To the Graduate Council:

I am submitting herewith a dissertation written by Michael Henry Bast entitled "Protein Kinase C and Control of the A System Amino Acid Transporter." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

John S. Cook

John S. Cook, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

PROTEIN KINASE C AND CONTROL OF THE A SYSTEM AMINO ACID TRANSPORTER

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Michael Henry Bast

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TO MY PARENTS WHO ENCOURAGED ME

AND MY WIFE, WHO SUPPORTED ME

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ABSTRACT

The activity of the A System amino acid transporter correlates with the growth state of the cell. Rapidly growing LLC-PK_1 cells have a high rate of A System transport activity, and confluent, differentiated LLC-PK_1 cells have a low rate of A System transport activity. Differentiated LLC-PK_1 cells treated with tumor promoters such as TPA will increase A System transport activity. Addition of the diphenolic compounds quercetin, phloretin and diethylstilbestrol to LLC-PK_1 cells will prevent the TPA stimulation of A System transport. This is shown to be due to inhibition of protein kinase C, part of a major signal transduction pathway, and not to an interaction with the transporter. Active protein kinase C is only required for approximately 10 % of the time needed to generate a TPA response, indicating a possible cascade reaction is involved in TPA stimulation.

Changes in transport were shown to be due to changes in the V_{max} of transport, indicating that transport is controlled by changing the number of active transporters, rather that individual transporter activity. This was shown for transport changes due to growth state, TPA stimulation of differentiated LLC-PK_1 cells, or inhibition of protein kinase C in rapidly growing cells by quercetin.

Polyclonal antisera were raised against the regulatory and catalytic domains of protein kinase C to determine if the calpain-generated kinase fragment, PKM, has a role in stimulation of A System transport activity. Although the antibodies react with protein kinase C in solution, and will react on immunoblots with protein kinase C from pig or rat brain, the antisera do not react with an appropriate

molecular weight band in LLC-PK_1 cells. Instead, bands were seen at positions similar to those reported for a newly discovered protein kinase C isotype, PKC ζ.

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CHAPTER 1

INTRODUCTION

The History of the A System Transporter

In 1913 Van Slyke and Meyer discovered animal tissues maintain significant intracellular pools of amino acids that can accumulate against a concentration gradient (Van Slyke and Meyer, 1913). Fifty years later, the existence of a specific molecule or molecular complex for transporting small, neutral amino acids across the cell membrane of Ehrlich Ascites Tumor cells was first postulated by Oxender and Christensen (1963). They named this the A System ("alanine preferring"), and differentiated it from the L System which transports large, branched chain neutral amino acids. They defined transport over the A System through the capacity of amino acids to inhibit the concentrative uptake of another amino acid, such as alanine. On the basis of competition experiments, alanine, glycine, serine, threonine, proline, asparagine, glutamine and methionine were assigned to the A System, while leucine, isoleucine, valine and methionine were assigned to the L System.

As was first indicated by the transport of methionine by both the A System and L System, many of the amino acid transporters have overlapping substrate specificities. The definition of the A System has since been refined to include

small, neutral, straight chained amino acids which are transported in a Na^+-dependent manner (for reviews, see Guidotti et. al.; 1978, Shotwell et. al., 1983). Na^+-dependent transport refers to a requirement of transport for both an inward directed

Na^+ gradient in the cells, and also of a membrane potential created and maintained by the established Na^+ gradient. Thus, this combination of membrane potential and chemical gradient is often referred to as the Na^+ electrochemical gradient.

Later work by Christensen et. al. (1967) led to the proposal of another amino acid transport system, System ASC, named for its role in the transport of alanine, serine and cysteine. It is also responsible for transport of threonine and hydroxyproline. System ASC can be distinguished from the A System by pH insensitivity and the inability of System ASC to transport N-methylated derivatives (Christensen et. al., 1967). System ASC is believed to be ubiquitous in avian and mammalian systems (For review, see Guidotti et. al., 1978).

Another amino acid transporter with a substrate preference overlapping that of the A System is System Gly (Vidaver et. al., 1964). System Gly appears to be a Na^+-dependent amino acid transporter specific for glycine and the N-methylated glycine derivative, sarcosine.

Asparagine and glutamine, originally described as A System substrates (Oxender and Christensen, 1963), as well as histidine, have been found to be transported by a different transport system in rat hepatocytes, called system N for

the nitrogen in the side chain of substrate amino acids (Kilberg et. al., 1980; Handlogten et. al., 1982). System N is also Na^+-dependent, and responds to substrate deprivation similarly to the A System, but appears to be a transport system found solely in liver (Kilberg, 1982).

Due to the broad and overlapping substrate specificity and widespread distribution of most amino acid transporters, the development of approaches to discriminate among transport systems in isolated cells or tissues was one of the primary aims in early research on the regulation of amino acid transport. Because amino acids can enter the cell through separate transport systems, it is only through the use substrates specific for a single transporter that problems of transporter specificity and transporter regulation can be easily addressed. The A System, unlike System ASC, will transport N-methylated amino acid derivatives (Christensen et. al., 1965); one of these, methylaminoisobutyric acid, (MeAIB) is a specific substrate for the A System (Christensen et. al., 1965). MeAIB will competitively inhibit the uptake of A System substrates (Christensen et. al., 1965). Through the use of MeAIB it is possible to investigate changes in transport by the A System alone, without having to correct for simultaneous transport through any of the other amino acid transport systems.

Direct examination of the A System transporter or its regulation is complicated by the fact that no specific probe for the transporter exists. While MeAIB is a specific transport substrate, it cannot be used to label the transporter molecule. The transporter itself has not been isolated and sequenced, and the gene

has not been found. However, progress is being made in both of these areas, with reports of reconstitution of A System transport in membrane vesicles (Columbini and Johnstone, 1974; Lever et. al., 1984; McCormick et. al., 1984; McCormick et. al., 1985) and recently, proteoliposomes (Bracy et. al., 1987; Quesada and McGiven, 1988; McCormick and Johnstone, 1988; Fafournoux et. al., 1989). An antibody generated against a major component of a preparation of membrane vesicles with active A System transport (McCormick and Johnstone, 1988) has been used to identify a protein with a molecular mass of 120-130 kDa as potentially a major part of the A System transporter, but explicit proof, or a protein sequence, has not yet been obtained.

An attempt at isolating the A System transporter cDNA by fractionation of total poly(A^+) mRNA and expression of the mRNA in *Xenopus* oocytes has been made by Kilberg's laboratory (Tarnuzzer et. al., 1990). Using mRNA from rat liver, CHO-K_1 cells (a subclone of a transport mutant, ala^r4, which overexpresses A System activity (Moffett and Englesberg, 1984)) and mRNA from human placenta, they found that transport activity correlated with a 2.0-2.5 kb mRNA fraction. This mRNA fraction gave the best correlation with transport activity, regardless of the tissue of origin, moreover, the degree of expression in the oocytes correlated with the A System activity of the tissue of origin of the mRNA. *In vitro* translation of the mRNA fraction resulted in synthesis of 40-60 kDa proteins, but since the A System transporter is believed to be a glycoprotein (Barber et. al.,

1983), this would represent only the protein portion and not the true molecular weight of the glycosylated protein.

The A System is dependent on the existence of an inward directed Na^+ gradient (Oxender and Christensen, 1963) and later work documented the ratio of Na^+ to amino acid in cotransport at 1:1 (Lever et. al., 1984). The A System has been documented to exist on the basolateral surface of LLC-PK_1 cells (Rabito and Karish, 1982), a pig kidney cell line which upon confluence shows some of the properties of proximal convoluted tubule (Hull et. al., 1976). Of all known amino acid transporters, the A System is the most responsive to changes in the cellular growth state (for reviews, see Guidotti et. al., 1978; Kilberg, 1982; Shotwell et. al., 1983; Saier et. al., 1988). Loss of contact inhibition leads to increased transport over the A System (Foster and Pardee, 1969; Villereal and Cook, 1978; Amsler et. al., 1983), and transport levels decrease when cells once again become confluent and differentiate (Amsler et. al., 1983).

In 1972 both Riggs et. al. (Riggs and Pan, 1972) and Gazzola et. al. (Gazzola et. al., 1972) reported the A System responds to deprivation of substrate by increasing transport. Cellular transformation has been shown to stimulate the A System in kidney cells (Boerner and Saier, 1985), as has treatment of cells with the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA, Baird and Boutwell, 1971; Becker and Cook, 1981; Amsler et. al., 1983). The A System has been described as a "possible determinant of growth rate" in MDCK cells (Saier et. al., 1988), and is known to be responsive to insulin, glucagon and

cAMP-stimulating hormones, as well as TPA and protein kinase C (PKC) (reviewed in Saier et. al., 1988).

Regulation of A System Transport

The A System is subject to regulation by hormones, growth factors and tumor promoters (for reviews, see Guidotti et. al., 1978; Kilberg, 1982; Shotwell et. al., 1983; Saier et. al., 1988). Regulation of the A System is also controlled by substrate availability through two different mechanisms, trans-inhibition and the adaptive response. Trans-inhibition decreases the activity of the A System in the presence of high internal amino acid pools. This decrease of activity is postulated to result from the slow movement of the amino acid-loaded binding site across the bilayer. In the presence of high internal amino acid pools, the binding site will be sequestered on the inside of the membrane and thus will be unavailable for internalization of more amino acids (Pall, 1970).

A model proposed by Guidotti and coworkers (Gazzola et. al., 1981) requires that the first step in trans-inhibition be binding of amino acids to the A System transporter. This binding sends a signal through an unknown mechanism to the nucleus to begin synthesis of an A System inactivating protein. They explicitly state that signal transduction takes place regardless of the orientation of the transporter, so that intracellular binding, favored by high intracellular substrate concentrations, is as effective as extracellular binding.

This model requires some revision, however, since more recent work (Moffett and Englesberg, 1986) has shown that not all A System substrates can trans-inhibit the A System, and some non-A System substrate amino acids can participate. This confirmed the results obtained by Boerner and Saier (1985) in MDCK cells, where they found that repressive amino acids were not necessarily substrates for the A System. For example, histidine, which is not an A System substrate, gave a greater trans-inhibition than alanine, glycine, proline, or serine, all of which are characteristic A System substrates (Boerner and Saier, 1985).

Trans-inhibition is very important in kinetic studies of the A System. If cells are removed from a complete growth medium and used immediately for an A System assay, the concentration of intracellular amino acid pools can be high enough that the A System will be trans-inhibited at the start. As the assay continues, the internal amino acid pools will decrease, and total transport can increase through a loss of trans-inhibition. To minimize the effect of trans-inhibition in my experiments, cells were preincubated for 90 min in a saline solution containing glucose as an energy source, but no amino acids. At the end of this period, intracellular amino acid pools should be depleted and trans-inhibition should not occur.

The other mechanism by which substrate availability controls the A System response occurs when the extracellular substrate concentration drops. In the absence of substrate, transport capacity over the A System increases. This response was first seen and referred to as the "adaptive response" by Gazzola et.

al. (1972) and was also discovered independently by Riggs and Pan (1972). Both groups found the transport capacity through the A System increases as cells or tissues are incubated in the absence of A System substrates.

Further work indicated that this adaptive response could be prevented if the incubation took place in the presence of RNA synthesis inhibitors (Gazzola, 1981), protein synthesis inhibitors (Tramacere et. al., 1977) or protein glycosylation inhibitors (Barber et. al., 1983). Since inhibition through protein synthesis and export pathways can prevent increases in A System transport, the adaptive response is believed to represent increases in the number of active transporters on the cell surface through increased synthesis of the transporter itself. When examined kinetically, the adaptive response was found by both Gazzola et. al. (1972) and Riggs and Pan (1972) to consist of changes in V_{max} with no effect on the K_m, further supporting the idea of changes in transporter number with no change in the A System transporter itself. This result was originally found in rat uterus and chick embryo heart cells, respectively, and has since been seen in human glial and glioma cells (Ronquist et. al., 1976), chick embryo fibroblasts (Tramacere et. al., 1977), cultured rat hepatocytes (Kelley and Potter, 1978), cultured human fibroblasts (Gazzola et. al., 1981), and fetal human fibroblasts (Gazzola et. al., 1984).

However, under some circumstances changes in K_m have also been reported to occur. If the K_m of transport is changing as well as the V_{max}, then control of transport would be more complex than a mere change in the number of transporters. The adaptive response would have to include changes in the

transporter itself which alter its affinity for amino acids, or synthesis of a different transporter with a different activity. Incubation of cells in the absence of A System substrates has been reported to result in changes in both K_m and V_{max} in MDCK-T_1 cells (Boerner and Saier, 1985) in L6 rat myoblast cells (Logan et. al., 1982) and fibroblast growth factor-treated G_0 3T3 cells (Hochstadt et. al., 1979).

Two models currently exist to explain the regulation of the A System. In the Kilberg model (Kilberg et. al., 1985), internal amino acids are positive regulators of a gene which negatively regulates the A System transporter gene directly. Hormones or substrate starvation positively regulate the A System transporter gene, through an unidentified mechanism. This model assumes that the A System transporter is being continuously inactivated at a high rate, and inactivation occurs regardless of amino acid concentration. Synthesis is increased by deprivation of substrate, or through hormone induced pathways. Increased transport results when synthesis occurs faster than degradation. Critical to this model is the existence of an as-yet-undiscovered intracellular amino acid binding protein, which senses levels of intracellular amino acids and alters gene transcription.

A more complex model has been proposed by Englesburg and Moffett (1986) who have based their proposal on work with CHO cells. They postulate the existence of two continuously synthesized regulatory genes, R_1 and R_2, in addition to the A System transporter gene. In their model, amino acids do not control synthesis of regulatory protein r_1, but once the protein has been synthesized, it must bind a repressive amino acid in order to inactivate the A System transporter

gene. They also assign the proposed r_1 repressor a role in the direct inactivation of the A System transporter in the cell membrane. In the absence of amino acids, the R_2 gene product, r_2, can inhibit directly the A System transporter gene. However, r_2 is inactivated, possibly by a protein kinase, after stimulation of cells by hormones such as insulin. With this model, the A System can be activated by deprivation of substrate, combined with some sort of hormonal signal, so that r_1 cannot form an active complex and r_2 is directly inactivated. To explain the high transport rates in proliferating cells, the cells must be using amino acids faster than they are being transported into the cell, so that the internal concentration is low. When cells decrease the rate of protein synthesis, the internal amino acid levels will increase, and r_1 will repress synthesis of the A System transporter gene and inactivate a portion of the transporter molecules in the membrane. This model is less effective at explaining the adaptive response, where incubation of cells in the absence of amino acids (and presumably effective hormones as well), results in an increase in transport. Under these conditions, r_2 should not be inactivated, and should prevent increased transcription of the A System gene.

TPA and the A System

In 1983 studies from this laboratory demonstrated that addition of the tumor-promoting phorbol ester, TPA, to confluent cultures of LLC-PK_1 cells increased the transport of amino acids over the A System (Amsler et. al., 1983).

Proliferating cultures of LLC-PK_1 cells have a high level of A System transport, which decreases over the time it takes for the cells to become confluent (usually around 5-10 days). Na^+-dependent hexose transport also begins to increase about the same time as the A System transport is decreasing (Amsler et. al., 1983). Amsler et. al. were primarily studying the regulation of the Na^+-dependent hexose transporter, which is expressed at a high level in differentiated cells, and has little activity in proliferating cells (Mullin et. al., 1980; Rabito and Karish, 1980; Amsler and Cook, 1982). The Na^+-dependent hexose transporter and the A System transporter, another Na^+-dependent process, are active under opposite growth conditions (Amsler et. al., 1983). Because part of the regulation of the A System is due to changes in the Na^+ electrochemical gradient (Villereal and Cook, 1978) which should alter transport in both systems, Amsler et. al. (1983) decided to examine the effects of differentiation inducers and mitogens on amino acid transport and hexose transport in the same cells.

The agents chosen for his study were the differentiation inducer hexamethylene bisacetamide (HMBA) (Reuben et. al., 1976), the phosphodiesterase inhibitors theophylline and methylisobutyl xanthine (MIX), and the tumor promoter and mitogen, TPA (Baird and Boutwell, 1971). HMBA, theophylline, and MIX all increase uptake of sugar via the Na^+-dependent hexose transporter, while decreasing amino acid transport via the A System (Amsler et. al., 1983). TPA, added to proliferating cells, will prevent cultures from acquiring a high level of Na^+-dependent hexose transport as they differentiate over 7-10 days

(Amsler and Cook, 1982). However, TPA does not have a direct effect on hexose transport in the short term (several hours), but acts to prevent increases in Na^+-dependent hexose transport over the long term (days to weeks). TPA also maintains a high level of amino acid transport activity via the A System, even in the presence of a differentiation inducer, or will increase A System transport in confluent, stepped-down cells (Amsler et. al., 1983). As seen for the adaptive response, this TPA stimulation of the A System can be blocked by addition of inhibitors of protein synthesis or RNA synthesis, and did not appear to be due to changes in membrane potential (Amsler et. al., 1983). Further, cells maintained in 10^{-7}M TPA maintained a high level of amino acid transport via the A System even after 11 days in culture (Amsler et. al., 1983). Castagna et. al. (1982) demonstrated protein kinase C (PKC) was directly activated by TPA and PKC is now believed to be the main site of action of TPA *in vivo* (Ashendel et. al., 1983a; Ashendel et. al., 1983b; Niedel et. al., 1983; Kikkawa et. al., 1983; Nishizuka, 1984); these results suggest the activation of PKC should stimulate the A System.

To investigate the link between PKC and the A System, Dawson and Cook (1987) examined the distribution and activity of PKC in LLC-PK_1 cells as the cultures aged, and also after treatment with TPA. They found the total amount of PKC activity in the cells remained constant, but the location of PKC changed from a membrane bound form when A System transport activity was high to a cytosolic form when A System transport activity was low. Addition of TPA to confluent, stepped-down cells produces a rapid shift in PKC from the cytosol to a membrane

bound form. Since Adamo et. al. (1986) have shown that the subcellular distribution of PKC in human fibroblasts depends on the growth state of the cells; only the membrane bound form is active, which suggests activation of PKC is followed by an increase in A System transport activity and strengthens the argument for PKC as the mediator of TPA stimulation of the A System.

Further, addition of TPA to cells is believed to replace the requirement of diacylglycerol for PKC activation (Yamanishi et. al., 1983). If PKC controls the level of A System transport, then addition of cell permeating diacylglycerols should mimic the effect of adding TPA to cultures. This experiment was performed by Dawson and Cook (1987), who showed diacylglycerols that activate PKC such as 1-Hexadecyl-2-oleoyl-sn-glycerol also stimulate increases in A System transport activity, while other diacylglycerols that do not stimulate PKC such as 1-Palmitoyl-2-acetyl-sn-glycerol have no effect on A System transport activity.

Since addition of TPA results in loss of PKC activity in many cell types (Kraft et. al., 1982b; Rodriguez-Pena and Rozengurt, 1984; Blackshear et. al., 1985; Chida et. al., 1986; Stabel et. al., 1987; Young et. al., 1987; Ase et. al., 1988), the role of PKC in the response of the A System to TPA could be disputed. Long term exposure of LLC-PK_1 cells to TPA (up to 11 days in Amsler et. al., 1983) should completely deplete the cells of PKC and prevent any A System response. However, in LLC-PK_1 cells, TPA-induced down regulation of PKC activity has not been observed (W. D. Dawson, personal communication). Further, the results of Ase et. al. (1988) showing different PKC isozymes are down regulated at different

rates within a given cell could also provide an explanation of the resistance of LLC-PK_1 cells to TPA-induced down regulation of PKC.

Regulation of Protein Kinase C

Protein kinase C was first described in 1977 as a proteolytically activated protein kinase (Inoue et. al., 1977). Later, it was found this kinase was also activated by phospholipids in the presence of Ca^{2+} (Takai et. al., 1979a) and probably involved in signal transduction. Protein kinase C is dependent on high Ca^{2+} and phospholipids for maximum activity; addition of diacylglycerol (DAG) results in a decrease in the Ca^{2+} requirement to physiological values (Takai et. al., 1979b; Kishimoto et. al., 1980; Kaibuchi et. al., 1981). Phosphatidylserine (PS) is an absolute requirement for active PKC, and other phospholipids can enhance or repress PKC activity in combination with phosphatidylserine (Kaibuchi et. al., 1981; Ashendel et. al., 1983b). PKC requires the binding of 4 molecules of PS, one molecule of DAG, and Ca^{2+} for complete activation (Hannun et. al., 1985; Hannun et. al., 1986).

The diacylglycerol needed to decrease the Ca^{2+} requirement of PKC is thought to be mainly derived by the action of phospholipase C, which degrades phosphatidylinositol-4,5-bisphosphate to diacylglycerol and 1,4,5-inositol trisphosphate (reviewed by Nishizuka, 1984; Berridge, 1987; Bell et. al., 1988; Rana and Hokin, 1990). An alternative pathway for DAG production involves

phospholipase D, which forms phosphatidic acid (PA) from phosphatidylcholine, and phosphatidate phosphohydrolase, which converts PA to DAG, has been reported (Billah et. al., 1989). Phospholipase C appears to be regulated by cell surface receptors in a G protein-coupled reaction. The diacylglycerol produced diffuses laterally through the membrane and can stabilize transitory complexes formed by Ca^{2+}, PS, and PKC. Once the complex is formed with DAG, PKC is activated. The 1,4,5-inositol trisphosphate formed results in an increase in cellular Ca^{2+} levels, presumably from endoplasmic reticular stores (Berridge and Irvine, 1984; Berridge, 1987). A model for PKC activation proposed by Bell's lab (Ganong et. al., 1986) states that the carboxyl groups on the serine residues of four PS molecules ligate Ca^{2+} and PKC associates with this PS/Ca^{2+} complex. DAG binding to this complex activates PKC. In the absence of DAG, Ca^{2+} levels required for full PKC activity are 100 times higher (Takai et. al., 1979b; Kishimoto et. al., 1980).

Addition of tumor promoters such as the phorbol esters also reduces the Ca^{2+} requirement of PKC to physiological levels by substituting for the diacylglycerol requirement (Yamanishi et. al., 1983). PKC has been shown to be the cellular receptor for these tumor promoting phorbol esters (Castagna et. al., 1982; Ashendel et. al., 1983a; Ashendel et. al., 1983b; Niedel et. al., 1983). In addition, the potency of individual phorbol esters as tumor promoters correlates with the potency of those phorbol esters as PKC activators (Castagna et. al., 1982).

Protein kinase C designates a family of calcium- and phospholipid-dependent kinases (for reviews, see Coussens et. al., 1986; Nishizuka, 1988; Kikkawa et. al., 1989). The four major isoforms , α, β_I and β_{II} and γ have been identified by cDNA library screening (for reviews, see Parker et. al., 1986; Coussens et. al., 1986), while the other, less homologous isozymes, δ, ϵ, ζ have been identified by lower stringency screening (for reviews, see Nishizuka, 1988; Kikkawa et. al., 1989). The isozymes of PKC differ in size, tissue distribution, and vary in their Ca^{2+} and phospholipid requirements for activity. It is thought different isozymes within a single cell may have different substrates and could respond to different signals (Nishizuka, 1988; Kikkawa et. al., 1989).

