REGULATION OF TIGHT JUNCTION BARRIER FUNCTION BY PHOSPHOLIPASE C

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ABSTRACT

Regulation of Tight Junction Barrier Function by Phospholipase C
(Under the direction of Dhiren R. Thakker, Ph.D.)

The intestinal epithelium presents a formidable barrier to the absorption of orally administered drugs and macromolecules. It is comprised of a monolayer of diverse epithelial cells connected via multi-protein junctional complexes at the apical membrane. The tight junction, the most apical component of the junctional complex, is generally considered to be the major barrier regulating the passage of molecules between adjacent cells and into the systemic circulation. Over the last fifteen years, evidence has emerged supporting a role for phospholipase C (PLC) enzymes, an important class of intracellular signaling molecules, in the regulation of tight junction function; however, unequivocal evidence to support such a role for any PLC isozyme has yet to be established. Studies in this dissertation were intended to develop a clearer understanding of the molecular mechanism involved in the regulation of intestinal epithelial tight junctions via the PLC-catalyzed signal transduction cascade. Specifically, the goal was to explore whether a cause-effect relationship exists between the inhibition of PLC activity and increased paracellular permeability via specific modulation of epithelial tight junctions.

The mRNA expression profile of PLC isozymes in Caco-2 cells, an *in vitro* model for the human intestine, was established and directly compared to the human intestine. Results demonstrated that PLCβ1, PLCβ3, PLCγ1, PLCγ2, PLCδ3, and PLCε were all expressed at the mRNA level in Caco-2 cells. Importantly, each of these isozymes was also detected in all

regions of the human small intestine. Further studies were intended to implicate PLC isozymes in the regulation of human intestinal tight junctions by determining the potency of previously reported PLC inhibitors to increase paracellular permeability and inhibit PLC activity in Caco-2 cells. The potency of a series of homologous alkylphosphocholines (APCs) to inhibit PLCβ activity varied forty five fold and correlated significantly with their potency to enhance paracellular permeability, suggesting that inhibition of PLCβ activity is associated with increased paracellular permeability. Further, structurally unrelated PLC inhibitor, U73122, also increased paracellular permeability and inhibited PLCβ activity.

In order to establish a cause-effect relationship between PLC inhibition and increased paracellular permeability in epithelial cells, RNA interference was used to suppress the expression of specific PLC isozymes in MDCK cells, and the effect on tight junction function and structure was evaluated. Surprisingly, depletion of PLCβ3 and PLCγ1 (the only isozymes of their respective families detected in these cells), alone or in combination, had no impact on either tight junction assembly or on the barrier function of already formed tight junctions, suggesting that a cause-effect relationship does not exist between inhibition of these PLC families and tight junction function in epithelial cells. These results further implied that putative PLC inhibitors, APCs and U73122, increase paracellular permeability via mechanisms independent of their effects on PLC enzymes.

Additional studies established that APCs disrupt apical membrane order at concentrations that also increase paracellular permeability in Caco-2 cells, providing an explanation for the observed effects on tight junction function. Unexpectedly, U73122 was found to increase the activity of hPLCβ3 in a cell free system, rather than inhibit its activity, providing an alternative hypothesis to explain the observed increase in paracellular

permeability following treatment of epithelial cells with this compound. This novel interaction between U73122 and PLC was due to alkylation of the protein at cysteine residues by the highly reactive maleimide moiety in U73122.

In summary, studies in this dissertation have significantly enhanced the current understanding of the role PLC enzymes play in the regulation of tight junction function, and have raised questions regarding previous reports that inhibition of these enzymes leads to increased paracellular permeability in epithelial cells.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3-NC	3-nitrocoumarin
APC	alkylphosphocholine
ATP	adenosine triphosphate
C10	decyl 2-(N, N, N,-trimethylamino) ethyl phosphate
C12	dodecyl 2-(N, N, N,-trimethylamino) ethyl phosphate (DPC)
C14	tetradecyl 2-(N, N, N,-trimethylamino) ethyl phosphate
C16	hexadecyl 2-(N, N, N,-trimethylamino) ethyl phosphate (HPC)
C18	octadecyl 2-(N, N, N,-trimethylamino) ethyl phosphate
C20	arachidyl 2-(N, N, N,-trimethylamino) ethyl phosphate
Cys	Cysteine
CPE	clostridium perfringens enterotoxin
CT	C-terminal
DAG	diacylglycerol
DDM	dodecylmaltoside
DPC	dodecyl 2-(N, N, N,-trimethylamino) ethyl phosphate
EC_{10X}	Drug concentration that increases mannitol permeability ten fold
$EC_{50\ LDH}$	Drug concentration that causes release of half the maximum LDH release from cultured cells
$EC_{50\ TEER}$	Drug concentration that decreases transepithelial electrical resistance to 50% of the control value
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
GI	gastrointestinal

GPCR	G-protein coupled receptor
HBSS	Hank's balance salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPC	hexadecyl 2-(N, N, N,-trimethylamino) ethyl phosphate (hexadecylphosphocholine)
hPLC	human phospholipase C
$IC_{50\ PLC\beta}$	Drug concentration that inhibits ATP-stimulated PLC activity in cultured cells to 50% of control value
$IC_{50\ hPLC\beta1}$	Drug concentration that inhibits hPLCβ1 activity in a cell free system to 50% of control value
$IC_{50\ PLC\beta3}$	Drug concentration that inhibits hPLCβ1 activity in a cell free system to 50% of control value
IP_3	1,4,5-inositol triphosphate
JAM	junctional adhesion molecule
LC	low calcium
MAGUK	membrane associated guanylate kinase
MDCK	Madin-darby canine kidney
MOPS	3-(N-morpholino) propane sulfonic acid
Mr	molecular radius
mRNA	messenger RNA
NC	normal calcium
NEM	N-ethylmaleimide
NC	normal calcium
NS	non-sense transfected cells
NT	non-transfected cells

