the membrane following phorbol ester treatment is unimpeded by hyperglycemic conditions. Moreover, PKC activation had no effect on myo-inositol uptake either in normal cells or in cells exposed to hyperglycemic conditions. This study suggests that the high ambient glucose-induced or galactose-induced attenuation in myo-inositol concentrating ability appears to be unrelated to PKC activity in cultured bovine lens epithelial cells.
However, the role of PKC in hyperglycemic BLECs is not a dead issue. This study should not be taken to imply that the PKC activity in intact cells will still remain unaltered under hyperglycemic conditions. In fact, this study has shown that protein kinase C activation is affected by ATP concentrations in reaction mixtures (Figure 9). It has been shown that ATP is depleted after prolonged exposure of tissue to high ambient sugar concentration, although it has not been directly tested in bovine lens epithelial cells. Among the other defects in hyperglycemic lens, the depletion of ATP would be expected to decrease protein kinase C activity and thus may alter cellular function.

The findings of this study should provide a beginning toward defining the precise role of protein kinase C in the hyperglycemic bovine lens epithelium.
BIBLIOGRAPHY


13. Cammarata, P.R., Tse, D. and Yorio, T. (1990) Sorbinil prevents the hypergalactosemic-induced reduction in


40. Lampe, P.D. and Johnson, R.G. (1989) Phosphorylation of MP26, a lens junction protein, is enhanced by


inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry, 23, 5036-5041.


59. Kraft, A.S. and Anderson, W.B. (1983) Phorbol esters increase the amount of Ca²⁺, phospholipid-dependent


pericytes by glucose and reversal by sorbinil.

Effect of galactose and glucose levels and sorbinil
treatment on myo-inositol metabolism and Na^+-K^+ pump
activity in cultured neuroblastoma cells. Diabetes, 38,
996-1004.

Inositol uptake in rat aorta. Life Sciences, 46, 1715-
1725.