The isozymes of protein kinase C are down regulated by proteolytic cleavage (Chida et. al., 1986; Young et. al., 1987). The active, membrane bound form of PKC is the protease susceptible form (Chida et. al., 1986; Nishizuka, 1986; Parker et. al., 1986; Suzuki and Ohno, 1990). Partial proteolysis by the calcium-activated neutral protease calpain (CANP) (reviewed in Murachi, 1983; Suzuki and Ohno, 1990), can generate an active protein kinase (PKM) which has lost the calcium and phospholipid requirements of the intact PKC molecule (Inoue et. al., 1977; Kishimoto et. al., 1983; Nishizuka, 1984; Melloni et. al., 1985; Melloni et. al, 1986; Nishizuka, 1986, Parker et. al., 1986; Chida et. al., 1986; Nakadate et. al, 1987; Pontremoli et. al., 1990; Suzuki and Ohno, 1990). Conditions that stimulate PKC activity also stimulate calpain, and therefore it has been suggested that calpain

cleavage of PKC is a normal part of PKC activation (Kishimoto et. al., 1983; Chida et. al., 1986; Parker et. al., 1986; Suzuki and Ohno, 1990).

Two forms of calpain, mCANP and μCANP, have been identified based on their Ca^{2+} dependence (Mellgren, 1980; Murachi, 1983). mCANP was first reported to require 10^{-3}M Ca^{2+} for activity, while μCANP required 10^{-5}M Ca^{2+} (Murachi, 1983). Although these Ca^{2+} concentrations are 1000-100,000 fold higher than physiological Ca^{2+} concentrations, recent work has shown that mCANP and μCANP are proenzymes, and association of either calpain with membranes and physiological Ca^{2+} concentrations result in activation by autolysis of either mCANP or μCANP (Suzuki and Ohno, 1990). Activation of calpain in this manner causes the active protease to be sequestered near the site of activation of PKC. Also, the promoter regions of the calpain genes contain sequences identical to those found in genes known to be activated by TPA (Angel et. al., 1987), indicating activation of PKC may result in increased calpain synthesis (Hata et. al., 1989).

As shown in Figure 1.1, PKC cleavage by calpain is believed to occur between the regulatory domain and the catalytic domain. Since the regulatory domain contains the structure responsible for binding phospholipids (Ono et. al., 1989b), and is not required for kinase activity, loss of this domain will release a constituitively active protein kinase into the cytosol (Kishimoto et. al., 1989). This could solve one of the remaining puzzles in PKC mediated signal transduction, namely, how does a membrane bound kinase transmit a signal to alter gene regulation? Once calpain cleaves PKC to form PKM the kinase is free to diffuse

Figure 1.1. Domain Structure of PKC and Activation by Calpain.

A. Domain structure of PKC. PKC can be divided into nine functional domains. Four of the domains are highly conserved within the major forms of PKC (α, β_I, β_{II}, and γ) and between species, and are termed C1, C2, C3, and C4. The other domains vary between isozymes and also between species. The variable domains are termed V1, V2, V3, V4, and V5. C1 and C2 are responsible for regulation of PKC. C1 contains the cysteine-rich zinc finger structures believed responsible for DAG or phorbol ester binding (Ono et. al., 1989), C2 appears to confer Ca^{2+} dependence, and C3 and C4 are responsible for the kinase's catalytic function. Calpain cleavage occurs in the V3 region, near the C-terminal end of C2 (based on data presented in Nishizuka, 1988 and Coussens et. al., 1986).

B. Calpain Activation of PKC. Active PKC is the substrate for cleavage by calpain, causing a loss of the regulatory subunits C1 and C2. The catalytic subunits, C3 and C4, retain catalytic activity, but are no longer subject to Ca^{2+} and phospholipid dependency. Also, since phospholipid binding is a function of the regulatory domain, the kinase fragment formed, (PKM), is now a soluble kinase, rather than a membrane bound kinase (based on data presented in Parker et. al., 1986).

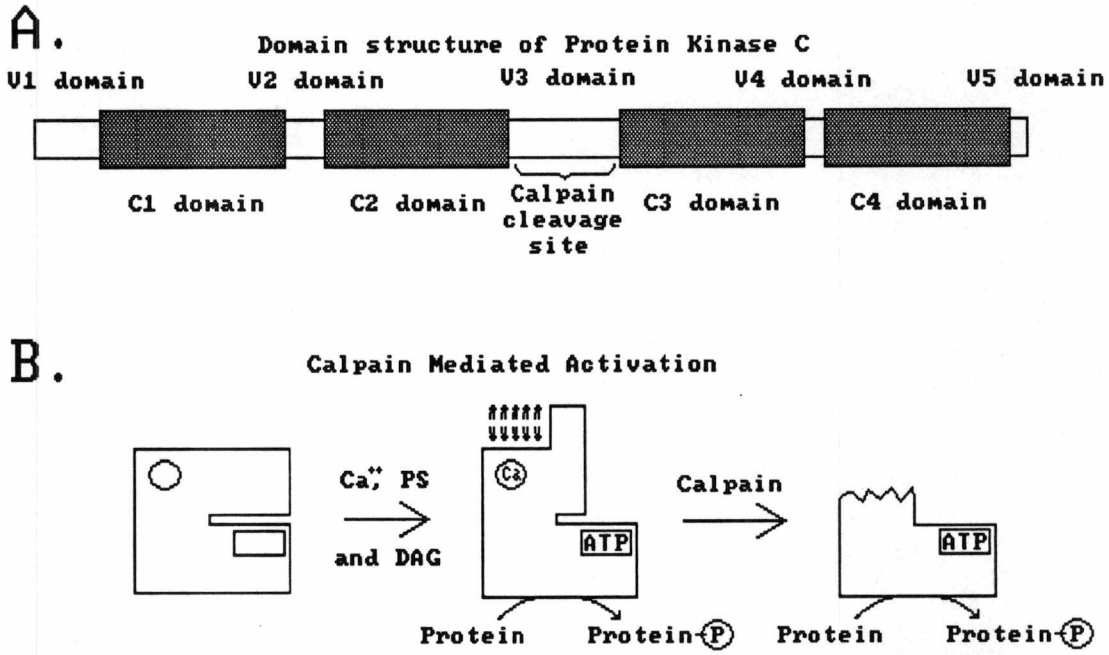
A.
Domain structure of Protein Kinase C
V1 domain
V2 domain
V3 domain
V4 domain
V5 domain
C1 domain
C2 domain
Calpain cleavage site
C3 domain
C4 domain
B.
Calpain Mediated Activation
Ca++ PS
and DAG
ATP
Calpain
ATP
Protein
Protein-P
Protein
Protein-P

Figure 1.1

throughout the cell. Also, loss of the regulatory domain is believed to result in formation of an active kinase with no requirement for Ca^{2+} or phospholipids (Kishimoto et. al., 1983; Melloni et. al., 1985; Parker et. al., 1986; Suzuki and Ohno, 1990).

Calpain is a Ca^{2+} dependent protease that cleaves proteins in a very restricted fashion (for review, see Suzuki and Ohno, 1990). Calpain will not cleave proteins to small peptides or amino acids. In multi-domain proteins, calpain cleavage occurs between domains rather than within a domain. The limited action of calpain on substrate proteins has led Suzuki and Ohno (1990) to postulate that calpain is a regulatory or processing protease, rather than a degradative or digestive enzyme. In agreement with these conclusions, Pontremoli et. al. (1990) documented that once PKC is converted to PKM it is no longer a substrate for proteolysis by calpain; down regulation of both PKC and PKM is performed by a separate neutral serine protease.

Some papers, however, have stated that the proteolytic fragment of PKC may not be physiologically significant in the regulation of cellular events (for reviews, see Nishizuka, 1988; Kikkawa et. al., 1989). These studies claim that the active kinase fragment generated during limited proteolysis merely is formed during the initial step in total degradation of PKC.

Addition of TPA has been shown to cause diverse biological effects in different cell types (reviewed in Nishizuka, 1986), e.g. acts as a secretagogue (Katakami et. al., 1984), activates T and B lymphocytes (Kaibuchi et. al., 1985; Nel

et. al., 1985), increases amino acid transport (Amsler et. al., 1983), as well as promoting tumors in mouse skin (Baird and Boutwell, 1971). TPA is also known to increase the transcription of specific genes, such as SV40 early, collagenase, metallothionein IIA, and stromelysin (Angel et. al., 1987). Furthermore, TPA increases the synthesis of a family of transcriptional activator proteins known collectively as AP-1, (Hunter et. al., 1988) and Fos, the product of the cellular oncogene c-*fos* (Barber and Verma, 1987).

A consensus sequence for the TPA-induced transcriptional increase has been identified in the promoter region of TPA regulated genes (Angel et. al., 1987). This sequence, 5'-TGACTCA-3', has been identified as an AP-1 binding site (Lee et. al., 1987). AP-1 is phosphorylated *in vivo* (Chiu et. al., 1988), which would seem to provide a simple regulatory mechanism for TPA transcriptional induction, but TPA treatment of cells expressing AP-1 does not change the phosphorylation pattern, indicating that AP-1 is not directly phosphorylated by PKC (Chiu et. al., 1988). Fos is also synthesized in response to TPA (Barber and Verma, 1987), and Fos had been implicated in the activation of the adipocyte aP2 gene (Distel et. al., 1987), a gene now known to contain an AP-1 binding site (Rauscher et. al., 1988). This implies that Fos and AP-1 may interact in gene regulation, an argument strengthened by the discovery that cells with an inducible c-*fos* gene will respond to c-*fos* induction by synthesizing the same gene products induced by TPA (Chiu et. al., 1988). Since Fos is also phosphorylated in response to TPA, Hunter et. al. (1988) have proposed a general model for activation of TPA responsive genes

(Figure 1.2). In their model, TPA stimulates formation of a complex between Fos and members of the AP-1 family. This complex can interact with RNA polymerase II at promoter regions in the DNA which contain the AP-1 binding site to stimulate transcription of TPA inducible genes. PKC does not directly phosphorylate AP-1 (Chiu et. al., 1988). Fos is a nuclear protein while PKC is a cytoplasmic and membrane-bound kinase, so PKC cannot be directly phosphorylating either AP-1 or Fos. To explain this, the model specifies a currently unknown kinase, activated as part of a cascade reaction, that migrates to the nucleus and activates Fos (and possibly AP-1 as well). I have included a possible role for PKM in the figure, since PKM is not a cytoplasmic membrane-bound kinase, and could therefore phosphorylate Fos directly.

Figure 1.2. Activation of TPA-Inducible Genes by PKC.

Activation of PKC at the membrane is suggested to cause activation of a currently unknown kinase which migrates to the nucleus and phosphorylates Fos, and potentially AP-1 as well. Phosphorylation of these proteins leads to complex formation and assembly with RNA polymerase II on DNA at the AP-1 binding sequence, TGACTCA. The latter results in increased synthesis of genes containing the TGACTCA sequence in their promoter region. The role of PKM in phosphorylation of Fos is not stated by the authors, but PKM could be the missing kinase (based on data and a model from Hunter et. al., 1988).

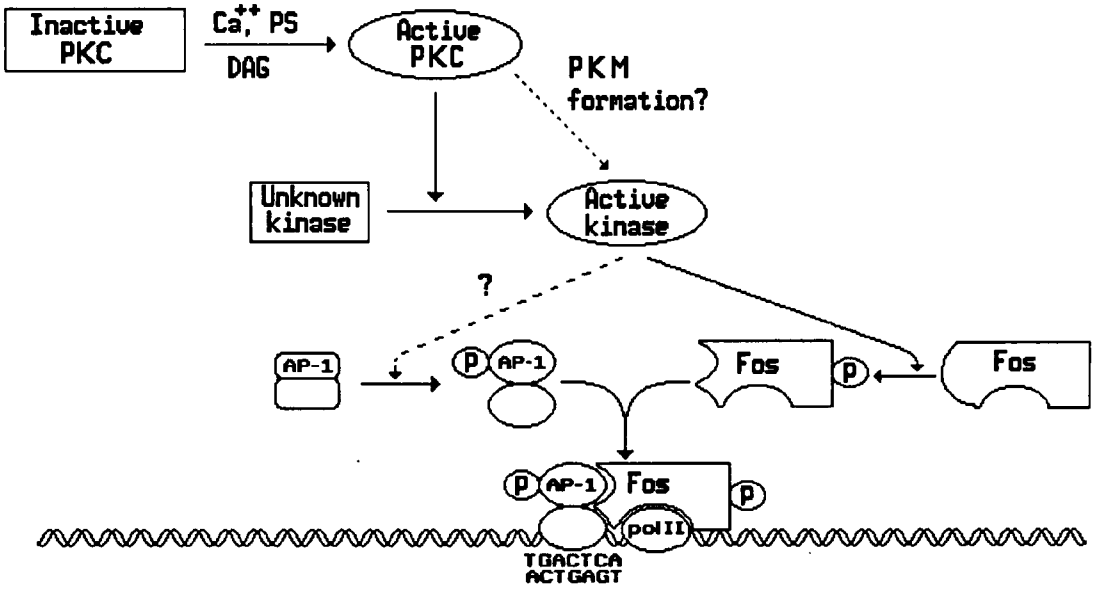
Inactive PKC
Ca++ PS
DAG
Active PKC
PKM formation?
Unknown kinase
Active kinase
?
AP-1
P
AP-1
Fos
P
Fos
P
AP-1
Fos
P
polII
TGACTCA
ACTGAGT

Figure 1.2

CHAPTER 2

DIPHENOLIC INHIBITORS MODULATE A SYSTEM TRANSPORT THROUGH MODULATION OF PROTEIN KINASE C

Abstract

Confluent LLC-PK_1 (pig kidney) cells respond to the tumor promoter TPA with up-regulation of the Na^+-dependent amino acid (A System) transport. A System activity was measured as Na^+-dependent uptake of the amino acid analog MeAIB. Three diphenolic compounds - quercetin, phloretin, and DES - that have little or no direct effect on A System activity, block the TPA stimulated response. The bioflavonoids quercetin and phloretin were maximally inhibitory at 150 μM, with respective $K_{1/2}$'s of ~ 60 μM and ~ 80 μM. DES inhibited maximally at 100 μM with a $K_{1/2}$ ~ 40 μM. Na^+-dependent uptake of α-methyl glucoside (α-MeGlc), driven by the same Na^+-electrochemical gradient in these cells, is not sensitive to the inhibitors at the same times and concentrations, indicating that inhibition of the A System is not due to disruption of the gradient. Incorporation of [^{35}S]Met into protein in the presence or absence of the inhibitors shows these compounds do not inhibit up-regulation of the A System transporter by inhibition of bulk protein synthesis.

Since Nakadate et al. (1988) had shown that quercetin can inhibit protein kinase C, the direct effect of all three diphenolics on protein kinase C was examined. All three of the compounds were found to be capable of inhibiting of protein kinase C, indicating that the inhibition of TPA stimulated A System activity is due to inhibition of protein kinase C and not to direct interaction with the A System transporter.

Introduction

LLC-PK_1 epithelial pig kidney cells (Hull et. al., 1976) have many of the characteristics of proximal tubule cells when cultured to confluence (Weiss et. al., 1985). Na^+-dependent amino acid transport via the A System drops, while Na^+-dependent hexose transport increases. Previous work in this laboratory (Amsler et. al., 1983; Dawson and Cook, 1987; Dawson and Cook, 1988a; Dawson and Cook, 1988b) has shown that treatment of LLC-PK_1 cells with the tumor promoter TPA causes an increase in amino acid transport via the A System. A System activity is assayed as the Na^+-dependent transport of MeAIB, a nonmetabolizable amino acid analog specifically transported over the A System.

Because the Ca^{2+}- and phospholipid-dependent protein kinase, PKC, is the main receptor of TPA (Castagna et. al., 1982; Ashendel et. al., 1983a; Ashendel et. al., 1983b; Niedel et. al., 1983; Kikkawa et. al., 1983; Nishizuka, 1984), and the stimulation of amino acid uptake can be inhibited by sphingosine (W. D. Dawson,

personal communication), a potent inhibitor of PKC (Hannun et. al., 1986). The TPA stimulated increase in A System transport activity can also be inhibited by cycloheximide, α-amanitin, and tunicamycin (Dawson and Cook, 1987 and unpublished). Therefore, it is believed that activating PKC results in increased synthesis of the transporter. Recently, it has been reported that quercetin, a bioflavonoid, also inhibits PKC activity (Nakadate et. al., 1988). Because quercetin is a close structural analog of the bioflavonoid phloretin, an inhibitor of Na^+-independent hexose transport, (LeFevre and Marshall, 1959, Salter et. al., 1978) we decided to study the effect of these compounds and the structurally related stilbene, DES, on the TPA stimulation of A System amino acid transport activity.

Materials and Methods

Cell Culture

Cells used were LLC-PK_1 pig kidney epithelial cells, obtained from R. N. Hull (Hull et. al., 1976) at passage 185, maintained in Eagles Minimum Essential Medium α-modification, supplemented with 10% fetal bovine serum, 2 mM glutamine and 60 U/ml penicillin, 600 μg/ml streptomycin. Cultures were maintained in T-75 flasks and grown to confluence on 25 mm collagen-coated polycarbonate filters for use in transport experiments. The medium was changed

every two days; fresh medium was never added on the day of an experiment. Cells were used between passage 200 and 220.

Chemicals

Unless otherwise indicated, chemicals were obtained from Sigma (St. Louis, MO). Radioisotopes were from DuPont/NEN (Boston, MA). TPA was from LC Services (Woburn, MA). [^{35}S]Met was purchased as Tran^{35}S-label from ICN (Costa Mesa, CA). Quercetin was purified by crystallization from EtOH before use.

Transport Assays

The assays used are based on methods reported previously (α-MeGlc transport assay, Amsler and Cook, 1982; MeAIB assay, Amsler et. al., 1983). Cells for uptake experiments were used between 10-14 days of growth in culture. Cells were washed and placed into Hank's Balanced Salt Solution containing 1 mM glucose, adjusted to pH 7.4 with 20 mM HEPES (HBSS/Glc). Cells incubated for 90 min at 37°C to deplete endogenous amino acids. At this time inhibitors and TPA (10^{-7}M) were added to the cells. The cells on the filters incubated in TPA in HBSS/Glc for 120 min, then were rinsed and placed in either HBSS (Na^+-replete buffer) or HBSS where Na^+ was replaced by N-methylglucamine (Na^+-free

buffer). Both media contained 100 μM MeAIB containing 0.1 μCi/ml [^{14}C]MeAIB for A System assays, or 100 μM α-MeGlc containing 0.1 μCi/ml [^{3}H]αMeGlc for hexose transport assays. After various periods of incubation as indicated in each figure, the cells were washed with ice-cold PBS then solubilized in 2 ml 0.2% SDS. (In TPA stimulated LLC-PK$_1$ cells, A System uptake is linear for > 60 min after addition according to Amsler et. al., 1983). The radioactivity of 1.0 ml of the lysate was measured with a liquid scintillation spectrophotometer. To determine protein concentration, the fluorometric method of Avruch and Wallach, (1971) was used on 0.5 ml of the lysate. Uptake of substrate in the absence of Na^+ was subtracted from total substrate internalized to yield Na^+-dependent uptake, all values were adjusted to nmol/mg cell protein/min.

Experiments with the Diphenolic Inhibitors

Confluent cultures of LLC-PK$_1$ cells were grown on collagen-coated filters for use in this experiment. Cells were initially transferred out of α-medium into HBSS/Glc and incubated at 37°C for 90 min. Half of the cell preparations were treated with TPA (10^{-7}M), while the other half received an equivalent volume of ethanol as a solvent control. At varying times (Figure 2.4), diphenolic inhibitors were added to the incubations at the concentrations indicated (Figures 2.2,2.5,2.6) for the TPA-treated cells and also the ethanol controls. After incubating the cells

for a total of 120 min in TPA, all cell preparations were used for an A System assay as above.

Protein Synthesis Assay

Cells were depleted of endogenous amino acids for 90 min at 37°C and then transferred to HBSS/Glc with or without TPA and inhibitors. At the end of 120 min incubation at 37°C half of the cell preparations were used for an A System assay, the other half was incubated with 6 μCi/ml [^{35}S]Met. After 30 min, the cells were washed with ice-cold PBS and dissolved in 0.2 % SDS. An aliquot was removed for protein determination, and trichloroacetic acid (TCA) was added to a final concentration of 10 %. Precipitates were washed three times with ice cold 10% TCA and three times with cold EtOH. The precipitates were counted for ^{35}S in a Packard liquid scintillation counter, and the results were graphed as cpm/mg protein.

Protein Kinase C Assays

The assay used for protein kinase C is a modification of the method of Kraft and Anderson (1982a). In brief, cultures of LLC-PK$_1$ cells were grown to confluence in 150 mm dishes. Cells were washed in homogenization Buffer A (20 mM Tris, 0.5 mM EGTA, 2.2 mM EDTA, 1 mM DTT) containing 600 mM

sucrose, scraped off the plates with a rubber policeman and concentrated by centrifugation. Addition of 600 mM sucrose to Buffer A was recommended to increase lysis of LLC-PK_1 cells in the homogenization protocol (W. D. Dawson, personal communication). Cells were resuspended and centrifuged three times in Buffer A to wash them thoroughly. Cells were homogenized in a Polytron at 17,000 rpm for 30 sec, then the homogenate was centrifuged at 100,000xg in an ultracentrifuge. The supernatant, containing cytosolic enzymes, was put on a 4 ml DEAE minicolumn and the column was washed with 2.5 ml Buffer A. The column was eluted with 2.5 ml fractions of 20 mM, 60 mM, 60 mM and 200 mM NaCl in Buffer A, and protein concentration was determined for all fractions. PKC activity was typically found in the second 60 mM fraction.

PKC was assayed as the difference in [^{32}P] transferred to histone from [γ-^{32}P]ATP in samples with phosphatidylserine (PS) and diolein vs. samples with EDTA lacking PS and diolein. The standard buffer used for all assays contained 300 μM Ca^{2+}; the 5 mM EGTA added to the background samples was assumed to chelate all available free Ca^{2+}. Reaction tubes contained 150 μl of premix buffer (50 mM magnesium acetate, 0.5 mg/ml histone Type IIIS (Sigma), 113 mM $CaCl_2$ in 20 mM Tris buffer, pH 7.7), 50 μl of sample and either 50 μl of lipids (0.5 mg/ml PS and 6.4 μg/ml diolein) or 50 μl of Tris-EGTA (5 mM EGTA in 20 mM Tris, pH 7.7). Reactions were started by addition of [γ-^{32}P]ATP (0.5 mg/ml ATP, 1.0 mg/ml delipidated BSA in 20 mM Tris buffer, pH 7.7 containing [γ-^{32}P]ATP (~10^6 cpm/ml)) and terminated after 5 min at 37°C by addition of ice-cold 10%

TCA containing 1% phosphotungstic acid. For PKC assays performed with inhibitors, the inhibitors were allowed to preincubate with the column eluate for 5 min before the start of the reaction. The data were converted to pmol ^{32}P transferred to histone/mg protein in assay/5 min. Values for kinase activity in the presence of EGTA and absence of added phospholipids was subtracted from the kinase activity values in the presence of Ca^{2+} and added phospholipids to calculate the Ca^{2+} and phospholipid dependent kinase activity defined as protein kinase C activity.

Because the specific activity varied in different protein kinase C preparations, inhibition data were adjusted to % control, where 100 % was defined as the kinase activity in the absence of added inhibitors.

PDBu Washout Experiments

Confluent cultures of LLC-PK_1 cells grown on collagen-coated filters were used for this experiment. Cells were transferred out of α-medium into HBSS/Glc and incubated at 37°C for 90 min. Phorbol dibutyrate (PDBu) was then added to the cells at a concentration of 10^{-7}M. After the indicated times, the medium containing PDBu was removed by aspiration, the cells on the filter were rinsed once in the dish with 2 ml ice-cold PBS with 1 % BSA (PBS/BSA) added, then washed three times in separate beakers of PBS/BSA before being placed in fresh

HBSS/Glc at 37°C. The cells were assayed for A System activity 120 minutes after addition of PDBu.