P_{app}	apparent permeability coefficient
PDGF	platelet derived growth factor
PDZ	postsynaptic density disc-large ZO-1
PEG	polyethylene glycol
PH	pleckstrin homology
PI	phosphatidylinositol
PIP	phosphatidylinositol phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	1,2-dioctanoylglycerol and phorbol-12-myristate-13-acetate
PPE	paracellular permeability enhancer
RNAi	RNA interference
RTK	receptor tyrosine kinase
siRNA	small interfering RNA
SH	src homology
TB	transport buffer (HBSS supplemented with 10 mM Hepes and 25 mM glucose)
TEER	transepithelial electrical resistance
TIM	triose phosphate isomerase
TJ	tight junction
U73122	1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione

VEGF	vascular endothelial growth factor
ZO	zonula occludens
ZOT	zonula occludens toxin

CHAPTER 1

INTRODUCTION

A. Introduction

The ability to deliver medicines orally to reach the systemic circulation has significant advantages and is a primary goal for the majority of therapeutic indications; this objective presents a formidable challenge in the discovery and development of new drug candidates. Components of both the intestinal lumen as well as the intestinal wall serve to protect the body from the external environment and regulate the access of orally ingested substances into the body. The following sections present a summary of some of the unique obstacles imposed by the human intestine, with significant focus on the composition and regulation of intercellular junctional complexes that provide a foundation for the structure and function of the intestinal epithelium.

B. Oral Drug Absorption

Oral administration is the preferred route of delivery for the majority of therapeutic agents; unfortunately, oral absorption is often limited due to the anatomical, biochemical, and physiological barrier imposed by the gastrointestinal (GI) tract. The small intestine is the longest segment of the GI tract, and is the primary site of absorption for orally administered nutrients, vitamins, and medicines. Composed of three ill-defined regions, *i.e.* duodenum, jejunum, and ileum, the small intestine incorporates special physical features such as mucosal folds on its inner surface, as well as the macro and microvilli of the epithelium, to provide optimal surface area for the absorption of ingested substance.

The intestinal epithelium consists of a continuous monolayer of diverse cell types that line the wall of the intestinal lumen, and presents a formidable barrier to the entry of exogenous molecules into the systemic circulation. Absorptive enterocytes are the predominant cell type of the intestinal epithelium; they act not only to limit the entry of

potentially harmful exogenous molecules and toxins, but also to facilitate the absorption and retention of essential vitamins, nutrients, ions, and water. Enterocytes are polarized cells that maintain distinct apical and basolateral cell membranes, each with unique lipid and protein composition. For example, only the apical membrane contains glycosphingolipids[1, 2] that serve to increase the stability of this lumenal-facing membrane through increased intermolecular hydrogen bonds[3-5]. Both membranes contain a variety of different influx and efflux proteins that facilitate movement of both endogenous and exogenous substrates into and out of the cytosol in both directions. Furthermore, enterocytes express a diverse array of metabolic enzymes, including cytochrome P450s and esterases, that metabolize orally administered drugs and limit their absorption into the systemic circulation.

Depending on their physico-chemical properties, drug molecules and nutrients move across the intestinal epithelium via one of three pathways: by passive diffusion through epithelial cells across cell membranes (transcellular pathway), by a transporter mediated process across cell membranes (transcellular carrier-mediated pathway), or by passive diffusion between adjacent epithelial cells through the intercellular space (paracellular pathway) (Figure 1.1). Lipophilic molecules are able to easily partition into the lipid rich environment of cell membranes and move across cell monolayers via the transcellular route, while hydrophilic molecules are not. As a result, hydrophilic molecules must move across cell monolayers via the paracellular route if they are not substrates for specific absorptive membrane transporters. Passive paracellular diffusion is severely restricted, not only by the extremely low surface area as compared to the transcellular pathway, but also by the presence of tight junctions, multi-protein complexes that connect adjacent cells and create a physical barrier within the intercellular space. Tight junctions limit the oral bioavailability of