Results

Figure 2.1 shows the increase in A System activity due to treatment with TPA. It also reveals that 150 μM quercetin added with the TPA prevents up-regulation of the A System over this time period. There is an immediate decrease in the level of transport at the zero point and over the course of this experiment there was no increase in A System transport with time in the presence of quercetin and TPA. Even though addition of quercetin yields an immediate but small decrease in A System activity, the level of A System activity in the presence of quercetin does not continue to drop wit incubation time. Since differentiation inducers were not used during any experiments, the cultures probably contained a small number of proliferating cells with a high rate of A System transport. As will be shown in Chapter 3, quercetin can inhibit the high level of transport in proliferating cells, thus potentially accounting for the small and variable inhibition of base line transport in these cells.

Figure 2.2 shows a dose-response curve for quercetin in TPA-treated and untreated cells. At 150 μM quercetin, TPA stimulation of the A System is completely inhibited. At the highest concentration used, 300 μM, quercetin gave a slight inhibition of αMeGlc uptake. At 150 μM quercetin gave no inhibition of

Figure 2.1. Addition of 150 μM Quercetin Can Prevent TPA Stimulation of the A System.

LLC-PK_1 cells were incubated for 90 min in HBSS/Glc to deplete internal stores of amino acids. Quercetin (150 μM) was added to half of the cells, then TPA (10^{-7} M) was added to all of the samples. At the times indicated in the figure, two sets of triplicate filters with cells were washed out of HBSS/Glc with TPA with and without quercetin and used for an A System assay. One set of filters was incubated with Na^+-replete medium, and the other incubated with Na^+-free medium. Amino acid uptake in the absence of Na^+ was subtracted from uptake in the presence of Na^+ to yield Na^+-dependent uptake. The points shown are means ± standard deviations.

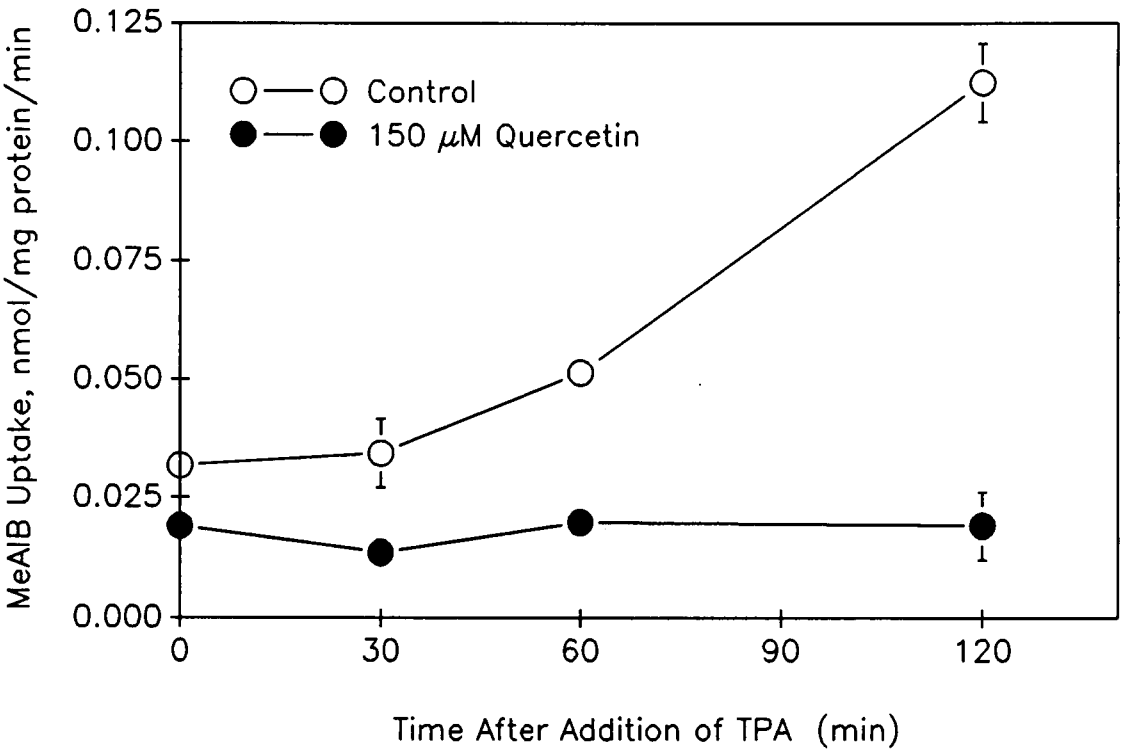
0.125
0.100
0.075
0.050
0.025
0.000
MeAIB Uptake, nmol/mg protein/min
Control
150 μM Quercetin
0
30
60
90
120
Time After Addition of TPA (min)

Figure 2.1

Figure 2.2. Dose-Response for Quercetin Inhibition of TPA Stimulated A System Uptake.

LLC-PK_1 cells were incubated in HBSS/Glc for 90 minutes to deplete the cells of internal stores of amino acids. Quercetin was then added to all cells at the concentrations indicated and TPA (10^{-7} M) was added to half of the cell preparations. After 120 min in TPA the cells were used in an A System assay. Some of the cells, treated with and without TPA, with and without quercetin, were used instead for an α-MeGlc uptake assay, as indicated in the inset. The points shown are means ± standard deviations.

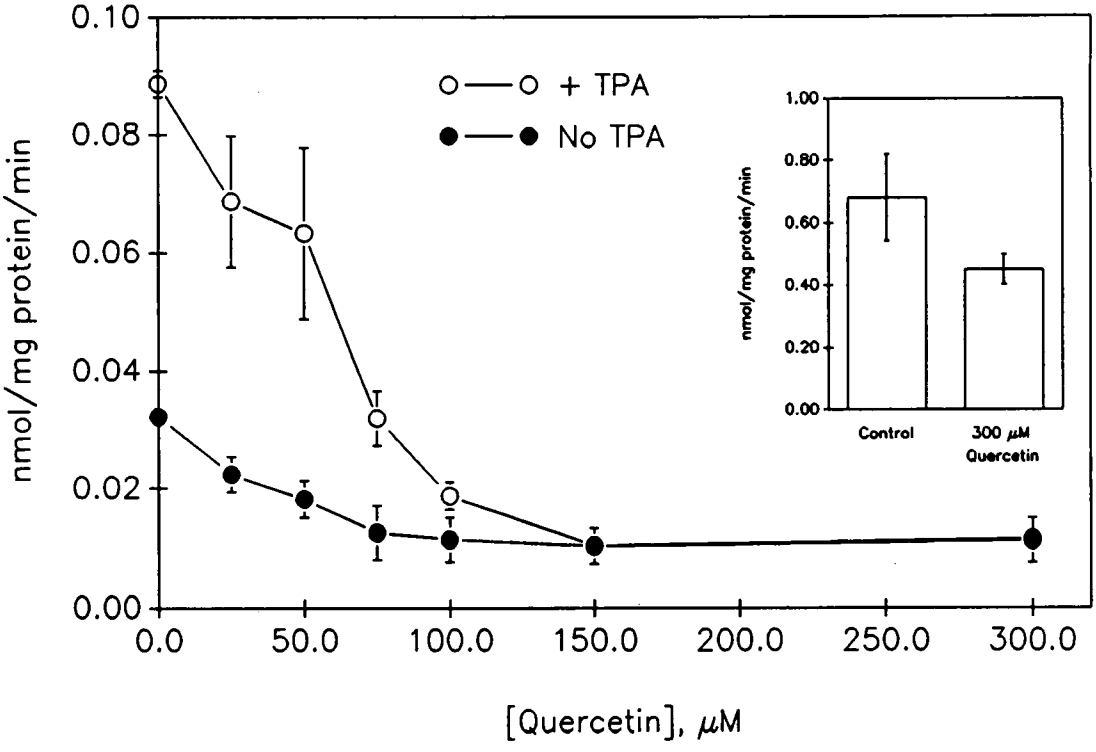
0.10
0.08
0.06
0.04
0.02
0.00
nmol/mg protein/min
+ TPA
No TPA
0.0
50.0
100.0
150.0
200.0
250.0
300.0
[Quercetin], μM
1.00
0.80
0.60
0.40
0.20
0.00
nmol/mg protein/min
Control
300 μM
Quercetin

Figure 2.2

αMeGlc uptake but was completely effective at inhibiting TPA stimulation of the A System. Therefore, in all further experiments quercetin was used at 150 μM. The half maximal dose for quercetin was approximately 80 μM.

Figure 2.3 shows that quercetin has little effect on the level of A System transport activity other than its inhibition of the TPA response. There is a small effect on the level of A System transport in control cells compared to the effect seen in TPA treated cells, probably due to the presence of proliferating cells in the culture, which is similar to the effect seen in Figure 2.1. Adding quercetin during the A System assay has no apparent effect on A System transport after cells have been treated with TPA. The major effect quercetin has in this experiment is during the 120 min TPA incubation period, where it blocks the TPA response.

To determine the time course for quercetin inhibition of TPA stimulated A System transport, quercetin was added to cultures of LLC-PK_1 cells at various times after addition of TPA or ethanol as a solvent control. Figure 2.4 shows that after the cells remain for 15 min in TPA, addition of quercetin doesn't prevent TPA stimulation of the A System transporter.

Figure 2.5 shows the dose-response curve for phloretin, a close structural analog of quercetin. Phloretin also reaches its maximal effective concentration at 150 μM, where it inhibits TPA stimulated A System transport activity close to control levels. The half maximal dose for phloretin was 60 μM.

Figure 2.3. Direct Effect of 150 μM Quercetin on the A System Transporter.

Triplicate samples of LLC-PK_1 cells grown on filters were incubated in HBSS/Glc for 90 min, then treated with either 150 μM Quercetin, TPA (10^{-7}M), Quercetin with TPA, or a solvent control (EtOH and DMSO). The last set of triplicate samples, (TPA + Quercetin, Direct Effect) was placed in TPA alone for the 120 min incubation period, then quercetin was added during the 15 min MeAIB uptake period. The points shown are means ± standard deviations for Na^+-dependent uptake of MeAIB.

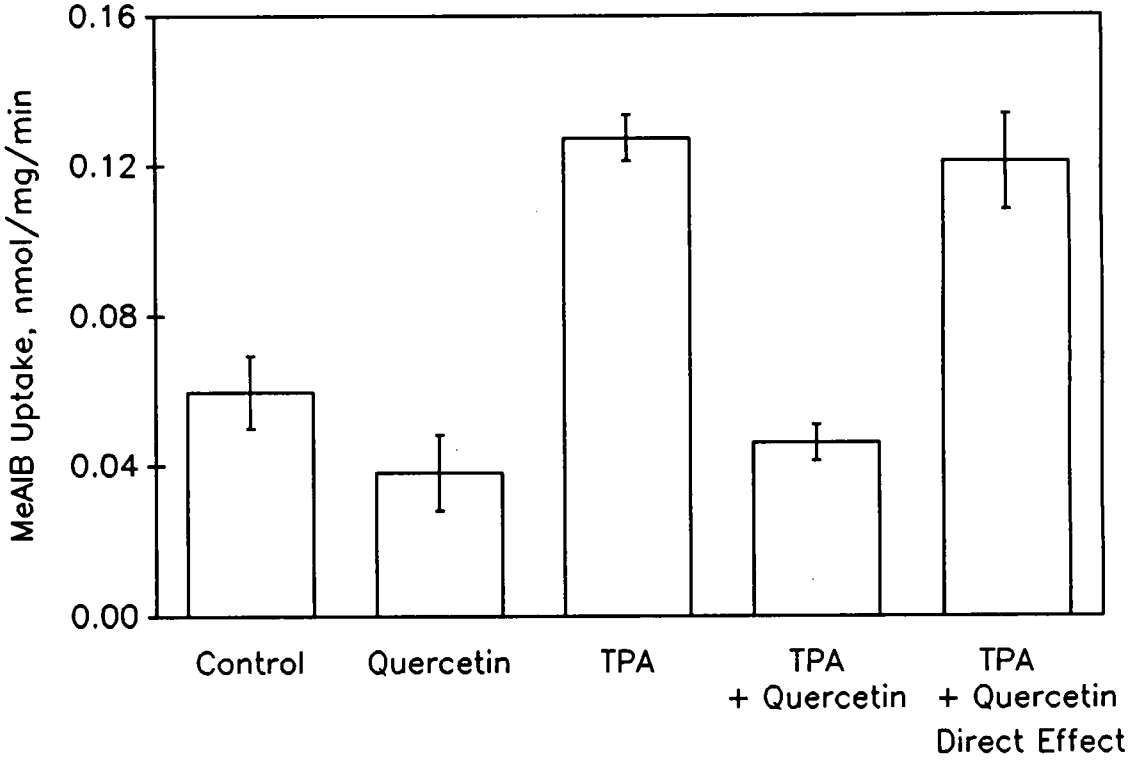
0.16
0.12
0.08
0.04
0.00
MeAIB Uptake, nmol/mg/min
Control
Quercetin
TPA
TPA
+ Quercetin
TPA
+ Quercetin
Direct Effect

Figure 2.3

Figure 2.4. Effect of the Time of Addition of Quercetin on A System Transport.

Confluent cultures of LLC-PK_1 cells were depleted of internal amino acids for 60 min, then half of the filters with attached cells were placed into TPA (10^{-7}M) for 120 min. At the times indicated in the figure, 150 μM quercetin was added to triplicate sets of cells on filters. After a total of 120 min in TPA the cells were used for an A System assay. The data were first adjusted for MeAIB uptake in the absence of Na^+ to give A System transport, and then values for A System transport in the absence of TPA was subtracted from values for A System transport with TPA to show the effect of TPA stimulation. Each point shown is the difference of means of four sets of triplicate filters, and the error bars are standard deviations.

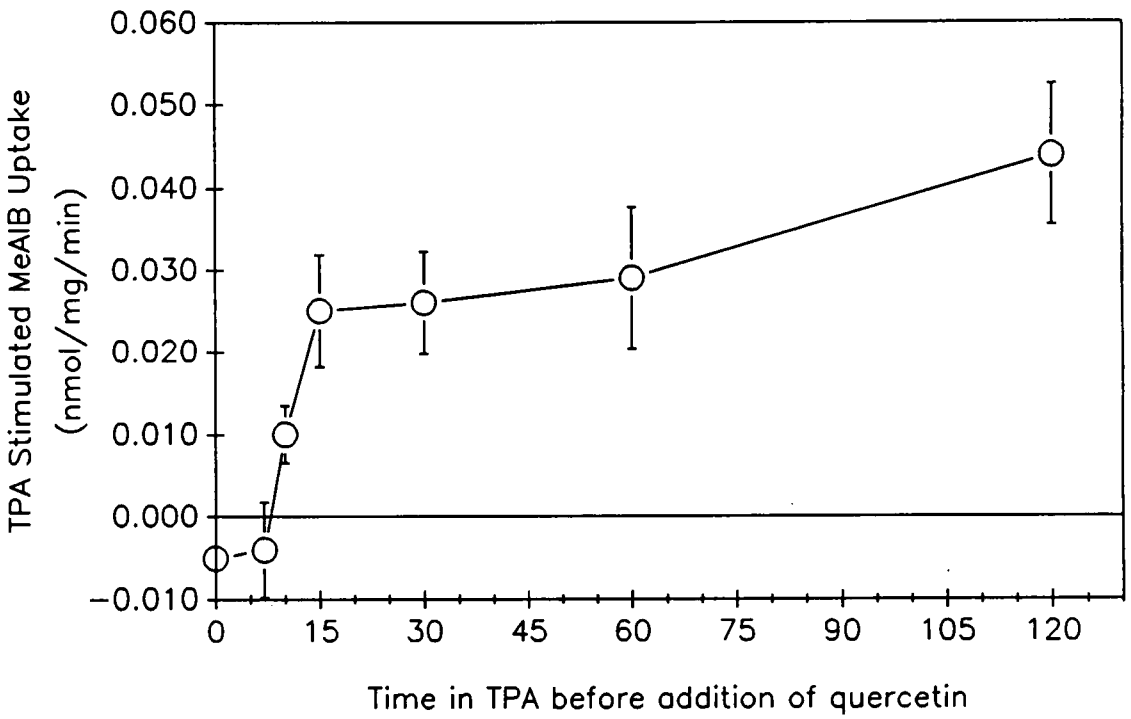
TPA Stimulated MeAIB Uptake
(nmol/mg/min)
0.060
0.050
0.040
0.030
0.020
0.010
0.000
−0.010
0
15
30
45
60
75
90
105
120
Time in TPA before addition of quercetin

Figure 2.4

Figure 2.5. Dose-Response for Phloretin Inhibition of TPA Stimulated A System Uptake.

LLC-PK_1 cells were incubated in HBSS/Glc for 90 minutes to deplete the cells of internal stores of amino acids. Phloretin was then added to all cells at the concentrations indicated and TPA (10^{-7} M) was added to half of the cells, with the remainder receiving an equivalent volume of EtOH. After 120 min in TPA the filters were used in an A System assay. The points shown are the difference in the means of triplicate samples with Na^+ and without Na^+. The error bars represent standard deviations.

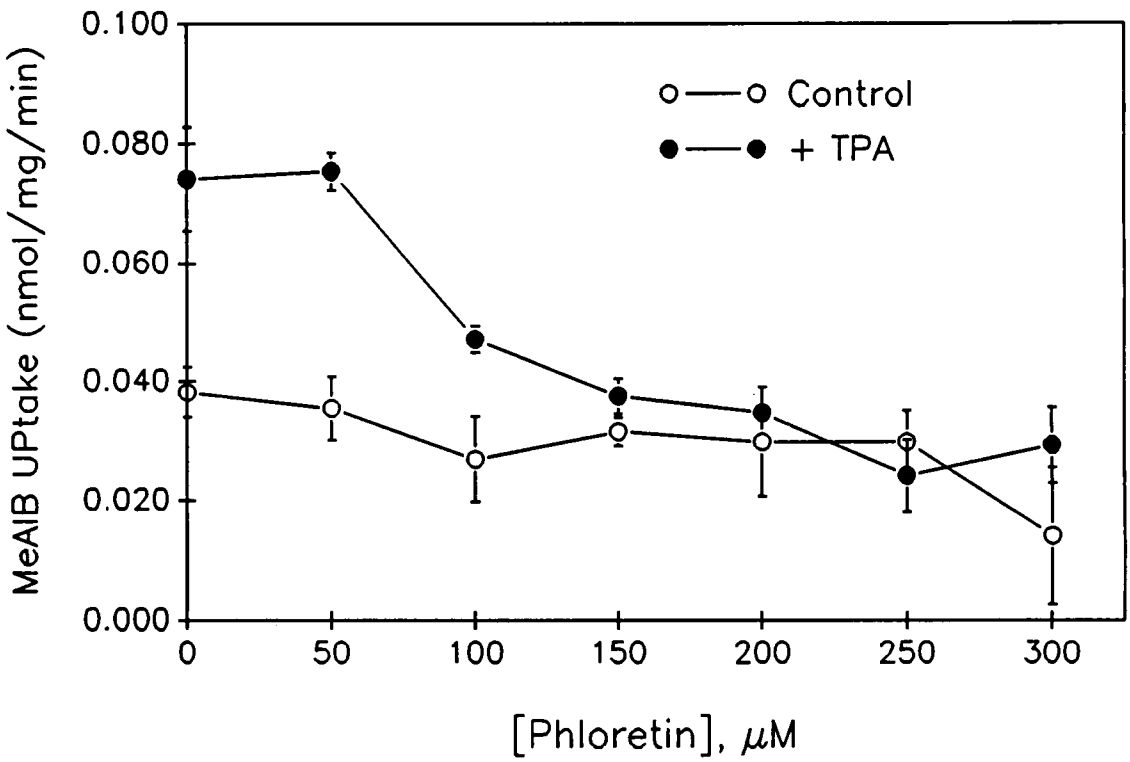
0.100
0.080
0.060
0.040
0.020
0.000
MeAIB UPtake (nmol/mg/min)
Control
+ TPA
0
50
100
150
200
250
300
[Phloretin], μM

Figure 2.5

Figure 2.6 shows the dose-response curve for DES, another diphenolic inhibitor. DES has a maximal effect at 100 μM, with a half maximal inhibition at 40 μM.

Quercetin is an inhibitor of many cellular ATPases, including the Na^+, K^+-ATPase, the enzyme responsible for maintaining the Na^+ gradient across the cell membrane. If this enzyme is inhibited, the Na^+ gradient diminishes and Na^+-dependent transporters like the A System decrease in activity. To test if the effect of these inhibitors involves dissipation of the Na^+-electrochemical gradient, transport of a different substrate over a different Na^+-dependent pathway was examined. α-MeGlc is a good indicator of the integrity of the Na^+ gradient because it is a nonmetabolizable glucose analog concentrated by these cells in a Na^+-dependent manner that is unaffected by TPA over the time course of these experiments (Weiss et. al., 1985). If the diphenolic inhibitors decreased Na^+-dependent amino acid transport by disrupting the Na^+ gradient, Na^+-dependent hexose transport should be similarly affected. Uptake of α-MeGlc is not affected by concentrations of phloretin, DES, and quercetin which maximally inhibit the A System (Figure 2.7). This indicates that the Na^+ gradient still exists and is capable of driving transport across the membrane. The reason for the significant increase in α-MeGlc uptake seen in the zero time point of DES treated cells versus either the control or the 120 min time point is unknown.

Figure 2.6. Dose-Response for Diethylstilbestrol Inhibition of TPA Stimulated A System Uptake.

LLC-PK_1 cells were incubated in HBSS/Glc for 90 minutes to deplete the cells of internal stores of amino acids. DES was then added to the cells at the concentrations indicated and TPA (10^{-7} M) was added. After 120 min in TPA the filters were used in an A System assay. Each point shown is the difference in the means of triplicate samples with Na^+ and triplicate samples without Na^+. The error bars represent standard deviations.

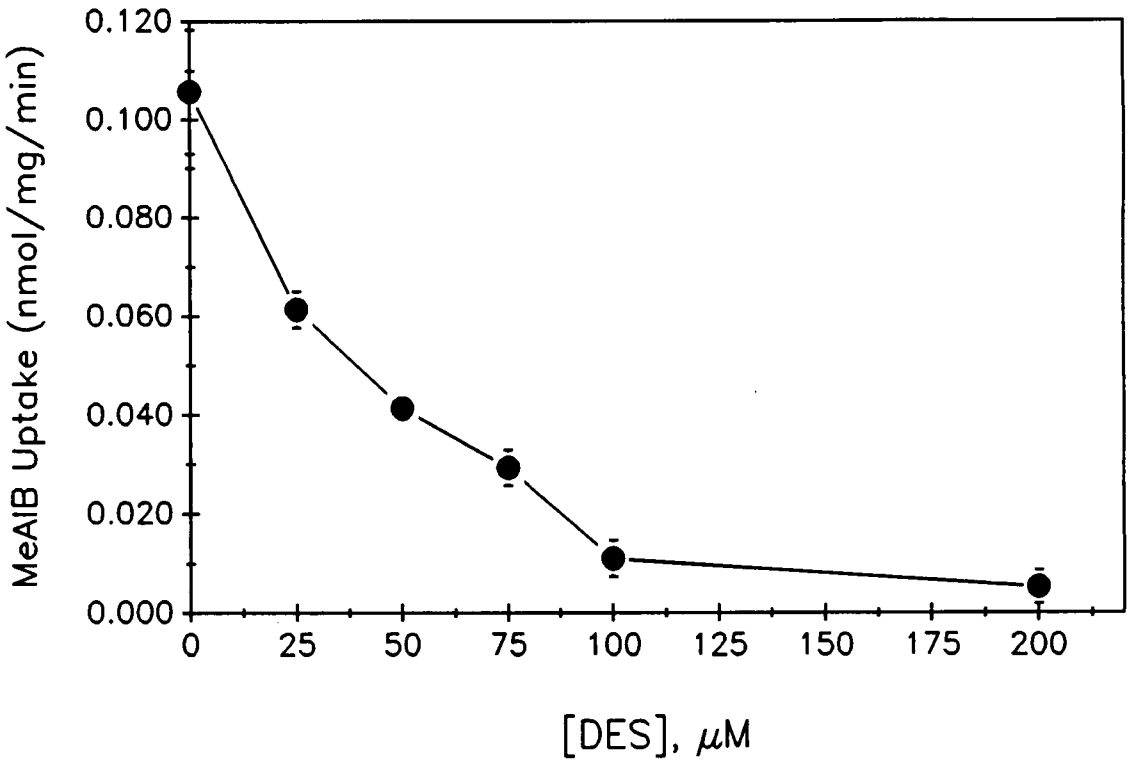
0.120
0.100
0.080
0.060
0.040
0.020
0.000
MeAIB Uptake (nmol/mg/min)
0
25
50
75
100
125
150
175
200
[DES], μM

Figure 2.6

Figure 2.7. Effect of the Diphenolic Inhibitors on Transport of α-MeGlc.

Cells were transferred to HBSS/Glc for 90 min as in a standard A System assay. Left Panel: Diphenolic inhibitors were added as indicated in the figure and half of the filters containing cells were used immediately for the time zero α-MeGlc transport rate. The remaining filters were incubated for 120 min in inhibitor. At the end of this time, filters were transferred into α-MeGlc and uptake with and without Na^+ was determined. Total transport was adjusted for protein content of the filters and time of transport to yield Na^+-dependent uptake as nmol/mg protein/min. The points shown are the difference in means of triplicate samples with Na^+ and triplicate samples lacking Na^+. Error bars represent standard deviations. Right Panel: The cells were incubated for 120 min in TPA (10^{-7}M) or inhibitor plus TPA. At the end of this time, all cells were transferred into MeAIB and uptake with and without Na^+ was determined. Total transport values were adjusted for protein content of the filters and time of transport to yield Na^+-dependent nmol/mg protein/min. Transport in the presence of inhibitors is displayed as a percent of control uptake ± standard deviations.

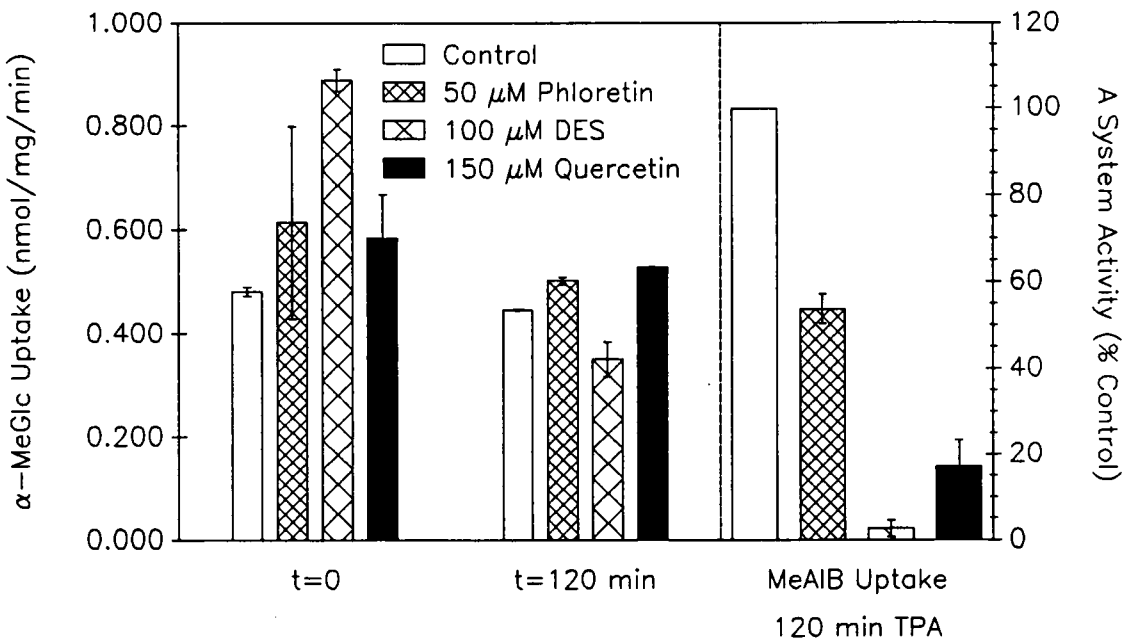
1.000
0.800
0.600
0.400
0.200
0.000
α-MeGlc Uptake (nmol/mg/min)
Control
50 μM Phloretin
100 μM DES
150 μM Quercetin
120
100
80
60
40
20
0
A System Activity (% Control)
t=0
t=120 min
MeAIB Uptake
120 min TPA

Figure 2.7

Since protein synthesis is required for TPA stimulation of the A System (Amsler et. al., 1983; Dawson and Cook, 1988b), one explanation for the elimination of TPA stimulated A System activity could be that these diphenolic compounds are disrupting protein synthesis. To assay protein synthesis directly in treated cells, incorporation of [^{35}S]Met into TCA precipitable material was examined.

Figure 2.8 shows that concentrations of quercetin and DES which prevent TPA stimulation of the A System (Figure 2.8A) do not perturb the sodium gradient as indicated by the lack of an effect on α-MeGlc transport (Figure 2.8A, inset), nor do these concentrations block bulk protein synthesis (Figure 2.8B). Also, there is no significant effect on protein synthesis in the absence of TPA, and protein synthesis increases with TPA addition, even in the presence of the inhibitors.

The cellular receptor responsible for mediating most effects of TPA and other phorbol esters is PKC (Castagna et. al., 1982; Ashendel et. al., 1983a; Ashendel et. al., 1983b; Niedel et. al., 1983; Kikkawa et. al., 1983; Nishizuka, 1984) and PKC can be inhibited by quercetin (Nakadate et. al., 1988). Previous work from this laboratory has shown that down regulation of the A System in confluent cells is accompanied by conversion of PKC from a membrane bound, active form to a cytosolic, inactive form (Dawson and Cook, 1987). Therefore, the effect of all three diphenolic inhibitors on PKC was examined.

Figure 2.8. Effect of Diphenolic Inhibitors on Bulk Protein Synthesis.

Cells were incubated for 90 min in HBSS/Glc, then the medium was removed and replaced by fresh HBSS/Glc ± TPA ± inhibitors for 120 min. One set of cells was used for an A System uptake, another set for an α-MeGlc uptake, and another set had [^{35}S]Met added to the HBSS/Glc at a concentration of 6 μCi/ml. The cells were incubated in the [^{35}S]Met for 30 min with or without TPA and inhibitors, then the cells were washed with ice-cold PBS, lysed in 0.2% SDS and proteins were precipitated with 10% TCA. Precipitates were counted for incorporation of [^{35}S]Met.

A. Quercetin and DES will inhibit A System uptake at concentrations which do not affect α-MeGlc transport. Quercetin inhibited ~ 80 % of the A System activity and DES inhibited ~ 60 % of the A System activity. Inset: No effect on α-MeGlc uptake is seen at these concentrations. The bars shown are the differences in means of triplicate samples with Na^+ and triplicate samples without Na^+. Error bars represent standard deviations.

B. Quercetin and DES do not inhibit protein synthesis at concentrations which inhibit A System transport. Addition of TPA increases protein synthesis even in the presence of diphenolic compounds at concentrations that inhibit TPA stimulated A System transport. The bars shown are means of triplicate samples. Error bars represent standard deviations.

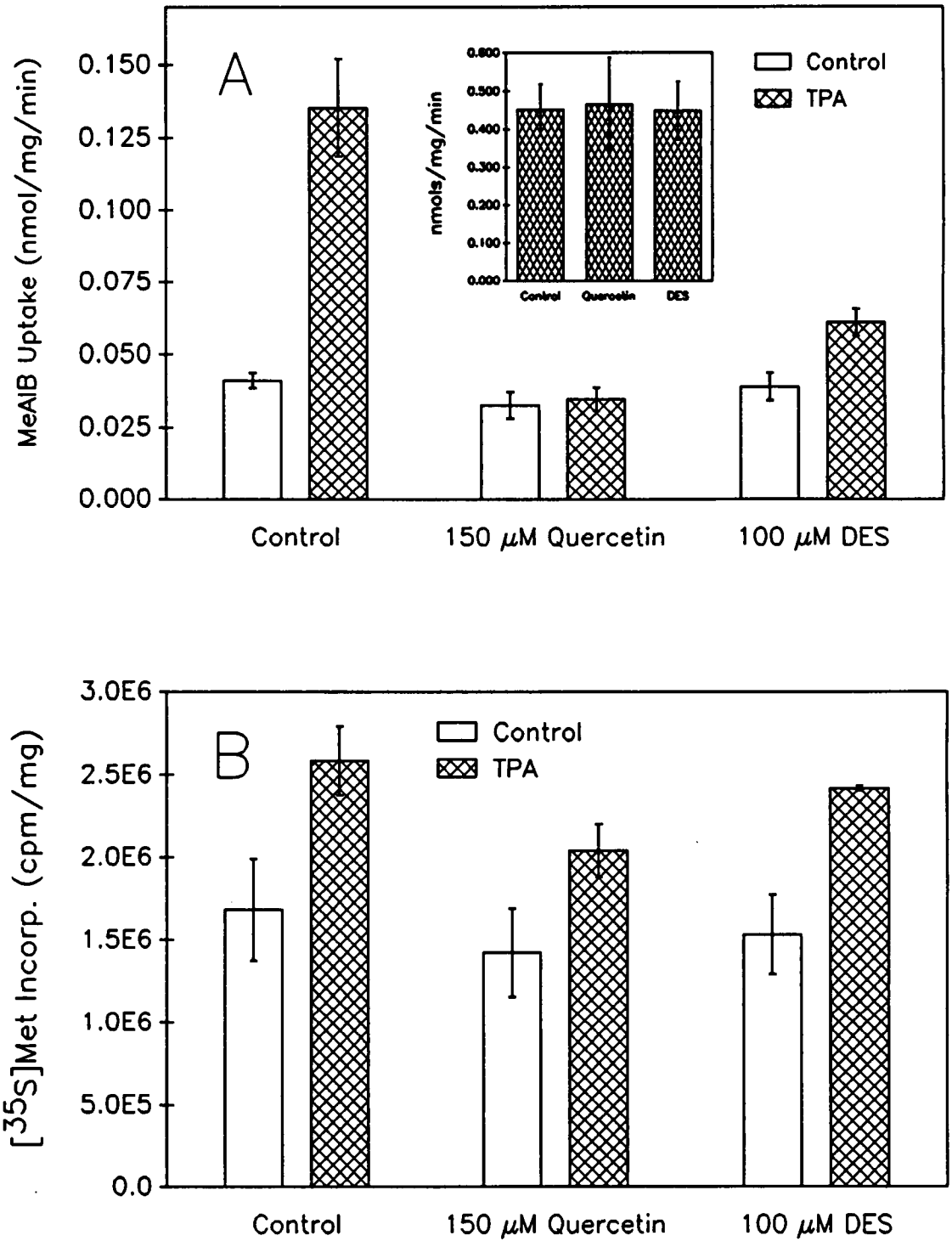
A
MeAIB Uptake (nmol/mg/min)
0.150
0.125
0.100
0.075
0.050
0.025
0.000
Control
TPA
nmols/mg/min
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Control
Quercetin
DES
Control
150 μM Quercetin
100 μM DES
B
[35S]Met Incorp. (cpm/mg)
3.0E6
2.5E6
2.0E6
1.5E6
1.0E6
5.0E5
0.0
Control
TPA
Control
150 μM Quercetin
100 μM DES

Figure 2.8

Figure 2.9. Effect of Diphenolic Inhibitors on Protein Kinase C.

All inhibitors were added to the final concentrations indicated in the figure. PKC activity was assayed as total [γ-^{32}P] transferred from [γ-^{32}P]ATP to histone minus [γ-^{32}P] transferred from [γ-^{32}P]ATP to histone in the absence of added phospholipid and presence of 5 mM EGTA to give Ca^{2+}- and phospholipid-dependent kinase activity. The points shown are the difference in means of triplicate samples with Ca^{2+} and lipids and triplicate samples with EGTA lacking lipids, adjusted to percent of uninhibited control. Error bars represent standard deviations.

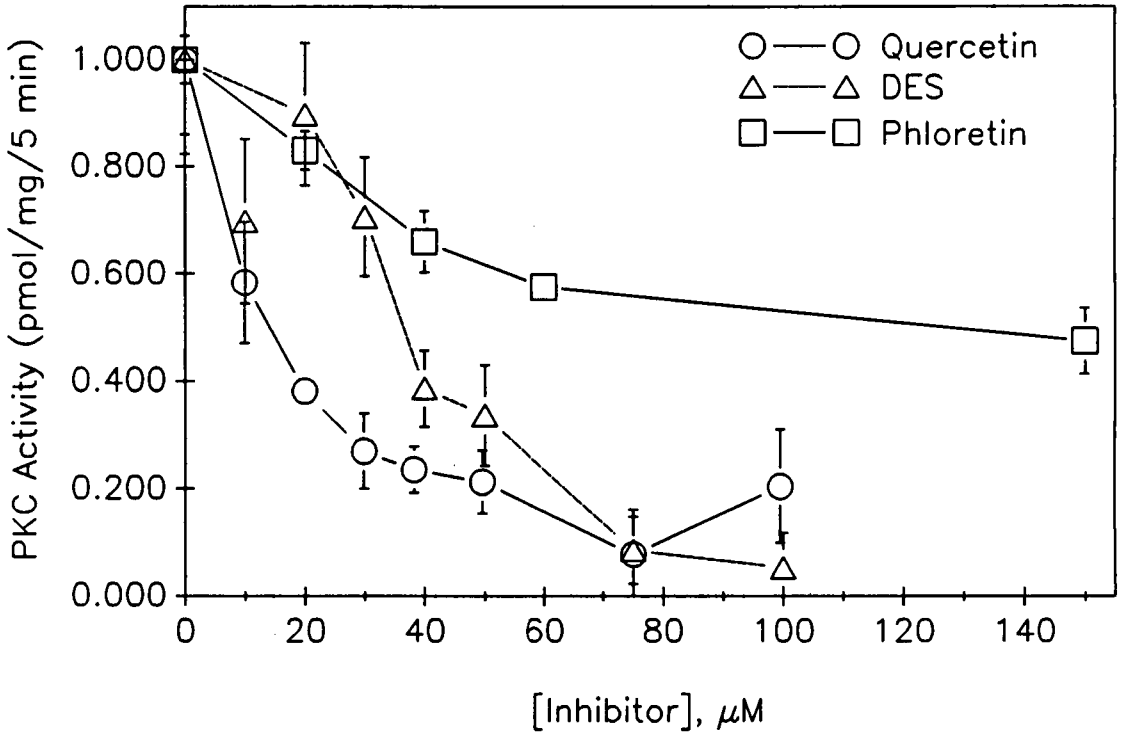
PKC Activity (pmol/mg/5 min)
1.000
0.800
0.600
0.400
0.200
0.000
0
20
40
60
80
100
120
140
[Inhibitor], μM
Quercetin
DES
Phloretin

Figure 2.9

All three diphenolic compounds inhibit PKC at concentrations lower than those which inhibit TPA stimulation of the A System (Figure 2.9). DES and quercetin are the most effective at inhibiting PKC (both will inhibit ≥ 90% of the PKC activity at 75 μM with $K_{1/2}$'s of ~ 15 μM and ~ 35 μM, respectively) while phloretin is a much less effective inhibitor of PKC (maximal inhibition is 50 % at 150 μM, with a $K_{1/2}$ of ~ 30 μM). This is the same degree to which these compounds are capable of inhibiting TPA stimulation of the A System, with quercetin and DES inhibiting almost all of the activity and phloretin inhibiting only about half of the activity.

Further, if PKC is the site of action of quercetin, then by removing the phorbol ester from the cell it should be possible to mimic the effect seen in Figure 2.4. In that experiment, quercetin was ineffective at inhibiting the TPA stimulation of the A System if an interval of 15 minutes or longer passed between addition of TPA and addition of quercetin to the cells. TPA cannot be used to perform washout experiments, because it is too lipophilic to wash out of the cell membrane quickly and completely. Instead, phorbol dibutyrate (PDBu), a much more hydrophilic compound, was used. Figure 2.10 shows that after 5 min in PDBu, washing the cells out of the PDBu does not decrease the extent of the A System response. Since quercetin, phloretin and DES are capable of preventing TPA stimulation of A System transport, and have no effect on the level of protein

Figure 2.10. Effect of PDBu Washout on A System Transport.

LLC-PK_1 cells were grown to confluence on collagen coated filters. Cells were depleted of internal amino acids for 90 min, then placed into PDBu in HBSS/Glc. At the times indicated in the figure, the HBSS/Glc containing PDBu was removed by aspiration, and the filter containing the cells was washed four times in PBS/BSA. All cells were assayed for A system activity 120 min after addition of the PDBu. The points shown are the difference in means of triplicate samples with Na^+ and triplicate samples lacking Na^+. Error bars represent standard deviations.

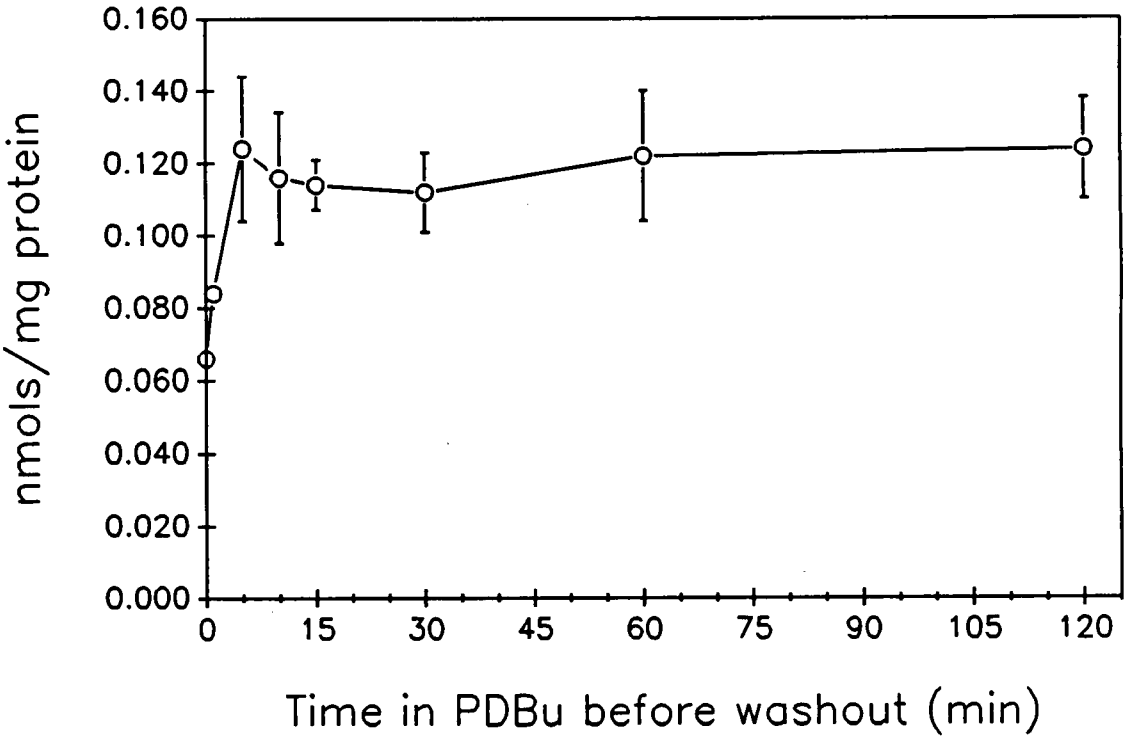
0.160
0.140
0.120
0.100
0.080
0.060
0.040
0.020
0.000
nmols/mg protein
0
15
30
45
60
75
90
105
120
Time in PDBu before washout (min)

Figure 2.10

synthesis (Figure 2.8), the Na^+ gradient as determined by α-MeGlc uptake (Figure 2.7), or the transporter directly (Figure 2.3), and do inhibit PKC *in vitro*, this indicates that PKC is indeed the site of action of quercetin, phloretin and DES in inhibiting the TPA stimulation of A System transport activity.

Discussion

Rapidly proliferating LLC-PK_1 cells need high levels of amino acids for protein synthesis. Amino acid transporters such as the A System are very active in proliferating cells. When cells reach confluence however, their need for new amino acids decreases, and the transporters are down regulated. Addition of TPA to confluent, down regulated cultures of LLC-PK_1 cells causes a reversal of this effect, and 2 hours after addition of TPA transport is stimulated (Amsler et. al., 1983). Protein synthesis is required for this long term stimulation of transport to occur, as is protein glycosylation (Dawson and Cook, 1987 and unpublished). This indicates that the addition of TPA to confluent cultures could cause increased transport through increased synthesis of the A System transporter.

Phloretin, DES, and quercetin are well known as inhibitors of the Na^+-independent hexose transporter (LeFevre and Marshall, 1959; Salter et. al., 1978); and quercetin is also known to inhibit protein kinase C (Nakadate et. al., 1988). Phloretin and DES have also been shown in this study to inhibit protein kinase C (Figure 2.9). All three of these compounds will prevent TPA from stimulating

A System transport activity and will also prevent the adaptive response (discussed in Chapter 1) from increasing transport (no increase in transport is seen over the course of a 120 minute incubation in Figure 2.1). If these compounds were directly inhibiting the A System transporter, then the degree of inhibition should be similar in TPA-treated and non-TPA-treated cells. This is clearly not the case. Addition of quercetin appears to decrease transport to a level slightly below the control, whether or not the cells have been exposed to TPA (see Figures 2.2, 2.3, 2.8A). Further, this level of amino acid transport does not continue to decrease with time in the inhibitor (Figure 2.1).

Transport over other Na^+ dependent pathways, such as the Na^+-dependent hexose transporter, is not affected under conditions which inhibit A System transport (Figures 27, 2.8A inset). If the diphenolics simply disrupt the Na^+ gradient, either through inhibition of the Na^+-, K^+-, ATPase or a direct effect on the membrane, then transport of α-MeGlc should be affected as well. Since these compounds have little or no effect on α-MeGlc uptake, the Na^+ gradient probably remains intact.

Protein synthesis is required for up-regulation of the A System (Amsler et. al., 1983; Dawson and Cook, 1988b), and therefore, inhibition of protein synthesis would block up-regulation of A System transport as well. To test if this happens, quercetin and DES were assayed to determine if they could inhibit protein synthesis under conditions that inhibit A System transport activity. Figure 2.8 shows that DES and quercetin do not inhibit protein synthesis under these conditions. In fact,

TPA causes a small increase in the level of bulk protein synthesis while TPA stimulation of the A System is blocked.