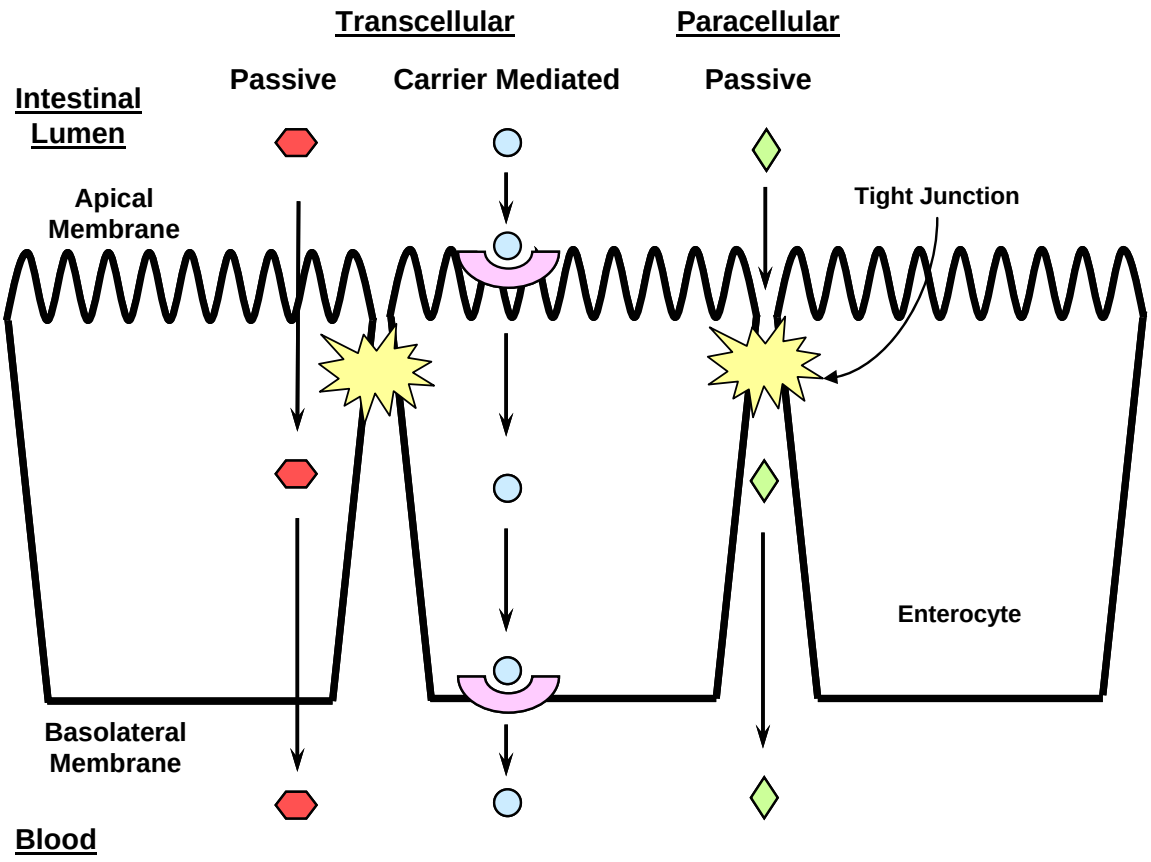

Figure 1.1. Transport across an epithelial cell monolayer. Drug molecules cross a monolayer of epithelial cells by one of three pathways: (1) by passive diffusion through epithelial cells across cell membranes (transcellular pathway), (2) by a transporter mediated process across cell membranes (transcellular carrier-mediated pathway), or (3) by passive diffusion between adjacent epithelial cells through the intercellular space (paracellular pathway).

hydrophilic drugs; therefore, this class of drugs often require alternative, less desirable, routes of administration that make them useful for physicians and patients. Numerous approaches have been devised with the objective of increasing the intestinal absorption of hydrophilic drugs and macromolecules. One such approach involves the co-administration of a paracellular permeability enhancer (PPE), or a molecule that specifically increases paracellular passive diffusion by modulating the structure and/or function of intestinal epithelial tight junctions. However, despite two decades of active research, a safe and effective PPE has not yet been discovered and approved for use in humans.

C. Tight Junctions

Neighboring epithelial cells are connected to one another via multi-protein junctional complexes near the apical membrane consisting of tight junctions, adherens junctions, and desmosomes. The tight junction, the most apical component of the junctional complex[6], is generally considered to be the major barrier to the passage of molecules between adjacent cells and through the intercellular space. The tight junction is comprised of transmembrane proteins that combine to form the physical barrier of the tight junction by spanning the intercellular space and interacting with transmembrane proteins on neighboring cells, as well as membrane scaffolding proteins that link the transmembrane proteins to the actin cytoskeleton and arrange them adjacent to important cytosolic regulatory proteins (Figure 1.2). There exists an abundance of evidence suggesting that these dynamic multi-protein structures are under an impressive degree of regulation, and that their barrier function can be modulated in response to physiological, pharmacological, and pathophysiological stimuli. The following sections will review both the protein composition of epithelial tight junctions, as well as regulatory mechanisms thought to influence their structure and function.