If these diphenolic compounds are not directly inhibiting the A System transporter itself and are not reducing the driving force for entry or stopping bulk protein synthesis, then their action must occur during the signal transduction pathway. All three inhibitors have been shown to have a direct effect on protein kinase C (Nakadate et. al., 1988; also Figure 2.9), the cellular receptor for tumor promoting phorbol esters (Castagna et. al., 1982; Ashendel et. al., 1983a; Ashendel et. al., 1983b; Niedel et. al., 1983; Kikkawa et. al., 1983; Nishizuka, 1984). If these diphenolic inhibitors directly inhibit protein kinase C, then TPA will not be able to stimulate up-regulation of the A System. Further, addition of quercetin, a proven PKC inhibitor, prevents stimulation of A System transport over the same time course as inhibition of PKC by removal of the phorbol ester (compare Figure 2.4 and Figure 2.10). Therefore, these three compounds are believed to prevent A System up-regulation by interrupting signal transduction at the level of protein kinase C and not by any direct effect on the A System transporter itself.

PKC appears to be required for only a brief time (< 15 min) to get maximal stimulation of the A System transporter, since either inhibition of PKC (Figure 2.4) or washing the phorbol ester out of the cells after this time (Figure 2.10) do not affect the overall level of transport. It is not apparent why PKC is required for such a short time, but this possibly could be due to down regulation of PKC in the course of signal transduction. Alternatively, PKC could be initiating a cascade

reaction which is the actual stimulus for enhanced A System transport, and after 15 minutes the signal has been passed and active PKC is no longer needed. Further discussion of these possibilities and the implications for control of A System transport can be found in Chapter 5.

In either case, it is very interesting to note that there are two other modulators of the A System that work in a similar time course. Glucagon (Fehlmann et. al., 1979a) and insulin (LeCam and Freychet, 1979, as cited in Kilberg, 1982) take about 2 hours to stimulate transport via the A System; both hormones can be removed from the cells after 15 min of exposure and still yield a significant stimulation of transport. It is not known if these hormone responses involve PKC.

CHAPTER 3

PROTEIN KINASE C AND KINETIC CONTROL OF A SYSTEM TRANSPORT

Abstract

The kinetics of A System transport in LLC-PK1 cells were examined in proliferating cultures, and again as those cultures became confluent. The decrease in A System transport seen in confluent cells was due to a decrease in the V_{max} of the transporter. TPA stimulation of confluent cultures caused over a four-fold increase in V_{max}, while the K_m (0.5 - 1.0 mM) did not change significantly. Treatment of proliferating cells with 150 μM quercetin also significantly decreased the V_{max}, to approximately half its original value; this occurred with no significant change in K_m. Changes in V_{max} with no change in K_m indicate that A System transport may be controlled by changes in the number of transporters in the membrane rather than modification of the transporter itself. The K_m was unaffected by varying growth rate, addition of phorbol esters to confluent cells or addition of quercetin to rapidly growing cells.

Introduction

Proliferating LLC-PK_1 cells have a much higher level of A System transport than confluent cells (Amsler et. al., 1983). As a culture of LLC-PK_1 cells becomes confluent, the cells differentiate, and A System transport decreases. This process is referred to as "step down". If confluent, stepped down cultures of LLC-PK_1 cells are treated with TPA, the level of A System transport increases (Amsler et. al., 1983, Figure 2.1). Previous experiments have shown this TPA stimulated increase is accompanied by translocation of PKC from an inactive, cytoplasmic form to an active, membrane-bound form (Dawson and Cook, 1989). PKC is the cellular receptor for TPA (Castagna et. al., 1982; Ashendel et. al., 1983a; Ashendel et. al., 1983b; Niedel et. al., 1983; Kikkawa et. al., 1983; Nishizuka, 1984). PKC is found in its membrane-bound, active form in proliferating cells (Adamo et. al., 1986). If PKC is a normal cellular controlling factor in stimulation of A System transport activity and PKC is active in proliferating cells, then inhibition of PKC should lead to a decrease in transport, even in proliferating cells not exposed to phorbol esters such as TPA. Therefore, addition of quercetin to proliferating cells should decrease transport in a manner similar to the decrease seen when cells stop proliferating, and opposite to the increase caused by TPA. By examining the kinetics of A System transport in TPA-stimulated cells and also in PKC-inhibited cells, it may be possible to determine how cells modulate A System transport activity.

The earliest discussion of modulation of A System transport activity came from Guidotti's laboratory (Gazzola et. al., 1973). They were studying the effect of deprivation of exogenous A System substrates on amino acid transport in chick heart cells, and found that cells respond to deprivation by increasing transport over the A System. They termed this response "starvation derepression", or the "adaptive response". The adaptive response has since been shown to be dependent on mRNA synthesis (Gazzola et. al., 1981), protein synthesis (Tramacere et. al., 1977), and protein glycosylation (Barber et. al., 1983). The adaptive response is characterized by an increase in V_{max} for transport with no change in K_m (Tramacere et. al., 1977; Kelley and Potter, 1978; Gazzola et. al., 1981; Gazzola et. al., 1984) and is believed to represent synthesis and insertion of new transporters into the cell membrane (Tramacere et. al., 1977).

Changes in K_m would be evidence that this system is more complex than regulation of transport through control of the number of active A System transporters in the cell membrane. Evidence for alteration of the A System K_m under some conditions, such as starvation of transformed MDCK-T_1 cells (Boerner and Saier, 1985) or of G_0 3T3 cells (Hochstadt et. al., 1979) reportedly alters both the K_m and V_{max} of the A System transporter.

In order to determine if TPA and quercetin induced changes in amino acid transport over the A System is due to changes in K_m, V_{max} or both, experiments were performed to determine K_m and V_{max} at different times as cells became confluent. Similar experiments were then performed on confluent cells treated with

TPA to determine if TPA treatment stimulates A System transport in a manner similar to induction of proliferation. Lastly, proliferating cultures of LLC-PK_1 cells were treated with quercetin to determine the effects of PKC inhibition on cells that have never been exposed to phorbol esters, yet maintain a high level of A System transport.

Materials and Methods

Cell Culture

LLC-PK_1 pig kidney epithelial cells, obtained from R. N. Hull (Hull et. al., 1976) at passage 185 were used for these experiments. Cultures were maintained as described in Chapter 2. Cells were used between passage 200 and 220.

Chemicals

Unless otherwise indicated, chemicals were obtained from Sigma (St. Louis). Radioisotopically-labelled compounds were from DuPont/NEN (Boston, MA). TPA and PDBu were from LC Biochemicals (Woburn, MA). Quercetin was purified by crystallization from EtOH before use.

A System Transport Assays

For kinetic studies on rapidly growing cells, a nearly confluent LLC-PK_1 stock culture was trypsinized from a single T-75 flask and plated onto collagen-coated filters. Two days later, the filters were removed from α-medium, washed once in HBSS/Glc and incubated in HBSS/Glc for 90 minutes at 37°C. In kinetic experiments with quercetin, the quercetin was added to a final concentration of 150 μM, during the last 30 minutes of the 90 minute incubation. The cells were then assayed for A system activity using the method described in Chapter 2, which was adapted from Amsler et. al. (1983). Concentrations of MeAIB used for these kinetic studies were 0.1, 0.5, 1.0, 3.0, and 5.0 mM. For the two lower concentrations of MeAIB, 0.1 μCi/ml [^{14}C]MeAIB was used, whereas 1.0 μCi/ml [^{14}C]MeAIB was used for all MeAIB concentrations of 1.0 mM or higher. Triplicate cell filters were assayed with and without Na^+ to determine Na^+-dependent uptake. After an uptake period of 30 min at 37°C, which is within the linear portion of the uptake reaction (Amsler et. al., 1983), the cells were washed with ice-cold PBS and solubilized in 2 ml 0.2% SDS. Radioactivity of the [^{14}C]MeAIB was measured on 1.0 ml of the lysate, while 0.5 ml of the lysate was used to determine protein concentration by the fluorometric method of Avruch and Wallach (1971). The uptake data were expressed as nmol MeAIB/mg protein/min, uptake values in the absence of Na^+ were subtracted from total uptake values to

calculate Na^+-dependent uptake. Results were graphed as an Eadie-Hofstee plot, i.e., velocity vs velocity divided by substrate concentration.

For kinetic analysis in confluent cells, LLC-PK_1 cells were again plated on collagen-coated filters. On the second day after plating, the filters with cells were transferred to individual petri dishes and cultured for an additional 8-12 days, with a medium change every 2 days. The medium was last changed two days before the cells were used. For the assay, filters with cells were first transferred into HBSS/Glc at 37°C for 90 min to deplete the intracellular amino acid stores, and then TPA was added for 120 min. At the end of this time, the cells were washed in Na^+-replete or Na^+-free buffer and used for a 30 min A System assay as above.

To determine the significance of changes in K_m or V_{max}, first order linear regression was used to generate the appropriate graphs. Student's t-test, adapted for use with first order regression equations (Zar, 1984) was used to test for differences in the slopes and Y-intercepts of the calculated regression lines.

Results

Figure 3.1 shows the decrease in A System activity as cells become confluent. The data are presented as an Eadie-Hofstee plot, where the Y-intercept of a set of data is the V_{max} for the reaction, while the slope of the line through the data is equal to $-K_m$ for the reaction. The V_{max} of the A System, approximately 0.5 nmols/mg protein/min in this assay, is almost two-fold higher in the 2 day old

Figure 3.1. The V_{max} of the A System Transporter Decreases as Cells Become Confluent.

LLC-PK_1 cells were plated on collagen-coated filters on day 0 and grown for 14 days. Cells at 2, 6 and 14 days of growth were assayed for A System transport using MeAIB at 0.1, 0.5, 1.0, 3.0 and 5.0 mM. The data are presented in the form of an Eadie-Hofstee graph, where the Y-intercept is the V_{max} of the reaction and the slope is equal to the negative K_m. All points are the differences in the mean of triplicate samples of MeAIB uptake with Na^+ and triplicate samples of MeAIB uptake without Na^+. Error bars represent standard deviations.

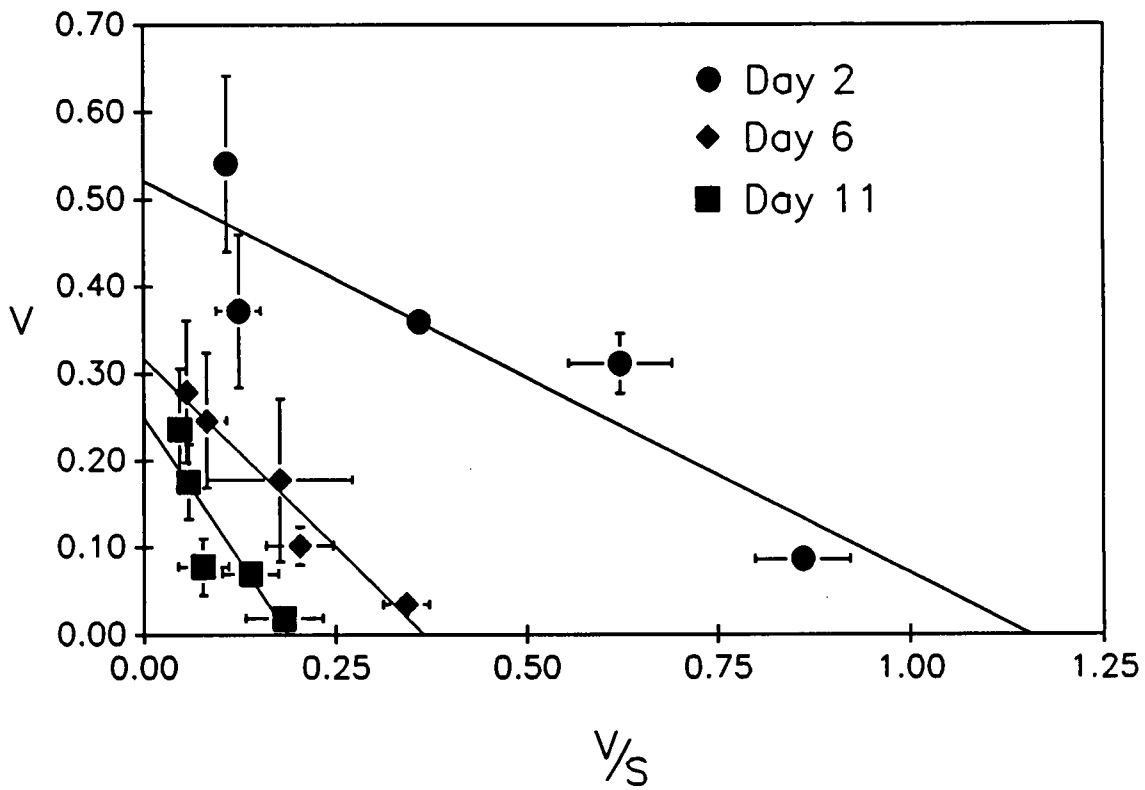
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
V
0.00
0.25
0.50
0.75
1.00
1.25
V/S
Day 2
Day 6
Day 11

Figure 3.1

Table 3.1 Summary of the data derived from Figures 3.1

Days in Culture	Number of samples*	K_m ± SE	V_{max} ± SE	% Δ in V_{max}	% Δ in K_m
2	15	0.5 ± 0.8	0.52 ± 0.08	1.00	100
6	15	0.9 ± 0.8	0.32 ± 0.08	0.61	180
14	15	1.3 ± 0.8	0.25 ± 0.08	0.48†	260

* 15 filters containing cells were used for Na^+-replete samples and 15 were used for Na^+-free samples; each data point on the graph represents two sets of triplicates which are subtracted to yield Na^+-dependent transport. A total of 30 samples are used to generate each line, but half are used to determine background. In t-test calculations, the value used for n was 15.

† significantly different from Day 2 V_{max} at $p < 0.015$

cells as in the 14 day old cells. Figure 3.1 also shows a two-fold difference in the K_m of the transporter, but while the increase in V_{max} is statistically significant at the $p < 0.015$ level, the apparent change in K_m is not. Table 3.1 summarizes the data plotted in Figure 3.1; all differences in K_m and V_{max} not indicated as significant in this and the following tables are not significant at the $p < 0.05$ level. The variance seen in the data can be explained by the fact that the means shown are the result of subtracting transport in the absence of Na^+ from total transport, and small variations in transport rate in different cells are magnified by the calculations required to obtain specific transport via the A System. While no significant change was found for the K_m in this assay, it must be noted, however, that other groups have reported modulation of the A System by a 4.5-fold change in transporter K_m in growth factor-treated 3T3 fibroblasts (Hochstadt et. al., 1979), or changes in both V_{max} and K_m in amino acid-starved MDCK-T_1 cells (an induced transformant of MDCK cells) (Boerner and Saier, 1985).

Figure 3.2 shows the effect of TPA on confluent cells. In the absence of TPA, the A System displays a stepped down transport rate as previously described (Amsler et. al., 1983). After 120 min in TPA the data show a greater than four-fold increase in the V_{max} of the transporter with no significant change in the K_m. The V_{max} of confluent cells in this experiment is 152 % of that seen for confluent cells in Figure 3.1, and after TPA treatment the V_{max} of the transporter in confluent cells reaches a value of 1.58 nmol/mg protein/min, which is approximately 300 % of the V_{max} of proliferating cells in Figure 3.1.

Figure 3.2. The Effect of TPA on the A System Transporter.

LLC-PK_1 cells grown in culture for 14 days were depleted of intracellular amino acids for 90 min at 37°C; half of the cells were then treated with TPA (10^{-7}M) for 120 min, and the other half were only treated with ethanol (Control). Cells were then assayed for A system transport activity at 0.1, 0.5, 1.0, 3.0, and 5.0 mM MeAIB. The data are presented as an Eadie-Hofstee graph. Each point on the graph represents a single sample of Na^+-replete uptake, with a single sample of Na^+-free uptake subtracted.

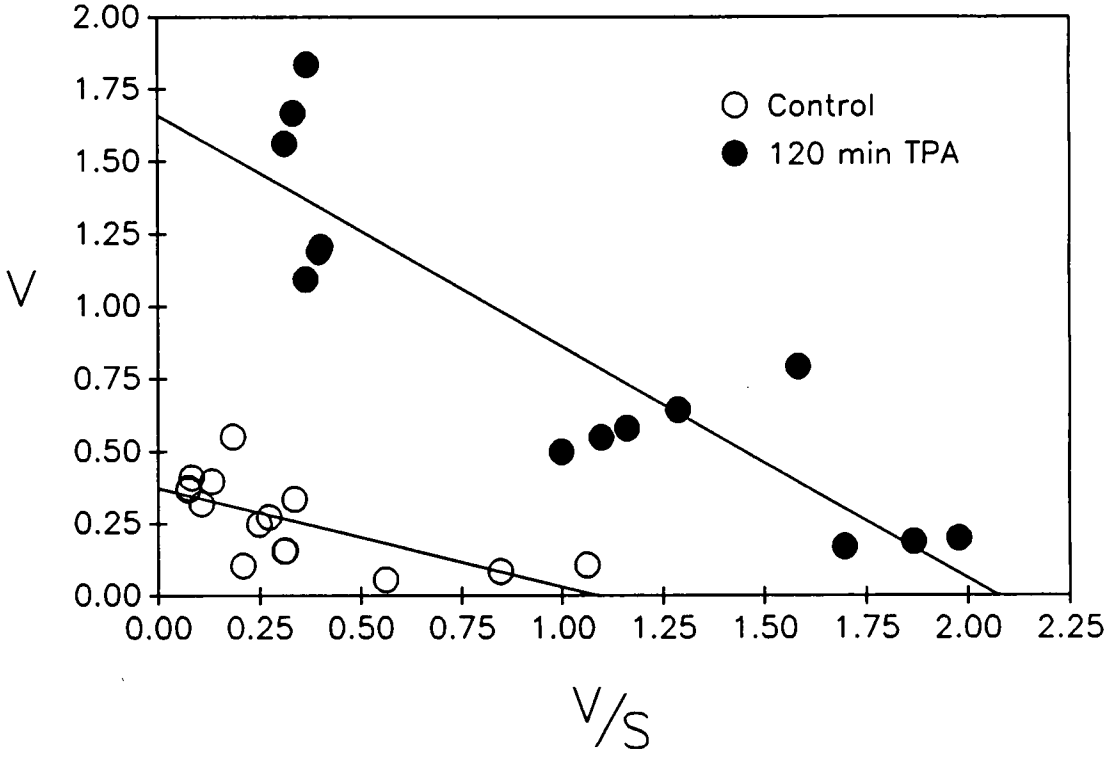
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
V
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
2.25
V/S
Control
120 min TPA

Figure 3.2

Table 3.2 Summary of the data presented in Figure 3.2

Age of Cells	Number of samples*	Treatment	K_m ± SE	V_{max} ± SE	% of V_{max}	% of K_m
14 days	15	EtOH Control	0.3 ± 0.4	0.38 ± 0.32	100	100
14 days	14	TPA (10^{-7}M)	0.8 ± 0.4	1.58 ± 0.32	424†	233

* 15 filters containing cells were used for Na^+-replete samples and 15 were used for Na^+-free samples; each data point on the graph represents two sets of triplicates which are subtracted to yield Na^+-dependent transport. A total of 30 samples are used to generate each line, but half are used to determine background. One filter from the samples treated with TPA was discarded. In t-test calculations, the value used for n was 15 for the control and 14 for TPA-treated cells.

† significantly different from control at $p < 0.001$

Figure 3.3 shows the effect of treating rapidly growing cells with 150 μM quercetin for 30 min. In rapidly growing cells, the K_m for transport appears to be in the same range (0.5-1.0 mM) as the K_m found in differentiated cells. Addition of 150 μM quercetin significantly decreases ($p < 0.025$) the V_{max} of A System transport to approximately half the normal level without significantly affecting the K_m of the transporter. In 3 day old cultures of LLC-PK_1 cells, the V_{max} for the A System transporter is approximately 1.6 nmol/mg protein/min, which is a value similar to the V_{max} observed in TPA-treated confluent cells (approximately 1.6 nmol/mg/min in Figure 3.2). This demonstrates quercetin decreases the rate at which proliferating cells accumulate MeAIB, which helps to explain the immediate decrease seen in A System transport in Figures 2.1 and 2.3. Since those cultures were grown without differentiation inducers, they would contain a small number of proliferating cells with a disproportionately high rate of A System transport activity. Quercetin inhibition of transport in those cells would lower the total amount of A System transport in the cell population, and once these cells were inhibited, there would be little additional effect on transport (Figure 2.1, Figure 2.3).

Discussion

On the basis of the data presented, it appears that modulation of A System transport activity by PKC stimulation or inhibition is carried out by altering the number of active transporters associated with the cell membrane. The K_m for the

Figure 3.3. The Effect of 150 μM Quercetin on A System Transport in Proliferating Cells.

LLC-PK_1 cells were used 3 days after plating onto collagen-coated filters. Cells were first depleted of intracellular amino acids for 60 min; then half of the cells were treated with 150 μM quercetin, and half with DMSO (Control). After an additional 30 min, the cells were assayed for A System transport activity at 0.1, 0.5, 1.0, 3.0, and 5.0 mM MeAIB. The results are presented as an Eadie-Hofstee graph. Each point on the graph represents a single sample of Na^+-replete uptake, with a single sample of Na^+-free uptake subtracted.

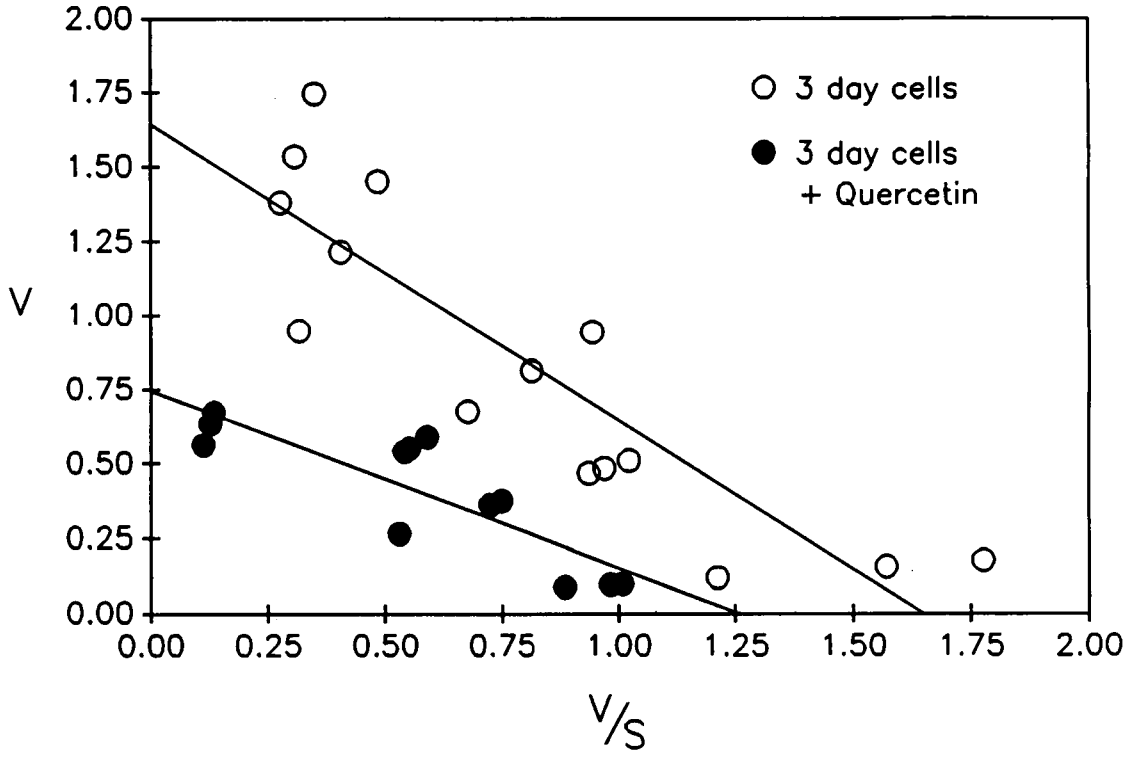
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
V
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
V/S
3 day cells
3 day cells
+ Quercetin

Figure 3.3

Table 3.3 Summary of the data presented in Figure 3.3

Age of Cells	Number of Samples*	Treatment (30 minutes)	K_m ± SE	V_{max} ± SE	% of V_{max}	% of K_m
3 days	15	DMSO Control	1.0 ± 0.4	1.63 ± 0.42	100	100
3 days	12	30 min Quercetin	0.6 ± 0.4	0.75 ± 0.42	44†	59

* 15 filters containing cells were used for Na^+-replete samples and 15 were used for Na^+-free samples; each data point on the graph represents two sets of triplicates which are subtracted to yield Na^+-dependent transport. A total of 30 samples are used to generate each line, but half are used to determine background. Three filters from the samples treated with quercetin were discarded. In t-test calculations, the value used for n was 15 for the control and 12 for quercetin treated cells.