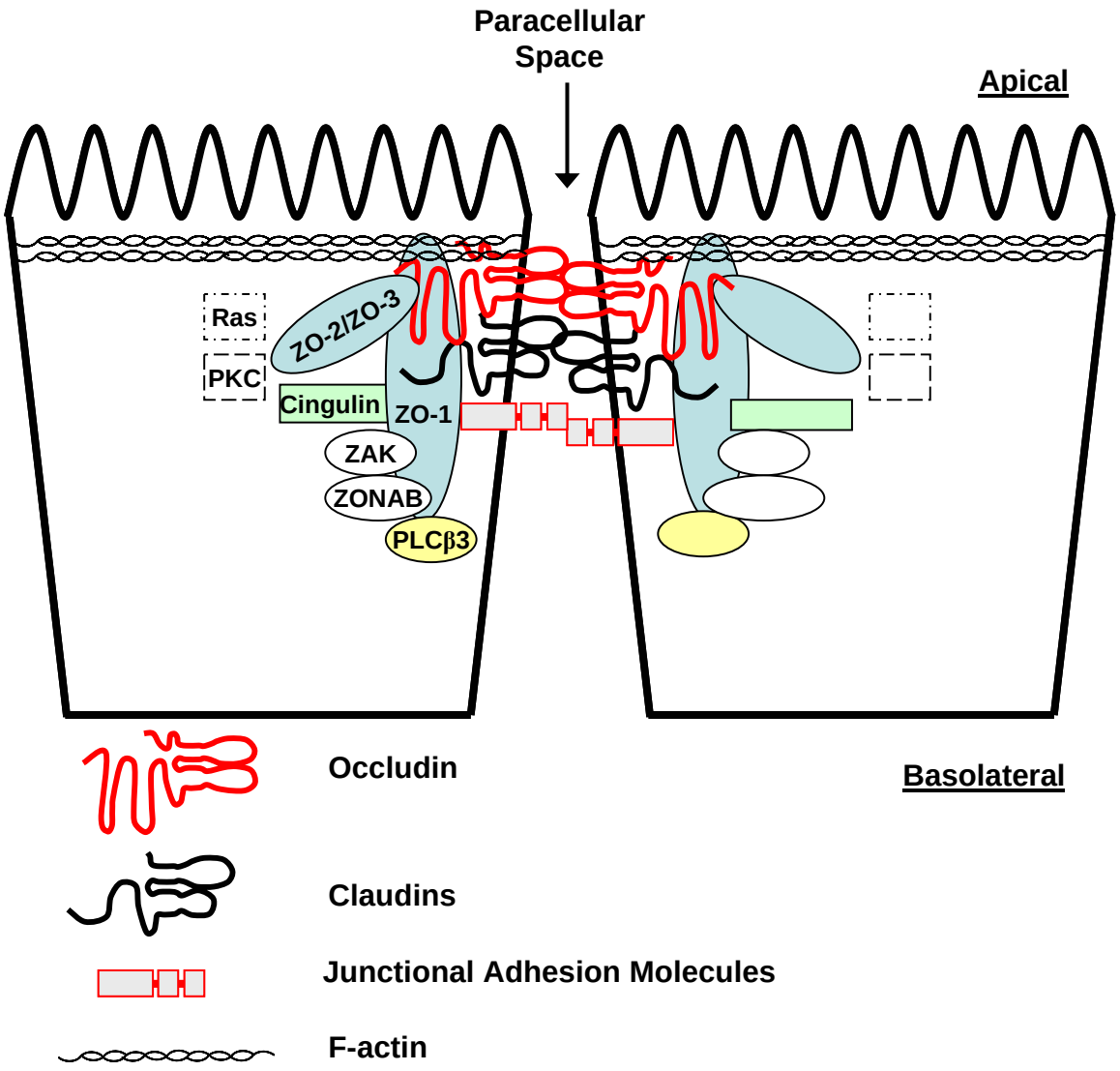

Figure 1.2. Schematic representation of an epithelial tight junction including major proteins and their interactions. Tight junctions are a multi-protein complex composed of several transmembrane and cytosolic proteins. Transmembrane proteins (e.g. claudins, occludin, junctional adhesion molecules) interact through their extracellular domains with transmembrane proteins in adjacent cells, as well as with cytosolic proteins and the actin cytoskeleton. Cytosolic proteins (e.g. zonula occludens) interact with each other, and serve as a scaffold to link the tight junction to the cytoskeleton, as well to organize regulatory proteins that control tight junction function. Adapted from a figure provided by Dr. Alan Fanning (School of Medicine, Department of Cellular and Molecular Physiology, University of North Carolina at Chapel Hill). Not all known tight junction proteins are depicted.

1. Tight Junction Structure

Tight junctions contain more than forty different proteins with numerous functions [7]. The current view of the tight junction[8] includes four transmembrane proteins: occludin (approximately 65 kDa), claudins (approximately 20-27 kDa), junctional adhesion molecules (approximately 40 kDa), as well as the recently identified tricellulin (approximately 64 kDa). The extracellular domains of these proteins are thought to interact homophilically and heterophilically with one another, and with the extracellular domains of the same set of proteins on neighboring cells. These interactions create continuous circumferential belts of anastomizing strands, often referred to as fibrils, that create a physical barrier separating the apical and basolateral cell membranes as well as a selective intercellular pathway for ions, solutes, and in some cases, whole cells[9-11].

Occludin was the first protein identified to be a critical component of tight junctions [12-14], and contains four transmembrane domains that form two extracellular loops that contain a high content of tyrosine and glycine residues[13]. The N-terminal cytosolic domain contains sequences predicted to bind to src homology 3 domains (SH3)[15], while the C-terminal cytoplasmic tail, highly concentrated with charged amino acids, binds directly to the cytoskeleton[16], as well as to tight junction-associated proteins (*i.e.* zonula occludens-1 (ZO-1), ZO-2, and ZO-3) that also interact with the cytoskeleton [17-20]. While the N-terminus and extracellular domains appear to be critical for normal tight junction barrier function[21-26], conflicting evidence exists as to whether the C-terminal domain is essential for proper targeting and localization of occludin to the cell periphery, as well as for maintaining normal barrier function[27, 28]. Consistent with these conflicting results, the importance of occludin as a component of the tight junction physical barrier remains controversial. For example, over-

expression of chick occludin in Madin-darby canine kidney (MDCK) cells initially decreased mannitol permeability compared to control; however, after an extended incubation, mannitol permeability increased to 50% of control and was accompanied by an unexpected and disconcordant increase in transepithelial electrical resistance (TEER)[29]. Some studies suggest that occludin does play a critical role in junctional formation and maintenance, as its exogenous expression in occludin-null fibroblasts leads to its co-localization with ZO-1 at points of cell-cell contact[30]. On the other hand, mounting evidence suggests that the role of occludin in tight junction barrier function may be less critical. For example, disruption of the occludin gene in embryonic stem cells did not prevent the formation of functional strands[31], and *ex vivo* permeability studies across intestinal tissues from occludin knockout mice maintained normal permeability function[32], despite observed phenotypic changes including gastric inflammation in these occludin null mice[33].