† significantly different from control at $p < 0.025$

transporter appears to be unchanged in cells as the total amount of transport is varied due to changes in growth state or treatment with TPA or quercetin. These results indicate that the actual K_m of the system probably lies in the range of 0.5-1.0 mM for LLC-PK_1 cells. This agrees with the published range of K_m values for MeAIB and the A System in many different cell types: 0.4 mM in Ehrlich ascites tumor cells (Christensen et. al., 1965), 1.2-1.4 mM in chick embryo fibroblasts (Tramacere et. al., 1977), 1.4-1.6 mM in fetal human fibroblasts (Gazzola et. al., 1984), 1.1-1.3 mM in cultured rat hepatocytes (Kelley and Potter, 1978), 0.6-0.8 mM in isolated rat hepatocytes (Fehlmann et. al., 1979b), and 1.6-3.0 mM in CHO cells (Bass et. al., 1981).

An earlier study from this laboratory (Villereal and Cook, 1978) indicated that changes in AIB transport in quiescent and growing HSWP cells, a human fibroblast cell line, could be attributed to changes in membrane potential. Modulation of the membrane potential resulted in immediate changes in the K_m. Subsequently, the V_{max} of the transporter increased, probably due to an increase in the number of transporters associated with the cell membrane. It was determined that in HSWP cells, growth-related membrane potential changes could account for the observed growth-related differences in AIB accumulation. In a later study, (Becker and Cook, 1981) indicated that in Friend erythroleukemic cells, TPA caused an increase in AIB accumulation through modulation of the membrane potential. Changes in the membrane potential result in K_m changes in the activity of the A System transporter (Villereal and Cook, 1978). No changes in the K_m of

the A System transporter were observed in LLC-PK_1 cells, and previous work (Amsler et. al., 1983) clearly shows that TPA stimulation of the A System transporter is not due to changes in membrane potential in LLC-PK_1 cells. Furthermore, the absence of any effect of growth state, TPA stimulation or quercetin addition on the K_m of the transporter indicate that changes in membrane potential may not be a major factor in modulation of A System activity under these conditions.

Direct comparisons of absolute values for V_{max} reported in the literature are difficult to make due to the many factors which affect the V_{max}. It is much more common to report the degree of stimulation or repression of the V_{max} produced by a given experimental treatment. In this regard, addition of serum to serum-deprived chick embryo fibroblasts resulted in a two-fold decrease in the V_{max} of the transporter activity (Tramacere et. al., 1977), which is similar in magnitude to the results from Figure 3.2 (V_{max} of quercetin treated cells is 44 % of control cells). Two studies on the effect of cell transformation on A System activity have reported increases of 3.6 fold in glioma cells (Ronquist et. al., 1976) or 3-7 fold in transformed MDCK-T_1 cells (Boerner and Saier, 1982), both of which are similar to the results of Figure 3.2, where TPA addition increased the V_{max} of four-fold.

Addition of TPA to confluent cells resulted in a significant increase in the V_{max} of greater than four-fold after 120 minutes, but the apparent change in K_m was not statistically significant. Also, the V_{max} for the A System transporter in TPA-treated cultures of confluent LLC-PK_1 cells was virtually identical to the V_{max}

seen in rapidly growing (3 day old) cultures (Figures 3.2 and 3.3). This indicates that A System transport activity in TPA-treated confluent cells is representative of A System transport activity in rapidly growing cells. TPA-treated confluent cells are preferable for experimental purposes because there is a much greater amount of protein per sample and all cells are at the same growth stage. LLC-PK_1 cells tend to grow in isolated islands, and recent experiments have shown that epithelial cell islands can consist of proliferating, nondifferentiated cells at the periphery and nonproliferating, differentiated cells in the center. Indications of this, specifically alterations in fodrin distribution (fodrin distribution is dependent on development of polarity in epithelial cells and is distinct in differentiated cells), have been seen in islands of as few as 25 MDCK cells (Nelson and Veshnock, 1986).

Addition of quercetin to proliferating cells decreased the V_{max}, with no significant change in K_m. The data in chapter 2 indicate that the site of action of quercetin in LLC-PK_1 cells is PKC. In proliferating LLC-PK_1 cells PKC exists in its membrane bound, active form (Adamo et. al.,1986; Dawson and Cook, 1987). Figure 3.3 shows that inhibition of PKC by quercetin will result in a decrease in transport over the A System. Previous work in H4 cells (Kilberg et. al., 1985) has indicated that continuous protein synthesis is required to maintain high levels of A System transport. If PKC is required to pass a signal to the nucleus to maintain the high rate of transcription of the A System gene, then inhibition of PKC should result in a decrease in transport over the A System. This would require, however, that transcription is tightly linked to PKC activity, and also that both the A System

mRNA and the transporter protein are degraded quickly enough that transport decreases to 50 % after a 30 minute pretreatment and a 20 min uptake period.

One other possibility is that PKC directly interacts with the A System transporter in proliferating cells. Phosphorylation/dephosphorylation of the transporter would serve as an absolute activator/deactivator of the molecule, rather than modulating the affinity, since no changes in K_m are seen with quercetin treatment of proliferating cultures (Figure 3.3). This hypothesis does not require rapid turnover of the A System mRNA or protein, or that PKC activity be tightly coupled to transcription. The data from Chapter 2 (Figure 2.4 and Figure 2.10) argue against such a tight coupling in confluent cells, since inhibition of PKC 15 minutes after stimulation gave no effect on transport rates 120 minutes after the original PKC stimulation. This also agrees with data gathered by W. David Dawson, which was consistent with the hypothesis that in proliferating cells, PKC directly affected the transporter (W. David Dawson, personal communication).

Whether the transporter is a direct target of PKC or not, it is clear that modulation of PKC activity in these cells, either through addition of PKC stimulators (Figure 3.2) or PKC inhibitors (Figure 3.3) results in modulation of the V_{max} of transport. This may be as simple as a change in the number of transporters in the cell membrane, or it may involve phosphorylation of the transporter in proliferating cells. Without a direct probe for the transporter protein, it is difficult to distinguish between these possibilities.

CHAPTER 4

LLC-PK_1 CELLS MAY EXPRESS PROTEIN KINASE C ζ

Abstract

Since active PKC is required for only ~ 15 minutes of the 120 minute TPA incubation period, it is possible that the kinase is being degraded or converted into PKM during this time. To examine this, three synthetic peptides were used to generate polyclonal antisera against protein kinase C. The published sequences of PKC α, ßI, ßII, and γ (Ohno et. al., 1988) were used to select two regions from the catalytic domain and one region from the regulatory domain of PKC for use in generating the antisera. Sequences were chosen on the basis of hydrophilicity and the number of charged amino acids in the region.

All three antisera were able to bind to pig brain PKC and immunoprecipitate PKC after addition of Protein A-Agarose. None of the antisera directly inhibited PKC in the standard *in vitro* assay. All three antisera showed some reactivity on Western blots against highly purified rat brain PKC (Calbiochem), though none of the antisera reacted with a similar molecular weight (78 kDa) band in LLC-PK_1 cells. Antiserum G59, which gave the strongest reaction on Western blots against rat brain PKC, reacted against a similar molecular weight band in pig brain PKC, but not pig kidney (LLC-PK_1) PKC.

Instead, The strongest reactions were with bands of ~ 70 kDa, ~ 35 kDa and ~ 30 kDa. Western Blots of cells expressing a recently discovered PKC isotype, PKC ζ, have bands at ~ 66 kDa and ~ 31 kDa (Ono et. al., 1989a), indicating LLC-PK_1 cells may express PKC ζ.

Introduction

Protein kinase C is the cellular receptor for tumor promoting phorbol esters (Castagna et. al., 1982; Ashendel et. al., 1983a; Ashendel et. al., 1983b; Niedel et. al., 1983; Kikkawa et. al., 1983; Nishizuka, 1984). After binding of phorbol esters or the natural substrate, diacylglycerol (Takai et. al., 1979b; Kishimoto et. al., 1980; Kaibuchi et. al., 1981), PKC changes from an inactive cytosolic protein to a membrane bound serine/threonine kinase (Nishizuka, 1984). It is this membrane bound, active form which is a substrate for calpain, the calcium-dependent neutral protease (Suzuki and Ohno, 1990). Calpain is believed to be responsible for down regulation of PKC after the initial stimulus (Suzuki and Ohno, 1990).

The translocation of PKC from its cytosolic form to the membrane bound form has been shown to occur in the same time course as TPA stimulation of the A System (Dawson and Cook, 1987). The data from Chapter 2 show that active PKC is required for less than the first 15 min of the 120 min TPA incubation period. Since active PKC is required for such a small fraction of the total time required for TPA stimulated increases in A System activity, it is possible that PKC

may not remain active during the entire incubation period. It is possible that a calpain-mediated down regulation of PKC is occurring in these cells. As an alternative hypothesis, it is also possible that PKC is converted to PKM, and PKM is responsible for signal transmission into the nucleus, as discussed in Chapter 1. To test if PKC is converted into PKM or degraded over the time course of TPA stimulation, antibodies were raised against the catalytic domain and also the regulatory domain of PKC. These antibodies were used on Western blots to look for loss of PKC following treatment of cells with TPA, or loss of an immunoreactive ~ 80 kDa band, with the appearance of an immunoreactive ~ 50 kDa band, indicative of PKM formation.

Materials and Methods

Cell Culture

LLC-PK_1 pig kidney epithelial cells, obtained from R. N. Hull (Hull et. al., 1976) at passage 185, were used for these experiments. Cultures were maintained as described in Chapter 2. Cells were used between passage 200 and 220.

Chemicals and Supplies

Unless otherwise indicated, chemicals were obtained from Sigma (St. Louis). Radioisotopically labelled compounds were from DuPont/NEN (Boston, MA). Diolein was from Avanti Polar Lipids (Pelham). The rabbits used were female New Zealand Whites from Hazelton Dutchland (Denver, PA). Synthetic peptides were prepared by Charles Murphy at the University of Tennessee Analytic Services (Knoxville, TN). Purified PKC, a mix of mostly PKC α and PKC γ from rat brain, was purchased from Calbiochem (San Diego, CA). Colloidal gold reagents were from Janssen Life Sciences Division/Amersham (Arlington Heights, IL). Buffer A contains 20 mM Tris, 0.5 mM EGTA, 2.2 mM EDTA, and 1 mM DTT. Buffer A/Sucrose also contains 600 mM sucrose.

Generation of Antisera

The sequence of the C3 (kinase) domain of rabbit protein kinase C γ (Ohno et. al., 1988) was typed into a hydrophobicity display program (Appendix) based on the algorithms of Kyte and Doolittle (Doolittle, 1987). Two regions were chosen for peptide synthesis based on hydrophilicity and charge density (Figure 4.1). These sequences were **KDVVIQDDDVE** (from the N-terminal end of the C3 kinase domain) and **KLENREIQPPFKPK** (from the C-terminal end of the C3 kinase domain). The sequence of the C1 regulatory domain (Ohno et. al., 1988)

Figure 4.1. Hydropathy plot of the C3 region of γ PKC.

The graph shows the calculated hydrophobicity of the amino acid sequences in the catalytic domain of γ PKC, indicating the sequences used to generate the two anti-catalytic domain antibodies, G48 and G59. The sequence used is from Ohno (Ohno et. al., 1988); the hydropathy values in the graph were generated by a computer program (Appendix) based on the algorithm and partition values of Kyte and Doolittle (Doolittle, 1987).

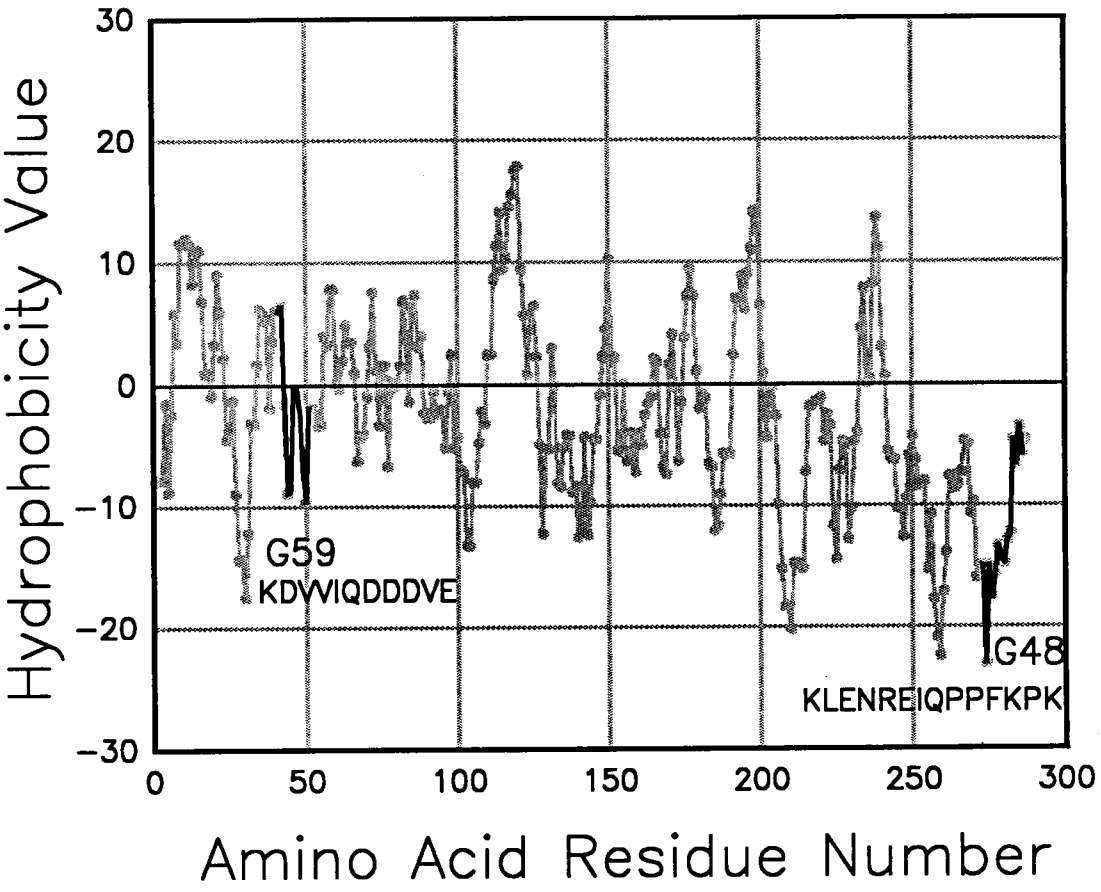
Hydrophobicity Value
30
20
10
0
-10
-20
-30
0
50
100
150
200
250
300
Amino Acid Residue Number
G59
KDVVIQDDDVE
G48
KLENREIQPPFKPK

Figure 4.1

was also entered into the hydrophobicity display program and a third peptide, **KGPDTDDPRS** was also synthesized and used to generate a polyclonal antisera (Figure 4.2). This peptide was from a region located in a short hydrophilic stretch between the lipid binding site and the calcium binding site (Ono et. al., 1989b).

These sequences were used to scan the Protein Sequence Database to look for homology to other known kinases. For the kinase domain N-terminal 11-mer, all PKC variants had a score >95%. No other kinases had a score above 65%; no other protein had a score above 67%. cAMP-dependent protein kinase from yeast had a score of 59%. For the kinase domain C-terminal 14-mer, PKC variant scores ranged from 66-100%; no other kinase had a score above 60%. cAMP-dependent protein kinase from yeast had a score of 56%. Sequence comparisons for the synthetic peptides and PKC ζ were performed using the GCG Sequence Analysis Software Package, Version 6.1 (Devereux et. al., 1984).

All three peptides were synthesized by UT Analytical Services. The synthetic peptides were coupled to Keyhole Limpet Hemocyanin (KLH) by the glutaraldehyde method of Kagen and Glick (Kagen and Glick, 1979) as modified by Doolittle (Doolittle, 1987). The extent of the reaction was monitored by running SDS-Page gels of coupled and uncoupled KLH, with the peptide coupled KLH running on the gel as a higher molecular weight smear due to the presence of multiple copies of the synthetic peptide on the KLH molecule. Initial injections of peptide coupled to KLH were performed in Freunds' Complete Adjuvant (Sigma), with all subsequent injections in Freund's Incomplete Adjuvant. Injections

Figure 4.2. Hydropathy plot of the C1 region of γ PKC.

The graph shows the calculated hydrophobicity of the amino acid sequences in the regulatory domain of γ PKC, indicating the sequence used to generate the anti-regulatory domain antibody, J. The sequence used is from Ohno (Ohno et. al., 1988); the hydropathy values in the graph were generated by a computer program (Appendix) based on the algorithm and partition values of Kyte and Doolittle (Doolittle, 1987).

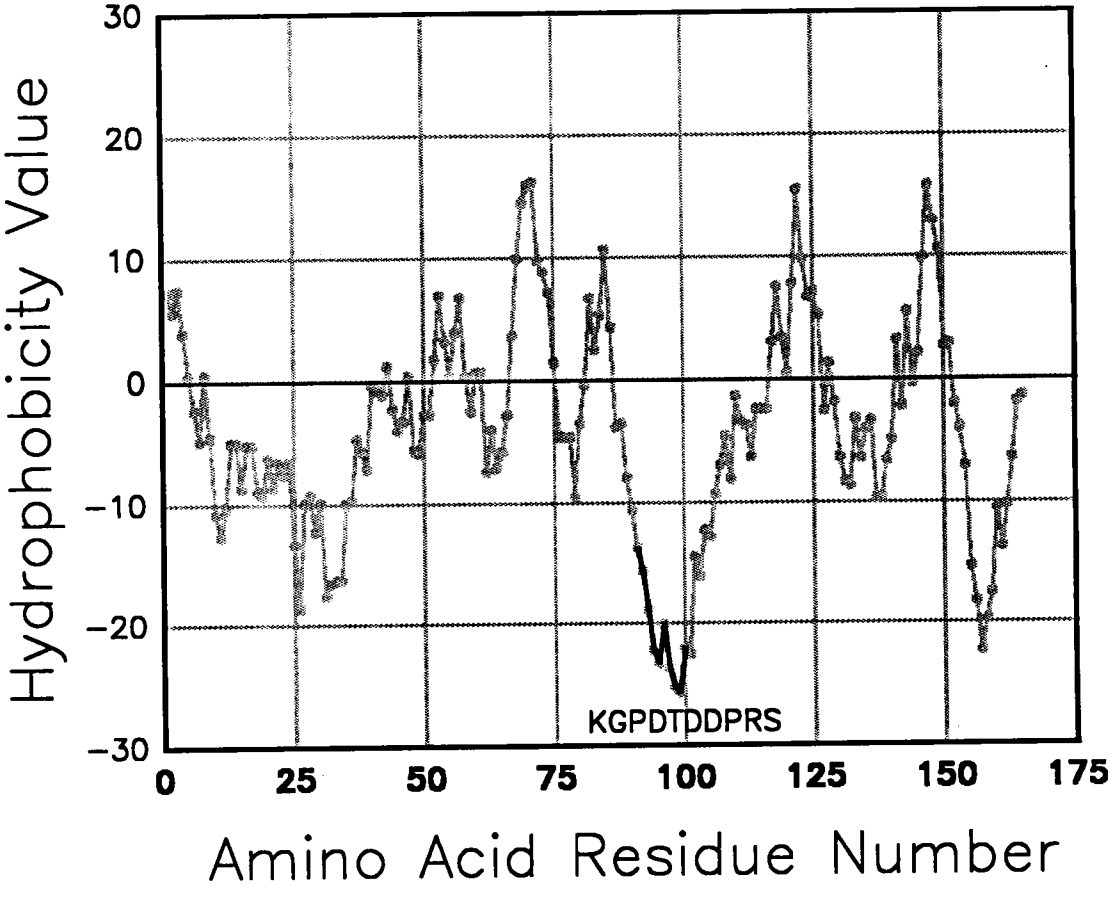
Hydrophobicity Value
30
20
10
0
−10
−20
−30
KGPDTDDPRS
0
25
50
75
100
125
150
175
Amino Acid Residue Number

Figure 4.2

contained 1.0 ml Adjuvant and 0.5 ml filter-sterilized KLH-coupled synthetic peptide at a final concentration of 1.0 mg/ml. The rabbits were bled 30 ml by cardiac puncture before any injections and the blood was allowed to clot overnight at 4°C to yield ~ 15 ml of preimmune serum after the clot was centrifuged. This was placed into eppendorf tubes as 0.5 ml aliquots and frozen.

The rabbits were injected 5 times at 4 week intervals, with 20 ml bleeds taken after the third, fourth, and fifth booster injections. The antiserum derived from the final bleed was used for these experiments.

Immunoprecipitation of PKC

The PKC used in this experiment was isolated from pig brain. Approximately 2 cc of frozen pig brain was thawed in Buffer A/Sucrose and centrifuged at 1000xg for 3 min. The supernatant was removed and the cells were resuspended in Buffer A/Sucrose and centrifuged again. The supernatant was again discarded, the cells resuspended to a final volume of 2 ml in Buffer A/Sucrose with protease inhibitors (5 μl Leupeptin and 20 μl PMSF). The final concentration of protease inhibitors was 25 μg/ml for Leupeptin and 1.0 mM for PMSF. The cells were transferred to a 10 ml thick walled ultracentrifuge tube and homogenized with a Polytron Tissue Homogenizer at 17,000 rpm for 30 sec. The homogenate was spun at 100,000xg for 30 min. The supernatant, containing soluble cytosolic proteins, was loaded onto a 4 ml DEAE minicolumn at 4°C. The column

was washed once with 3.75 ml of Buffer A, then eluted with 3.75 ml fractions of Buffer A containing 20 mM, 60 mM, 60 mM and 200 mM NaCl. These fractions were designated 20, 60.1, 60.2, and 200. All column fractions were assayed for protein kinase C activity as described in Chapter 2.

Fraction 60.1 contained the highest level of total PKC activity as well as specific PKC activity (Figure 4.3), and was used for the immunoprecipitation. Each of 12, 1.5 ml Eppendorf tubes received 300 μl of fraction 60.1, to which was added 30 μl of 4 % BSA in Buffer A, 10 μl of PMSF (final concentration 1 mM), 5 μl of leupeptin (final concentration 25 μg/ml) and finally 30 μl of either specific antisera or preimmune sera. The tubes were shaken on a low speed vortex mixer (Vortex Genie-2, Scientific Instruments, Bohemia, NY) at 4°C overnight (16 hrs), then 75 μl of Protein A-Agarose (Sigma) was added to each tube. The tubes were shaken for 60 min on a low speed vortex mixer at room temperature, then the protein A-Agarose was centrifuged 5 min in an Eppendorf microfuge, resulting in a firm pellet of protein A-Agarose. From each tube a 350 μl aliquot of the supernatant was removed and assayed for protein kinase C activity, as described in Chapter 1.