The functional role of occludin may instead be more organizational in nature, as its coiled-coil cytoplasmic domain has been shown to interact with a wide variety of intracellular proteins in addition to the aforementioned scaffolding ZO proteins, including JEAP, VAP-33, protein kinase C (PKC) ζ, c-Yes, and phosphatidylinositol (PI) 3-kinase[34-37]. Consistent with reported interactions with protein kinases, numerous phosphorylation sites have been identified in both cytoplasmic domains of occludin[38] that may serve to regulate its localization and function. Indeed, it has been reported that highly phosphorylated occludin is concentrated to tight junctions in MDCK cells[39], and phosphorylation is likely a key mechanism regulating the physiological function of occludin. To date, an unambiguous role for occludin in the maintenance and regulation of tight junctions has not been clearly defined.

While occludin was the first tight junction protein identified, it is the claudin family of proteins that are believed to constitute the primary physical components of tight junctions[40, 41]. Claudins, like occludin, contain four transmembrane domains, although they bear no sequence similarity to occludin. To date, more than twenty members of the claudin family have been identified[10, 42-44], and evidence to date suggests that claudins are responsible for creating the variable barrier properties (*i.e.* permeability selectivity and transepithelial electrical resistance) that exist between tight junctions in different tissues[45, 46], and even in different regions of the same tissues[47, 48]. For example, the longitudinal (*i.e.* duodenum to colon) and radial (*i.e.* villous to crypt) expression of claudins in the mouse intestine has been established and found to vary[49, 50], suggesting that previously observed differences to ion flux along the human small intestine may result from notable differences in claudin expression between each region[51]. An abundance of *in vitro* studies have further implicated claudins as the primary determinants of tight junction barrier properties. Expression of claudin-1 and claudin-2 in fibroblasts, cells that lack constitutive tight junction strands, induced tight junction formation[52]. Furthermore, expression of claudin-2 in high resistance MDCK strain I cells that do not normally express this isoform, dramatically reduced the resistance to that of MDCK strain II cells that typically do express this isoform[53]. In addition, overexpression of claudin-4 in MDCK strain II cells induced a decrease in conductance likely related to the concomitantly observed decrease in sodium permeability[54]. More recent studies have confirmed the importance of individual claudins in the charge selectivity of the paracellular pathway[55-58], firmly establishing these proteins as the unique functional differentiating components of tight junction barrier properties.

The continuing emergence of data suggesting claudins as the variable regulators of tight junctions between different tissues make claudins intriguing targets in human disease. In fact, several human diseases are thought to be caused by mutations in specific claudin isoforms. Mutations in the gene encoding claudin-14 cause autosomal recessive deafness[59], mutations in the gene encoding claudin-16 lead to excessive loss of magnesium and calcium and subsequent autosomal recessive renal hypomagnesemia and hypercalciuria[60], while mutations in claudin-19 lead to chronic renal failure and severe visual impairment[61]. Further, changes in claudin expression levels have been noted in conjunction with onset of intestinal diseases such as irritable bowel syndrome[62], Crohn's disease[63], Barret's Esophagus[64], as well as esophageal, gastric, and colon carcinomas[65-68]. In addition to notable changes to claudins in human disease, knockout and transgenic mice have confirmed the important role for these proteins in the barrier function of tight junctions in different tissues, including the brain[69] and skin[70-72]. Data from these and other studies provide support for a crucial role for claudins in creating and regulating selective channels through the tight junction barrier.

A third family of transmembrane proteins, junctional adhesion molecules (JAM)[11], has also been localized to tight junctions strands of both endothelial and epithelial cells[73] and has been shown to interact with junctional proteins occludin, cingulin, ZO-1[74], Par-3[75], MUPP-1[76], MAG-1[77], as well as atypical PKC isozymes[75]. JAMs are single transmembrane proteins with a relatively short cytoplasmic C-terminal domain and an N-terminal extracellular domain composed of two Ig-like loops. The exact function of JAMs at the tight junction is not clearly defined; evidence suggests they play a role in early tight junction assembly[78] as well as in direct interactions of junctional complexes with immune cells (*e.g.* leukocytes), including their transmigration across cell monolayers[73] specifically through the

intercellular space[79]. More recent evidence implicates these proteins in regulation of tight junction permeability in both the kidney and intestine[80], although their reported expression in platelets[81] and cells of the immune system[82] obscures their potential importance in specifically regulating tight junction barrier function in epithelial cells.

Most recently, another tetraspanning protein, tricellulin, has been localized to tight junction strands[83], and appears to be concentrated at points of tricellular contact. Early studies suggest an important role for tricellulin in epithelial tight junctions, as RNAi mediated depletion compromised epithelial barrier integrity[83]. In addition, recessive mutations in the gene encoding tricellulin cause nonsyndromic deafness in the inner ear, likely related to the loss of a conserved region in the cytosolic domain of the protein that binds ZO-1[84]. Additional studies are required to fully appreciate the specific role this novel protein may play in the assembly and regulation of tight junctions, specifically at tricellular contacts.