Immunoblotting

LLC-PK_1 cells used for immunoblots were grown on 150 mm tissue culture dishes for 10 days to complete confluence. The cells were rinsed once in the dish with ice-cold PBS, then scraped off the plate in fresh PBS with a rubber policeman.

Figure 4.3. Total and Specific Activity of the Pig Brain PKC used for Immunoprecipitation.

Frozen pig brain was used to isolate protein kinase C. Cells were thawed in Buffer A with Sucrose, 1 mM PMSF and 25 μg/ml leupeptin were added, and cells were homogenized in a Polytron. The homogenate was spun at 100,000xg in an ultracentrifuge for 30 min, then the supernatant was placed on a DEAE minicolumn. The column was washed with Buffer A, then eluted with Buffer A plus 20 mM NaCl (Fraction 20); Buffer A plus 60 mM NaCl (Fraction 60.1); Buffer A plus 60 mM NaCl (Fraction 60.2), and finally Buffer A plus 200 mM NaCl (Fraction 200). All four fractions were assayed for PKC activity as indicated in Methods. A. Total PKC activity in each fraction. B. Specific Activity of PKC in each fraction.

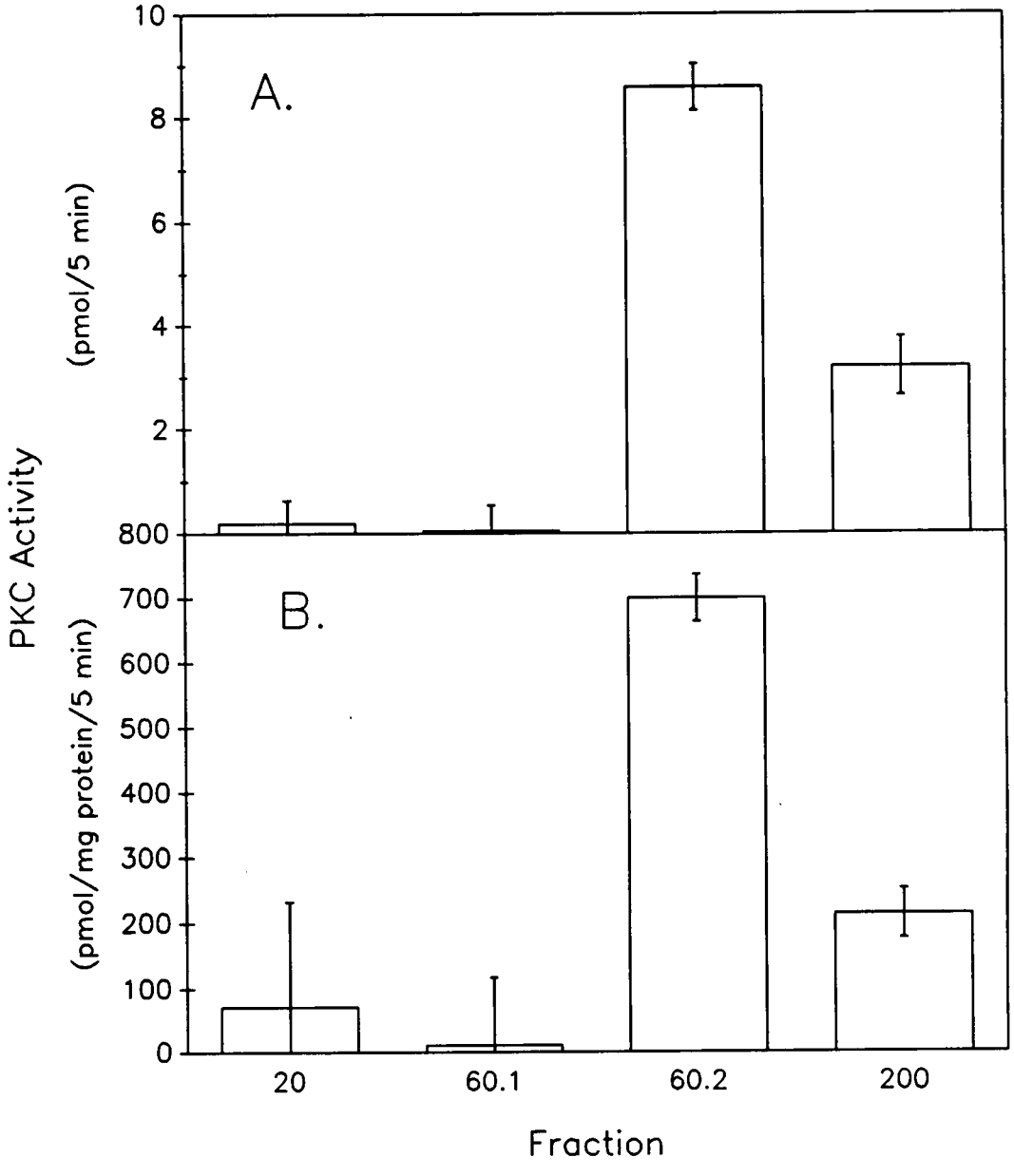
PKC Activity
(pmol/5 min)
(pmol/mg protein/5 min)
A.
B.
10
8
6
4
2
800
700
600
500
400
300
200
100
0
20
60.1
60.2
200
Fraction

Figure 4.3

The cells were transferred to a 15 ml centrifuge tube and spun at 1000xg for 3 min to pellet the cells. The supernatant was removed and the pellet resuspended in PBS. The cells were pelleted, resuspended and pelleted once more. Protease inhibitors were added to the pellet (final concentration 25 μg/ml Leupeptin, 1 mM PMSF), and the pellet was then dissolved in 1.0 ml SDS sample buffer (30 % glycerol, 15 % 2-mercaptoethanol, 3 % SDS, 0.01 % Bromophenol Blue, in 187.5 mM Tris, pH 6.8; from Laemmli, 1970). The lysate was boiled for 5 min, then centrifuged in an Eppendorf microfuge for 1 min. Samples for loading the gel were withdrawn from the top of the tube.

Gradient gels of 8-18% polyacrylamide were run at 40 mA constant current with cooling in a BioRad Protean gel box for 5 hours. The gel was transferred onto nitrocellulose in a Genie Western Blotting apparatus using a Tris/glycine buffer (Towbin et. al., 1979). The electrotransfer ran for 4 hours at 4°C, using a constant voltage of 12 V. The nitrocellulose was removed and allowed to air dry overnight (Harlow and Lane, 1988) while the gel was Coomasie stained and destained.

The nitrocellulose blot was then rehydrated by soaking in distilled water for 2 minutes. The blot section containing the molecular weight markers was cut off of the rest of the blot, and stained for protein using 0.1 % Amido Black in 45% MeOH/10% Acetic Acid. The rest of the blot was incubated in 5 % BSA in PBS for 30 minutes to block remaining protein binding sites, then clamped into a Surf-Blot manifold (Idea Scientific, Corvallis, OR). The liquid in each lane was

removed by aspiration, and 150 μl of primary antiserum mix (10 % Normal Goat Sera (Janssen), 0.1 % BSA, and the indicated dilution of specific antiserum in PBS) was added to each lane. The primary antiserum mix was allowed to react with the blot for 30 min at room temperature on a rocking platform. Each lane was then washed three times with 0.1 % BSA in PBS, and the blot was removed from the manifold. The blot was transferred to a Seal-A-Meal bag, and processed according to the instructions in the Janssen Auroprobe BL+ kit. Briefly, 5 ml of a secondary antibody mix (1:100 dilution of gold-labelled Goat-Anti-Rabbit serum, 1:20 dilution of the supplied gelatin solution, and 0.1 % BSA in PBS) was added. This was allowed to react overnight with the blot, then the blot was rinsed twice with 0.1 % BSA in PBS for 5 minutes and once with distilled water for 1 minute. The blot was then silver-enhanced using the Janssen IntenSE BL kit (Amersham, Arlington Heights, Il).

Results

Figure 4.4 shows that all three sera raised against synthetic peptides can bind PKC. This result is not due to simple inhibition of PKC by the antisera because none of the antisera block PKC activity in the standard *in vitro* PKC assay (Figure 4.5). Antiserum G59 was found to give the best reaction of all three sera with purified PKC (subtypes α, βI, βII, and γ) on Western blots (Figure 4.6). Antiserum G59 was therefore used in all further experiments.

Figure 4.4. Reaction of Antisera with Pig Brain PKC.

Protein kinase C was isolated from frozen pig brain. Specific antisera or preimmune sera from the same rabbit was added to a final concentration of 1:100. The mixture was shaken and Protein A-agarose beads were added. After centrifugation of the mixture an aliquot was removed from the supernatant and assayed for protein kinase C activity. Each bar represents the difference of means of triplicate samples with Ca^{2+} and lipids and triplicate samples with EGTA, lacking lipids. Error bars represent standard deviations.

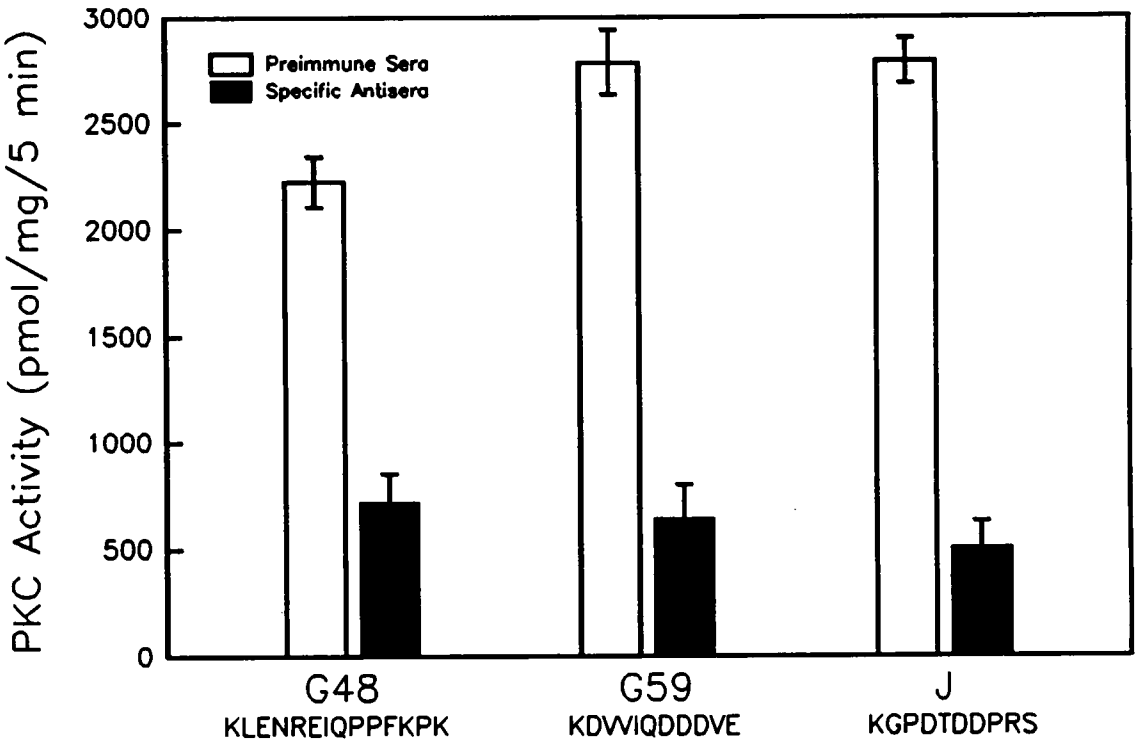
PKC Activity (pmol/mg/5 min)
3000
2500
2000
1500
1000
500
0
Preimmune Sera
Specific Antisera
G48
KLENREIQPPFKPK
G59
KDVVIQDDDVE
J
KGPDTDDPRS

Figure 4.4

Figure 4.5. The Antisera Do Not Directly Inhibit PKC Activity

Protein Kinase C was isolated from cells as described in Materials and Methods. Specific Antisera were added to a final concentration of 1:100, and allowed to react with the PKC-containing column fraction for 15 minutes at 4°C. The fractions were assayed for Protein Kinase C activity. Each bar represents the difference of means of triplicate samples with Ca^{2+} and lipids and triplicate samples with EGTA, lacking lipids. Error bars represent standard deviations.

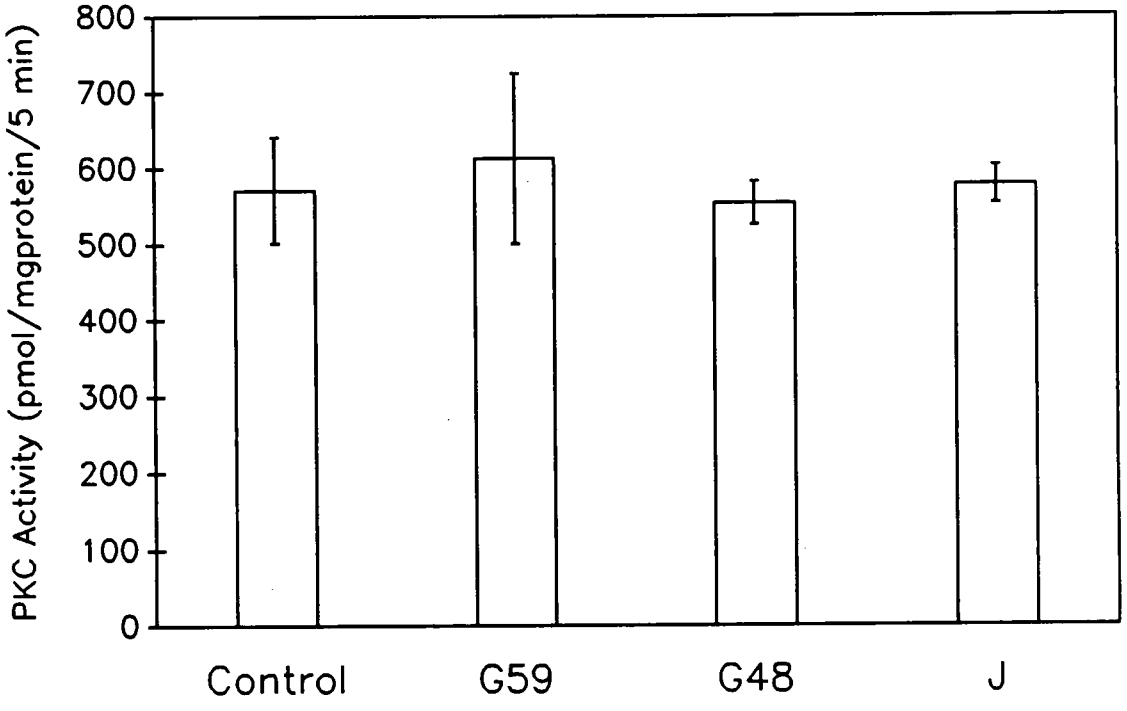
PKC Activity (pmol/mgprotein/5 min)
800
700
600
500
400
300
200
100
0
Control
G59
G48
J

Figure 4.5

Figure 4.6. Western Blot of LLC-PK_1 PKC with Antisera.

Purified PKC was obtained from Calbiochem. LLC-PK_1 cells were treated with leupeptin and lysed directly into SDS sample buffer, then boiled for 5 min. Samples were run on an 8-18 % PAGE gel, then transferred to nitrocellulose, blocked in 5% BSA, and probed with antisera at concentrations from 1:10-1:500 on a Surf-Blot manifold. The secondary Ab was gold-labelled GAR, followed by silver enhancement. a). Molecular Weight Markers. b). Total Protein Stain. c-e). G48 antisera at a concentration of 1:10, 1:50, 1:100. f-h). Pre-Immune sera from G48 at a concentration of 1:10, 1:50, 1:100. i-k). G59 antisera at a concentration of 1:10, 1:50, 1:100. l-n). G59 pre-Immune sera at a concentration of 1:10, 1:50, 1:100. o-q). J antisera at a concentration of 1:10, 1:50, 1:100. r-t). J pre-Immune sera at a concentration of 1:10, 1:50, 1:100. u). Purified PKC, 1:10 G48 Pre-Immune sera. v). Purified PKC, 1:10 G48 antisera. w). Purified PKC, 1:10 G59 Pre-Immune sera. x). Purified PKC, 1:10 G59 antisera. y). Purified PKC, 1:10 J Pre-Immune sera. z).Purified PKC, 1:10 J antisera.

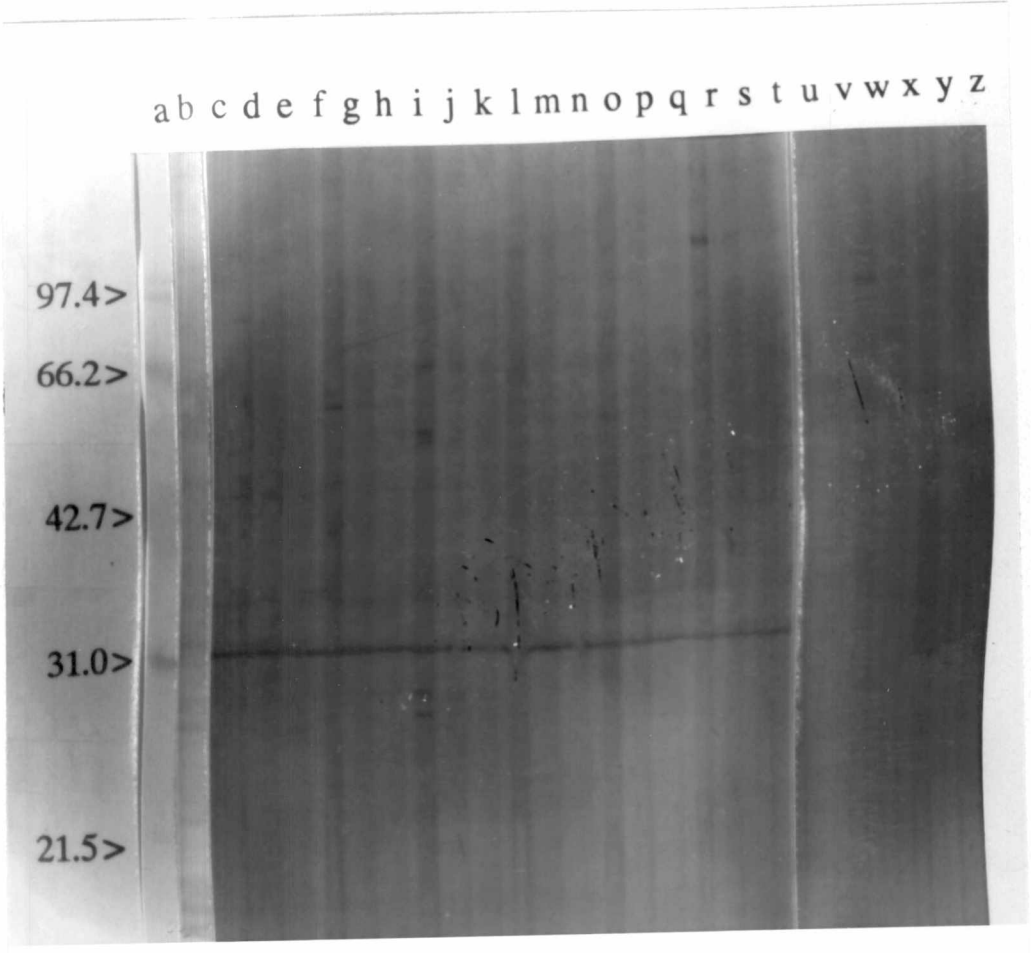

Figure 4.6

Antiserum G59 gave a strong reaction with purified PKC (MW 78-79 kDa), yet no reaction was seen with a 78-79 kDa band in extracts of LLC-PK_1 cells. This cannot be attributed to a lack of reactivity of the sera on Western blots because the sera will react to PKC isolated from pig brain with the same reagents, run on a gel at the same protein concentrations, and allowed to react under the same conditions as the LLC-PK_1 whole cell extract (Figure 4.7).

Major bands are seen in LLC-PK_1 whole cell extracts at approximate molecular weights of 70, 35 and 30 kDa, with minor bands at 97, 52, 46 and 25 kDa. Of the major bands that are reactive in LLC-PK_1 cell extracts, none have the expected molecular weight of PKC, 78 kDa. Recently, a new PKC subtype (PKC ζ) has been identified by probing a rat brain library with mixed α, βII and γ cDNAs at low stringency (Ono et. al., 1988). PKC ζ has modifications in the regulatory region of PKC, apparently lacking the entire C2 region; its molecular mass is calculated to be 67 kDa. From Northern blot analysis, the RNA is expressed mainly in brain, kidney, and lung (Ono et. al., 1988). Immunoblots with a polyclonal antibody directed against a synthetic PKC ζ specific peptide show a reaction with a band at ~ 66 kDa and another at 31 kDa (Ono et. al., 1989a). LLC-PK_1 cells exhibit a strong reaction with bands at ~ 70 kDa and also at 31 kDa (Figure 4.7). Comparing the published amino acid sequence of PKC ζ to the synthetic peptides used for antibody generation led to the results in Table 4.1. Antiserum G59, which gives the strongest reaction in Western Blots of LLC-PK_1

Figure 4.7. Western Blot Analysis of PKC from Pig Brain and LLC-PK_1 Cells with Antiserum G59.

Purified PKC was obtained from Calbiochem. Frozen pig brain was thawed and washed in homogenization buffer at 4°C, treated with leupeptin and lysed directly in SDS sample buffer. Samples were boiled for 5 min, then loaded on an 8-18% SDS PAGE gel. LLC-PK_1 samples were treated as in the previous figure. The gel was transferred to nitrocellulose, blocked in 5% BSA and probed with antiserum G59 at concentrations from 1:10-1:50 on a Surf-Blot manifold. The signal was detected with gold-labelled protein A, followed by silver enhancement. The arrow shows the position of the ~ 70 kDa band in the LLC-PK_1 samples. a). Molecular Weight markers. b). Total protein stain, pig brain. c-e). G59 Pre-Immune sera and pig brain lysate at concentrations of 1:100, 1:50, 1:10. f). blank lane. g-i). G59 sera and pig brain lysate at concentrations of 1:100, 1:50, 1:10. j). PKC Control with G59 sera at 1:10. k-m). G59 Pre-Immune sera and LLC-PK_1 cell lysate at concentrations of 1:100, 1:50, 1:10. n-p). G59 sera and LLC-PK_1 cell lysate at concentrations of 1:100, 1:50, 1:10. q). Total protein stain of LLC-PK_1 cell lysate.