Three cytosolic proteins, referred to as tight junction-associated proteins or zonula occludens (ZO) proteins, link the transmembrane proteins of the tight junction to the actin cytoskeleton, and also serve as a scaffold on which to organize various regulatory proteins (*e.g.* kinases, phosphatases, and transcription factors) near their cellular substrates. ZO proteins are all phosphoproteins, suggesting they may be phosphorylation targets for some of the signaling proteins they serve to organize. They are members of the membrane associated guanylate kinase family (MAGUK) and are the major constituents of the cytosolic domain of the tight junction. MAGUK proteins are characterized by having one to five N-terminal PDZ repeats (each ZO protein contains three), an SH3 domain, and a C-terminal region homologous to guanylate kinases[85]. PDZ domains are known to mediate protein-protein

interactions and typically bind to short amino acid motifs, usually ending in valine, at the C-termini of interacting proteins[86].

ZO-1 is the first tight junction associated protein identified with a mass of approximately 210 kD[87]; ZO-1 is also observed at some types of adherens junctions in non-epithelial cells[88]. The N-terminal half of ZO-1 interacts with the C-terminal tail of occludin[13, 89], claudin[19], ZO-2[89], and ZO-3[19], while the C-terminal half of ZO-1 interacts with F-actin, cortactin, and protein 4.1, and anchors the protein to the cellular cytoskeleton[89-92]. It has also been shown to interact via its SH3 domain with a serine protein kinase (ZAK)[93], a transcription factor, ZONAB, which plays a role in epithelial cell proliferation[94], and small G-proteins, such as $G\alpha_{12}$ [92]. ZO-1 has also been recently reported to form homodimers via its second PDZ domain[95]. Knockdown experiments in both mouse and canine epithelial cells have demonstrated that ZO-1 is not essential for the formation of tight junctions; however, both studies revealed a pronounced delay in the assembly of functional tight junctions following calcium switch[96, 97].

ZO-2, a 160 kDa protein, has also been found to interact with the C-terminal half of occludin[98] in addition to the N-terminal half of ZO-1[89], cingulin[99], and the C-terminal domain of claudins through its PDZ1 domain[19]. ZO-2 also associates with actin as well as the actin binding protein 4.1 through its C-terminal praline rich domain[16, 19, 100, 101]. The sub cellular localization of ZO-2 appears to be dependent on the degree of cell-cell contact in epithelial cells; in confluent monolayers, ZO-2 appears primarily at the tight junction, while in sub-confluent cells it demonstrates speckled nuclear localization, where it has been shown to interact with DNA binding proteins[102], and co-localize with nuclear splicing factors[103], suggesting a potential role for ZO-2 in the transmission of regulatory signals from the

nucleus to the cell periphery. Nuclear localization of this MAGUK protein is consistent with the identification of several nuclear localization and export signal sequences[104, 105]. More recently, ZO-2 has been hypothesized as a candidate tumor suppressor protein due to its high homology to other tumor suppressors[106], as well as its lack of expression in a number of cancers[107, 108]. A very recent report highlights the importance of ZO-2 in the formation and maintenance of tight junctions, as RNAi-mediated depletion in MDCK cells caused significant abnormalities in the barrier function of tight junctions in these cells[109]; although results from other recent cellular knockdown studies contradict this finding[96, 110], obscuring the absolute requirement of this protein for tight junction function and maintenance.

The most recently identified ZO-3, a 130 kDa protein, is the only ZO expressed exclusively in epithelial cells[111]. It interacts with ZO-1, occludin, claudins, as well as actin filaments, but not with ZO-2[16, 18, 19]. Independent ZO-1•ZO-2 and ZO-1•ZO-3 complexes have been found *in vivo*[16]; therefore, the tight junction associated proteins do not exist in a single trimeric grouping (i.e., ZO-1•ZO-2•ZO-3 complex). ZO-3 knockout mice and RNAi depleted mouse teratocarcinoma F9 cells appear normal with no obvious abnormalities in tight junction function and architecture[110]; however, overexpression of the N-terminal half of ZO-3 in MDCK cells caused marked changes in actin organization and delayed tight junction assembly[112, 113]. Additional studies will be necessary to clarify the role of ZO-3 in tight junction assembly and maintenance.

In addition to the ZO family of proteins, several other tight junction specific peripheral membrane proteins have been identified, including membrane associated guanylate kinase inverted proteins (MAGI-1, 2, 3)[114-117], the partitioning defective proteins (PAR-3 and PAR-6), the multi-PDZ domain protein-1 (MUPP-1), the MAGUK protein

associated with Lin-7 (PALS-1), cingulin[118, 119], 7H6 antigen[120, 121], symplekin[122] and others. All have been identified as cytosolic components of the tight junction, yet their functional role in tight junction structure and function has not been extensively evaluated and will not be discussed in detail. The function of some of these proteins has been recently reviewed[123].

2. Tight Junction Function

Research over the past 50 years has exposed the tight junction as a highly dynamic and complex multi-protein structure, selectively permeable to certain hydrophilic molecules including ions, nutrients and drugs. The current analogies for the tight junction are as a "gate," because it allows passage of small hydrophilic molecules but acts as a barrier to larger hydrophilic ones, and as a "fence," because it forms an intramembrane diffusion barrier that restricts the intermixing of apical and basolateral membrane components[124, 125]. The outer leaflet of the "fenced in" apical membrane is preferentially enriched in glycosphingolipids and sphingomyelin[1, 2, 126, 127]. The tight junction prevents the diffusion of such phospholipids out of the exoplasmic leaflet of the apical membrane, whereas phospholipids in the cytoplasmic leaflet of the apical membrane are free to diffuse into the basolateral membrane[128]. For example, when asymmetric liposomes containing over 85% of a fluorescent lipid in the exoplasmic leaflet were fused with the apical plasma membrane of MDCK cell monolayers, the fluorescent lipid did not move to the basolateral side. When symmetric liposomes, which contained the fluorescent lipid in both leaflets, were fused to the apical membrane, redistribution of the fluorescent lipid to the basolateral membrane occurred immediately[125]. The fence function further restricts to movement of apical membrane components to the apical membranes of neighboring cells. For example, an endogenous glycolipid (Forssman antigen), present in the exoplasmic leaflet of the apical membrane of

MDCK strain II cells was unable to pass to the apical membrane of MDCK strain I cells (which lack this glycolipid) under conditions where these cells are co-cultured and connected by tight junction strands. In addition, fluorescent lipids, which have been fused into the plasma membrane of one MDCK cell, do not diffuse to neighboring cells while the tight junction between cells is intact[129].

This fence function of the tight junction maintains the polarity of enterocytes, and is related to the gate function, as the maintenance of distinct membrane environments is directly related to the passage of molecules between adjacent cells and the subsequent exposure of such molecules to distinctly different membranes. However, the gate and fence function of the tight junction can be uncoupled, as ATP depletion increased the flux of the hydrophilic compound inulin across MDCK cell monolayers (*i.e.* disrupted gate function); however, a fluorescent phosphatidylcholine that was incorporated into the exoplasmic leaflet of the apical membrane remained confined to that membrane (*i.e.* maintained fence function)[130]. Undoubtedly, the biological function of the tight junction is quite complex, and the proteins and signal transduction cascades involved in maintaining and regulating these functions remain to be clearly defined.

The gate function of the tight junction is highly dynamic and regulates the movement of hydrophilic molecules across the intestinal epithelium. The tight junction restricts movement of non-charged solutes on the basis of molecular radius; however, this size-dependent restriction is still not well understood. Experiments have shown that tight junction permeability of polyethylene glycols (PEG) with different molecular radii is markedly reduced at a molecular radius of approximately four angstroms across epithelial cell monolayers[131], implying that the size restriction is not linearly related to molecular radius.

Instead, these data suggest the existence of two distinct sets of tight junction pores, small restrictive pores accessible to molecules less than four angstroms, and larger non-restrictive pores accessible to molecules of increasingly larger radii. This *in vitro* observation is similar to the *in vivo* PEG absorption profiles produced for rat, dog, and human, suggesting the tight junction has a molecular radius of approximately five angstroms *in vivo*[132]. Interestingly, increased tight junction permeability by depletion of extracellular calcium or treatment with sodium caprate had different effects on the size restriction of permeability of PEGs across Caco-2 and T84 cell monolayers. Although both treatments markedly increased permeability, depletion of extracellular calcium abolished the size restriction imposed by untreated cell monolayers, while treatment with sodium caprate did not[131], implying that these two approaches to modulate barrier function may be mediated via distinct molecular mechanisms. The gate function of epithelial tight junctions is clearly complex, and assessing changes to this functional aspect of junctional complexes in response to physiological and pharmacological stimuli should be performed with multiple and detailed approaches.

3. Assessing Changes to Tight Junction Barrier Function

The barrier function of epithelial tissues is typically assessed via measurement of two parameters, the transepithelial electrical resistance (TEER) and the monolayer permeability of hydrophilic molecules. Tight junctions restrict the movement of ions across monolayers of epithelial cells, giving rise to an electrical potential difference, measurable as electrical resistance. The paracellular space has a significantly lower resistance and accordingly higher conductance to ion flow compared to cell membranes, even in the presence of ion channels. Studies suggest that as much as 75% of the ionic conductance across moderately leaky epithelia, such as those found in the small intestine, is through the paracellular pathway [133,

[134]. Therefore, an increase in ionic conductance, and a concordant decrease in TEER, is thought to be a reflection of an increase in paracellular permeability, as opposed to transcellular permeability, of ions across a cell monolayer. A number of studies have confirmed this hypothesis. For example, treatment of Caco-2 cells with an ionophore specifically increased the cellular membrane conductance to ions rather than the paracellular conductance, and had no effect on TEER[135]. However, changes in TEER are not always reflective of an increase in monolayer permeability to solutes, as increased levels of cAMP and cGMP in intestinal epithelia led to significant and long lasting transepithelial potential changes, but no increase in permeability of macromolecules[136]. In addition, manipulation of the expression of specific claudins has been shown to alter TEER[55, 56] without measurable changes to solute flux[54]; therefore, in addition to the measurement of TEER, it is crucial to also include an evaluation of changes to the permeability of non-charged solutes, preferably of varying molecular radii.