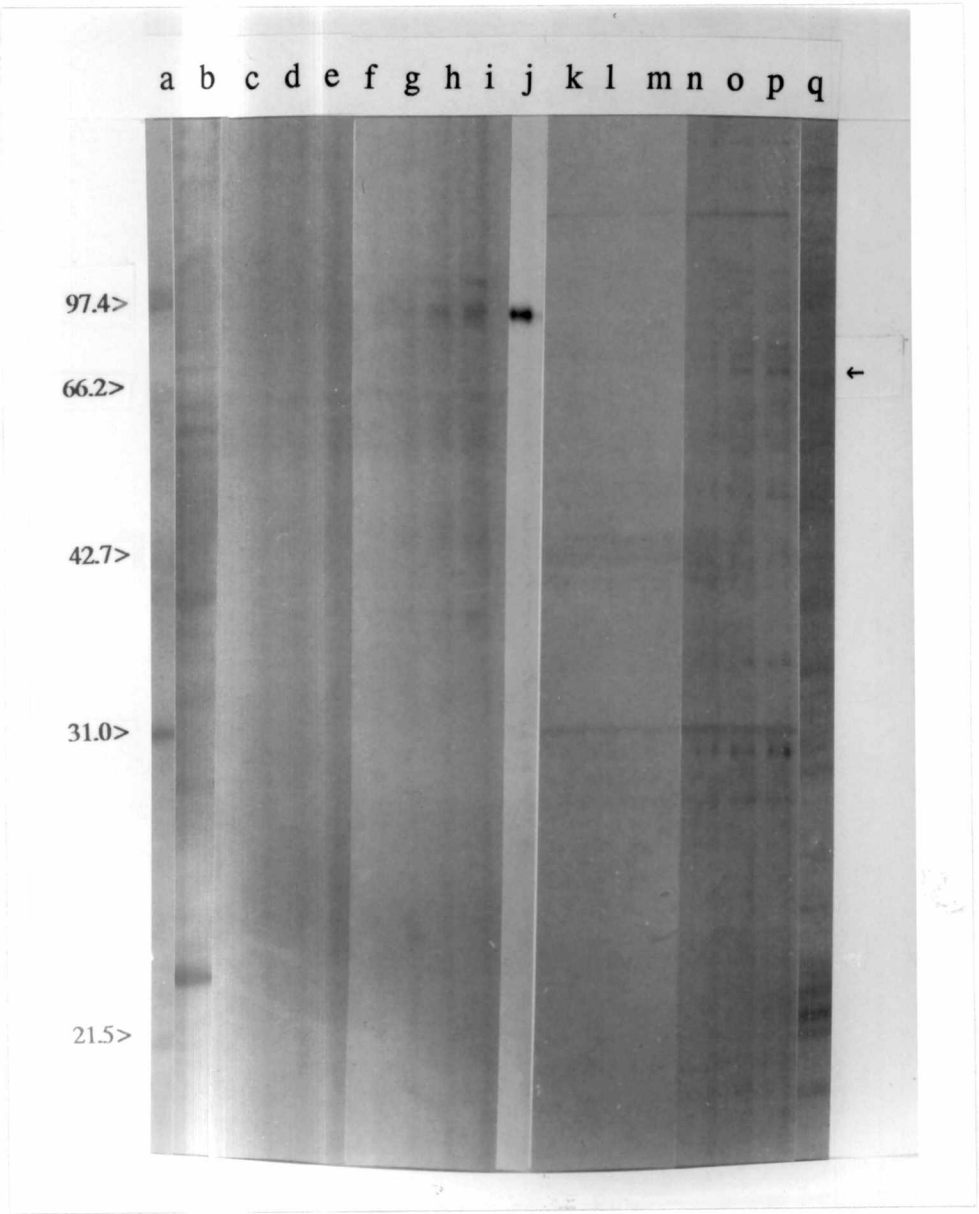

Figure 4.7

cells was generated using the peptide of highest degree of similarity to the published sequence for PKC ζ (Ono et. al., 1989a).

Table 4.1

Antibody	PKC Domain	Best Fit Sequence*	Similarity
J	C1	KGPDTDDPRS .¦ :.:¦. ¦ EGLGPGDTTS (PKC ζ)	50.0 %
G48	C3	KLENREIQPPFKPK ¦¦.:¦. ¦¦¦.¦. LLEKKQTLPPFQPQ (PKC ζ)	57.1 %
G59	C3	KDVVIQDDDVE ¦::¦ :¦:¦:: KELVHDDEDID (PKC ζ)	90.9 %

* Symbols used in comparison between sequences:

"¦" = Identical amino acid

":" = Conservative change

"." = Semiconservative change

" " = Nonconservative change

Discussion

The original intent of raising antibodies against PKC was to test whether a calpain-induced down regulation of PKC occurred within the TPA stimulation period for A System uptake. Three antisera were generated which reacted with PKC in solution (Figure 4.4), although none interfered with the kinase activity of PKC. Interestingly, all of the sera reacted with purified PKC on a Western blot (Figure 4.6) but none of the sera reacted with a band of the appropriate size in whole cell extracts of LLC-PK_1 cells. Since the antisera reacted on a Western blot with PKC isolated from pig brain, lack of reactivity with LLC-PK_1 cells is not due to species differences, or loss of epitopes during the Western blotting procedure. Because there was no immunoreactive band at ~ 78-79 kDa, it was not possible to examine the role of calpain in these cells.

Antiserum G59, which gave the best reaction against purified PKC, also reacted against a band in pig brain whole cell extract with the same molecular weight as purified PKC. No bands were visible in LLC-PK_1 cell extracts at the same molecular weight, but bands were seen at ~ 70 kDa and ~ 30 kDa. Recently, Ono et. al. (1989a) have published the sequence of a new subspecies of the PKC family with a calculated molecular weight of 67 kDa, which they call PKC ζ. PKC ζ has been shown to be expressed in rat kidney (Ono et. al., 1988). COS-7 cells which were transfected with the cloned PKC ζ gene contain proteins of MW 64 kDa and 30 kDa which react on immunoblots with an anti-PKC ζ antisera (Ono

et. al., 1989a). Of the three antisera, G59, the one which reacted best against bands in LLC-PK_1 cells had been generated with a sequence closest to that found in PKC ζ (Table 4.1). This implies that LLC-PK_1 cells may contain PKC ζ.

PKC α is the major form of PKC, and is found in most tissues (reviewed in Kikkawa et. al., 1989). In LLC-PK_1 cells, no PKC α was detected with G59, an antisera which reacted against purified α and γ PKC (see lane w, PKC control on Figure 4.6). Bands were seen at ~ 70 kDa and ~ 30 kDa (Figure 4.7), a pattern similar to that reported for cells expressing PKC ζ, a subspecies known to be expressed in kidney (Ono et. al., 1989a). This indicates that LLC-PK_1 cells may be expressing PKC ζ. In order to confirm if PKC ζ is expressed in LLC-PK_1 cells, future experiments must include Northern blot analysis with a PKC ζ specific probe, isolation and sequencing of the ~ 70 kDa protein, or Western blots with an anti-PKC ζ antibody.

CHAPTER 5

DISCUSSION

Summary of the Data

From the data in Chapter 2 it is apparent that quercetin, at a concentration of 150 μM, will prevent TPA stimulation of A System transport through its effects on PKC. Under the conditions used in this assay, quercetin had no demonstrable direct effect on the A System transporter (Figure 2.3), the Na^+ gradient, as assayed by α-MeGlc uptake (Figure 2.7, Figure 2.8), or bulk protein synthesis (Figure 2.8). However, quercetin did have a direct effect on PKC in the standard *in vitro* assay (Figure 2.9). Also, in the absence of TPA treatment, quercetin had little (Figure 2.1, Figure 2.2) or no significant effect (Figure 2.3, Figure 2.8) on A System transport. These data are consistent with the hypothesis that PKC is the site of interaction of quercetin in TPA-treated LLC-PK_1 cells.

It is also apparent that active PKC is required during the first < 15 min of the 120 min incubation period (Figure 2.4, Figure 2.10); this result is very similar to results reported for two other modulators of A System transport activity, insulin and glucagon (Barber et. al., 1983; Fehlmann et. al., 1979a; Kilberg, 1982). Since PKC participates in an early part of a major signal transduction pathway

(Nishizuka, 1984), it is probable that after the first 15 minutes a cascade reaction has been initiated, and active PKC is no longer required for induction.

Stimulation of LLC-PK_1 cells with TPA results in an increase in transport over the A System (Amsler et. al., 1983). In most cells, modulation of A System activity occurs as a result of changes in the V_{max} of transport, with no change in K_m (Tramacere et. al., 1977; Kelley and Potter, 1978; Gazzola et. al., 1981; Gazzola et. al., 1984). In some cells, however, changes in both V_{max} and K_m have been reported (Boerner and Saier, 1985; Hochstadt et. al., 1979).

To determine if TPA stimulation of A System transport activity is due to changes in V_{max} alone or both V_{max} and K_m, MeAIB uptake assays were performed at substrate concentrations of 0.1, 0.5, 1.0, 3.0, and 5.0 mM. The results were graphed as Eadie-Hofstee plots (velocity versus velocity divided by substrate concentration), in order to yield a first order regression line where the slope equals $-K_m$ and the Y-intercept is the V_{max} for the reaction. Figure 3.2 shows that addition of TPA to confluent cultures of LLC-PK_1 cells causes a four-fold increase in V_{max} ($p < 0.001$) with no significant change in K_m value.

LLC-PK_1 cells that are rapidly growing have a high A System transport rate (Amsler et. al., 1983). Upon confluence, these cells decrease transport via the A System (Amsler et. al., 1983). When these cells reach confluence, PKC translocates from a membrane-bound, active form to a cytosolic, inactive form (Dawson and Cook, 1987), as has reported for other cell types (Adamo et. al., 1986). Treatment of confluent cultures of LLC-PK_1 cells with 10^{-7}M TPA results

in redistribution of PKC to the membranes, mimicking the rapidly growing state. If PKC is critical to the high level of A System transport seen in rapidly growing cells, then addition of quercetin to young cultures with a high A System transport rate should decrease transport, in a manner similar to the decrease seen in differentiating cells and opposite to the TPA stimulation effect. Figure 3.3 shows that this is indeed the effect. Treatment of rapidly growing cultures of LLC-PK_1 cells with 150 μM quercetin for 30 min causes a decrease in V_{max} to less than half of the control level significant change in K_m. These data indicate PKC is indeed a vital part of the constitutive regulation of the A System. In rapidly growing cells active PKC is constantly required for maintaining a high level of A System transport. This is in contrast to the effect seen in confluent cells, where active PKC is only needed for the first ~ 15 min of TPA treatment. Results from another laboratory investigating starvation derepression has shown that continual protein synthesis is required to maintain increasing A System activity in H4 hepatoma cells (Kilberg et. al., 1985). If PKC is assumed to be inactivated instantly by addition of quercetin to the cells one-half of the A System transporters are lost from the cell surface within 50 minutes, although it is not possible to determine if they have been degraded, masked, or merely internalized and no longer able to function.

Previous work from this laboratory has indicated that A System transport activity could be directly controlled by PKC in rapidly growing cells through specific phosphorylation of the transporter (Dawson and Cook, 1988a). This hypothesis proposes the phosphorylated transporter molecule is fully active, and if

dephosphorylation reverts the transporter to an inactive state. If this is indeed true, the data from Figure 3.3 could be interpreted as representing the inactivation of A System transport by dephosphorylation of the transporters. Since no specific probe for the transporter itself exists, it is difficult to distinguish between transporter inactivation and transporter degradation at this time.

Since PKC activity is required for such a short time after addition of TPA to cells, and since PKC is known to stimulate the neutral protease involved in its degradation (Suzuki and Ohno, 1990), we hypothesized that PKC might be cleaved by calpain during the TPA stimulation to yield a new kinase (PKM). Alternatively, PKC could be degraded completely. To investigate this problem, a specific antiserum was raised against the regulatory domain of PKC (lost in the PKC→PKM transition) and antisera were also raised against the catalytic domain (unchanged in the PKC→PKM transition). While all three antisera had an affinity for PKC isolated from pig brain, as well as commercially available PKC from rat brain, none reacted with an appropriate molecular weight band from LLC-PK_1 cells. LLC-PK_1 cells have been shown to contain PKC by direct assay (Dawson and Cook, 1987; Dawson and Cook, 1988a; Dawson and Cook, 1988b), and by the effects of TPA in these cells. Recently, a new subspecies of PKC, PKC ζ, has been discovered by probing rat brain DNA with PKC specific sequences (Ono et. al., 1988; Ono et. al., 1989a). This subspecies has a calculated molecular weight of 67 kDa (Ono et. al., 1989a), similar to the band seen in LLC-PK_1 cell lysates. Moreover, in Northern blot analysis of various rat tissues a PKC ζ probe hybridized to brain, kidney and

lung tissue RNA (Ono et. al., 1988), indicating that PKC ζ is expressed in the rat kidney, in addition to brain and lung. While this does not constitute proof that LLC-PK_1 cells express PKC ζ, it would explain the lack of reactivity of LLC-PK_1 cells with anti-PKC antibodies at the appropriate molecular weight and the presence of an immunoreactive 67 kDa band.

Modelling

Based on the results presented in this dissertation and material published previously, a generalized model for the PKC control of A System transport can be proposed. In differentiated cells, binding of phorbol esters or generation of DAG by phospholipase C results in activation of PKC. PKC may be proteolytically processed to PKM, or may activate another kinase, but after 10-15 minutes of stimulation active PKC is no longer required for enhancement of the A System (Figures 2.4 and 2.10). By some still unknown mechanism, possibly involving phosphorylation of Fos protein or transcription factor AP-1 (Angel et. al., 1987), a signal to synthesize new A System transporter molecules is transmitted to the nucleus. Approximately 60 minutes later (Figure 2.1), new transporters begin arriving at the cell surface and transport increases, as indicated by the increase in V_{max} of transport (Figure 3.2).

In proliferating cells, PKC is already in its active, membrane-bound form (Adamo et. al., 1986; Dawson and Cook, 1987). This should provide for continuous

signaling of transporter synthesis, with a very high level of activity. Interruption of the PKC signal would result in cessation of transporter mRNA synthesis, and as the A System mRNA is depleted, A System transport should decrease based on the half-life turnover of the transporter molecule in the cell membranes. Since turnover of the A System transporter is believed to increase with availability of substrate amino acids (Gazzola et. al., 1981), rapidly growing cells with a high level of amino acids in their environment should have a high turnover rate for the A System transporter. If the continuous synthesis signal from PKC is interrupted, then the transporter should be down regulated at the membrane, resulting in a decrease in the V_{max}. This effect is seen in Figure 3.3.

The initial step in PKC down regulation within the cell is thought to occur via proteolytic cleavage by calpain (Kikkawa et. al., 1989). Calpain cleaves PKC in the V3 region, near the C-terminal end of the C2 region (Kishimoto et. al., 1989). There is no common sequence in the cleavage site (Kishimoto et. al., 1989), therefore the constant C2 and C3 regions on either side of the variable V3 region may provide the targeting information. Since PKC ζ lacks the C2 region completely (Ono et. al., 1989), it should be less susceptible to calpain mediated down regulation. If LLC-PK_1 cells contain PKC ζ as their major PKC isoform, it could explain why no TPA-inducible down regulation of PKC is seen in these cells.

Future Directions

There are still very important questions to be answered in exploring the role of PKC in controlling the activity of the A System amino acid transporter. First, is PKC ζ the dominant isotype in LLC-PK_1 cells? When this project was started, PKC ζ was unknown, but the failure to identify a standard PKC isoform in these cells, when enzymatic assays document PKC activity is present, is troubling. Discovery of a PKC isozyme in rat kidney (Ono et. al., 1988), which has the same approximate molecular weight as the major anti-PKC immunoreactive band in LLC-PK_1, indicates that LLC-PK_1 cells might express PKC ζ activity. However, this evidence is not proof; therefore future experiments should test this hypothesis. Nishizuka's group has recently employed a polyclonal anti-PKC ζ antibody to examine expression of the PKC ζ gene in COS cells (Ono et. al., 1989a). Provided this antibody has low cross-reactivity with other PKC isotypes, it would be very useful in determining if LLC-PK_1 cells do indeed express PKC ζ. Also, most PKC variants display slightly different activity requirements for Ca^{2+}, phospholipids, and DAG (for review, see Kikkawa et. al., 1989; Nishizuka, 1988). The standard PKC assay used in this study was not designed to look for small differences in Ca^{2+} or DAG sensitivity, but this information could help identify the correct isotype if, as the Chapter 4 data indicate, the LLC-PK_1 PKC isotype is not the standard PKC α.

Second, questions on the role or existence of PKM in these cells remain unanswered. In fact, if PKC ζ does exist in these cells, there is no information published on whether this isotype is even a calpain substrate. Along these lines, a recent very interesting paper by Pontremoli et. al. (1990), where an unidentified PKC isotype (nonreactive with anti-γ, slightly reactive with anti-α and anti-ß antisera) was found to be activated by calpain *in vivo*, since the other known subtypes, δ, ε and ζ all lack the C2 region (Ono et. al., 1988). Also, the different isozymes have different rates of cleavage by calpain (Ase et. al., 1988; Pontremoli et. al., 1990), in addition to differing activation conditions (Kikkawa et. al., 1989; Nishizuka, 1988), strengthening the argument that activation by calpain can be a well regulated event.

Third, assuming the existence of a nonstandard (not α, ß, or γ) PKC in these cells, will addition of calpain inhibitors before adding TPA change the A System transport activity response? Cell permeating inhibitors (tripeptidyl aldehydes) of the two calpains are available (Tsujinaka et. al., 1988; Sasaki et. al., 1990) that are more specific for calpains than the leupeptin and PMSF used previously, and if a PKM transition is necessary for the increased A System transport response then inhibition of calpain should diminish or abolish the response.

Fourth, the exact role of PKC is still unknown. The active enzyme is only required for 10-15 minutes (Figures 2.4 and 2.10). The targets that are phosphorylated in that time are unknown, as well as whether any of them are

kinases. Since Fos is known to be phosphorylated in response to TPA (Barber and Verma, 1987), does this occur in these cells? Questions of whether the A System transporter protein gene contains a AP-1 binding site must be deferred until some sort of probe for the gene itself is found. On a cruder level, if the antiserum G59 proves suitable for use in immunofluorescence, does the signal move from a cytoplasmic localization in confluent, nonstimulated cells to a membrane bound, or even nuclear location after addition of TPA? If a nuclear localization of PKC can be shown after stimulation of the cell by TPA then the need for invoking unknown kinases in a cascade pathway diminishes.

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Appendix

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10  SCREEN 9:KEY OFF:CLS
20  OPTION BASE 1
30  DIM TS$(1000),FACT(20),AA$(20),VALUE(1000),VAL2(1000)
40  FOR K=1 TO 20:READ FACT(K):NEXT K
50  DATA 4.5,4.2,3.8,2.8,2.5,1.9,1.8,-0.4,-0.7,-0.9,
-0.8,-1.3,-1.6, -3.2,-3.5,-3.5,-3.5,-3.5,-3.9,-4.5
60  FOR K=1 TO 20:READ AA$(K):NEXT K
70  DATA I,V,L,F,C,M,A,G,T,W,S,Y,P,H,E,Q,D,N,K,R
80  PRINT:PRINT "Make sure CAPS LOCK is on before running
this program.":PRINT
90 PRINT"This program will accept either data entered from
the keyboard (1 letter amino"
100 PRINT"acid codes with no spaces between the letters,
ending with a letter 'O') or a"
110 PRINT"sequence from an ASCII text file (1 letter
codes, 1 letter per line, first line"
120 PRINT"is the number of residues in the sequence).  The
program will graph the data on"
130 PRINT"the screen, then asks about saving the data
file.  The best hardcopy comes from"
140 PRINT"importing the saved file into SigmaPlot, then
drawing a graph as usual.":PRINT
150  INPUT"Retrieve old data Y/N  ",ASW$
160 IF ASW$="Y" THEN INPUT"What is the name of the data
file";REF$:GOTO 660 ELSE PRINT"Type one letter
abbreviations for the amino acid sequences, and type 'O'
to end sequence entry."
170 I=1
180 K$=INKEY$:IF LEN(K$)=0 THEN GOTO 180
190 PRINT K$;
200 FOR J=1 TO 20:IF AA$(J)=K$ THEN VALUE(I)=FACT(J):GOTO
220
210 NEXT J
220 IF K$<>"O" THEN TS$(I)=K$:I=I+1:GOTO 180
230 CLS:LOCATE 1,1:INPUT"Do you want to edit the data? Y/N
",AS$:IF AS$ <> "Y" THEN GOTO 360
240 FOR J=1 TO I STEP 20
250 LINE(0,1)-(600,60),0,BF
260 LOCATE 1,1:PRINT"AA residues ";J;"thru ";J+19;" ."
270 LOCATE 2,1:PRINT"1234567890  1234567890":LOCATE 3,1
280 FOR K=J TO J+9:PRINT TS$(K);
290 NEXT K
300 PRINT "  ";
310 FOR K=J+10 TO J+19
320 PRINT TS$(K);
330 NEXT K:LOCATE 3,25:INPUT"Change residue number
(RETURN= no change)",ANS2
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340 IF ANS2=0 THEN GOTO 350 ELSE INPUT"What is the correct
residue?";AN$:TS$(ANS2)=AN$:GOTO 260
350 NEXT J
360 I=I-1
370 FOR J=1 TO I
380  TOT=0:IF J-4<1 THEN X=1 ELSE X=J-4
390  IF J+4>I THEN Y=I ELSE Y=J+4
400   FOR L=X TO Y
410   TOT=TOT+VALUE(L)
420   NEXT L
430  VAL2(J)=TOT:NEXT J
440 SCREEN 9:CLS:LINE(0,0)-(650,200),15,BF:LOCATE
1,35:PRINT"DATA PREVIEW"
450 LINE(10,100)-(610,100),2:LINE(10,20)-(10,180),2
460 IF I>300 THEN
LINE(10,269)-(610,269),2:LINE(10,189)-(10,349),2
470 FOR X=-60 TO 60 STEP 20
480 LINE(5,100-X)-(15,100-X),2
490 IF I>300 THEN LINE(5,269-X)-(15,269-X),2
500 NEXT X
510 LINE(10,20)-(610,20),2:FOR X=30 TO 590 STEP
20:LINE(X,20)-(X,25),2:NEXT X
520 LINE(10,180)-(610,180),2:LINE(610,20)-(610,180),2
530 FOR X=210 TO 410 STEP 200:LINE(X,15)-(X,20),2:NEXT X
540 FOR X=1 TO I
550  IF X>300 THEN
CIRCLE(10+X,269-VAL2(X)*2),1,1:LINE(10+X,269-VAL2(X)*2)-(1
1+X,269-VAL2(X+1)*2),1,1:GOTO 580
560  CIRCLE(10+X+XX,100-VAL2(X)*2),1,1
570
LINE(10+X+XX,100-VAL2(X)*2)-(11+X+XX,100-VAL2(X+1)*2),1
:XX=XX+1
580 NEXT X
590 LOCATE 16,1:PRINT"Store sequence? Y/N":LOCATE 17,30
600 ANS$=INKEY$:IF LEN(ANS$)=0 THEN GOTO 600
610 IF ANS$<>"N" THEN INPUT"Store as:";REF$ ELSE SYSTEM
620 OPEN "O",#1,REF$:PRINT #1,I
630 FOR J=1 TO I:PRINT #1,TS$(J):NEXT J
640 FOR J=1 TO I:PRINT #1,VAL2(J):NEXT J
650 CLOSE #1:SYSTEM
660 OPEN "I",#1,REF$
670 PRINT"Please wait while the data loads."
680 INPUT #1,I
690 FOR K=1 TO I:INPUT #1,TS$(K)
700 FOR J=1 TO 20:IF AA$(J)=TS$(K) THEN VALUE(K)=FACT(J)
710 NEXT J:NEXT K
720 CLOSE
730 GOTO 230
```

VITA

Michael Henry Bast was born July 23, 1962 in Princeton, New Jersey. He subsequently lived in Orange City, Iowa, and Chicago, Illinois before settling down in Grand Rapids, Michigan. He attended Ottawa Hills High School, and graduated in June, 1980. He then attended Hope College in Holland, Michigan. He graduated in May, 1984 with a Bachelor of Science degree in a self-designed composite major of Biology and Chemistry. In September, 1984 he entered the Ph.D program at the University of Tennessee - Oak Ridge Graduate School of Biomedical Sciences, located in the Biology Division of the Oak Ridge National Laboratory, Oak Ridge, Tennessee. He received a Ph.D in Biomedical Sciences with an emphasis on Cell Biology in December, 1990 under the direction of Dr. John S. Cook.