Hydrophilic solutes are unlikely to move across lipid rich cell membranes; therefore, unless they are substrates for membrane transporters, they must rely on movement through the paracellular pathway between adjacent cells. As a result, increased epithelial monolayer permeability to hydrophilic solutes is typically a reflection of changes in paracellular permeability, rather than transcellular permeability. A number of small and large hydrophilic solutes have been utilized as markers for changes in paracellular permeability. Small molecules typically include neutral compounds such as mannitol (Mr = 4 Å) or urea (Mr = 2.7 Å)[137], cations such as atenolol (Mr = 4.8 Å)[137], ranitidine[138], or creatinine (Mr = 3.2 Å)[139], or anions such as fluorescein (Mr = 5.5 Å) or ^{51}Cr-EDTA (Mr = 5.4 Å)[140]. Mannitol is clearly the most often used paracellular marker, as a number of convincing studies support the

hypothesis that mannitol traverses epithelial monolayers via the paracellular route[141-143]. Positively charged hydrophilic solutes are typically more permeable than neutral or anionic solutes of similar size, presumably due to the anionic nature of cell membranes and the intercellular space[137, 144]. Hydrophilic macromolecules such as inulin (Mr = 7.5 Å) and fluorescein isothiocyanate dextrans (Mr = 14-60 Å)[140] are also frequently used to assess changes in paracellular permeability of epithelial monolayers, often in concert with small molecules in order to more accurately assess the extent of changes to the paracellular space. Recently, a number of groups have employed the use of polyethylene glycol polymers of increasing size to completely characterize the size restrictions of the paracellular pathway[131, 145], although the linear structure of PEGs allows significant flexibility that may cloud interpretations made using these polymers[145].

The relative tightness of epithelial tight junctions varies both among and within individual tissues. For example, intestinal tight junctions vary in tightness longitudinally, consistent with absorptive functions in different regions. The upper small intestine is significantly leakier than the colon, in accordance with the role of the small intestine in the rapid absorption of orally ingested vitamins and nutrients, and the role of the colon in regulating and retaining total body water as well as preventing entry of bacterial toxins. Typically, a leaky epithelium has a low TEER (high permeability), 10-50 ohm*cm^2, while a tight epithelium has a high TEER (low permeability), > 1000 ohm*cm^2. Regardless of the relative tightness of the epithelia under evaluation, both TEER and hydrophilic solute flux should be used in concert to characterize both baseline paracellular permeability, as well as PPE induced changes to the permeability barrier.

4. Physiological and Cellular Regulation of the Tight Junction

Tight junctions are not static; instead they are a highly dynamic but stable cellular structures maintained by numerous protein-protein interactions that respond to various physiological, pharmacological, and pathophysiological stimuli from both inside and outside the cell. Physiological regulation of the tight junction was first reported by Pappenheimer and Reiss[139], who proposed that "solvent drag" through the paracellular space is responsible for a significant proportion of total nutrient absorption following a meal. It has since been postulated that this apparent increase in absorption is the result of activation of the sodium dependent glucose transporter leading to contraction of the actin myosin ring via PKC-mediated phosphorylation of myosin light chain[146, 147]; although this hypothesis remains controversial[148], as subsequent studies have reported conflicting results with respect to increased absorption of paracellular permeants co-administered with glucose[51, 149, 150].

In addition to nutrients such as glucose, a number of other endogenous molecules have been reported to alter the barrier function of both endothelial and epithelial tight junctions. For example, hormones and neurotransmitters such as vasopressin, angiotensin II and epinephrine[151], nucleotides such as ADP and ATP[152], and growth factors such as hepatocyte growth factor[153], platelet derived growth factor (PDGF)[154], and epidermal growth factor (EGF)[155], have all been reported to modulate tight junction permeability in a number of cellular models. Cytokines, such as tumor necrosis factor-α[156], interferon-γ[157], and numerous interleukins[158, 159] have emerged in recent years as important factors regulating tight junction function, with particular relevance to a number of intestinal inflammatory diseases including Crohn's, ulcerative colitis, and inflammatory bowel disease[160], in which intestinal tight junction permeability is compromised. More recently, interferon-γ and

interleukin-4, cytokines that are known to negatively regulate one another, have been shown to have opposing effects on tight junction permeability in T84 cells mediated via claudin-2 expression[161]. Apical treatment with interferon-γ decreased claudin-2 expression and decreased paracellular permeability, while apical treatment with interleukin-4 increased claudin-2 expression and increased paracellular permeability, suggesting intricate and opposing physiological mechanisms controlling the barrier function of intestinal tight junctions. Interestingly, basolateral treatment of the same cell line with interferon-γ for extended time periods has been reported to increase, rather than decrease, paracellular permeability[162], suggesting membrane dependent effects of this cytokine on tight junction function. Consistent with these recently described compensatory cytokine-mediated regulatory mechanisms, pre-incubation of T84 cells with transforming growth factor-β has been reported to attenuate the effects of interferon-γ on barrier function[163], supporting a highly complex physiologic regulation of tight junction function in the human intestine.

Two primary intracellular molecular mechanisms are typically associated with alterations to the function of mature epithelial tight junctions, namely, reorganization of the actin cytoskeleton[9] and changes to the phosphorylation state of tight junction proteins[164] (Figure 1.3). It is well established that the apical junctional complex, including the tight junction, is located adjacent to a dense band of actin and myosin, typically referred to as the perijunctional actin myosin ring. The actin cytoskeleton associates with the plasma membrane, specifically through this dense band of filaments just beneath the tight junction at the level of the adherens junction[165]. As indicated previously, actin binds directly to cytosolic as well as transmembrane proteins of the tight junction[16, 90]; therefore, the stability of actin filaments directly impacts the structure and function of tight junctions. It has long been

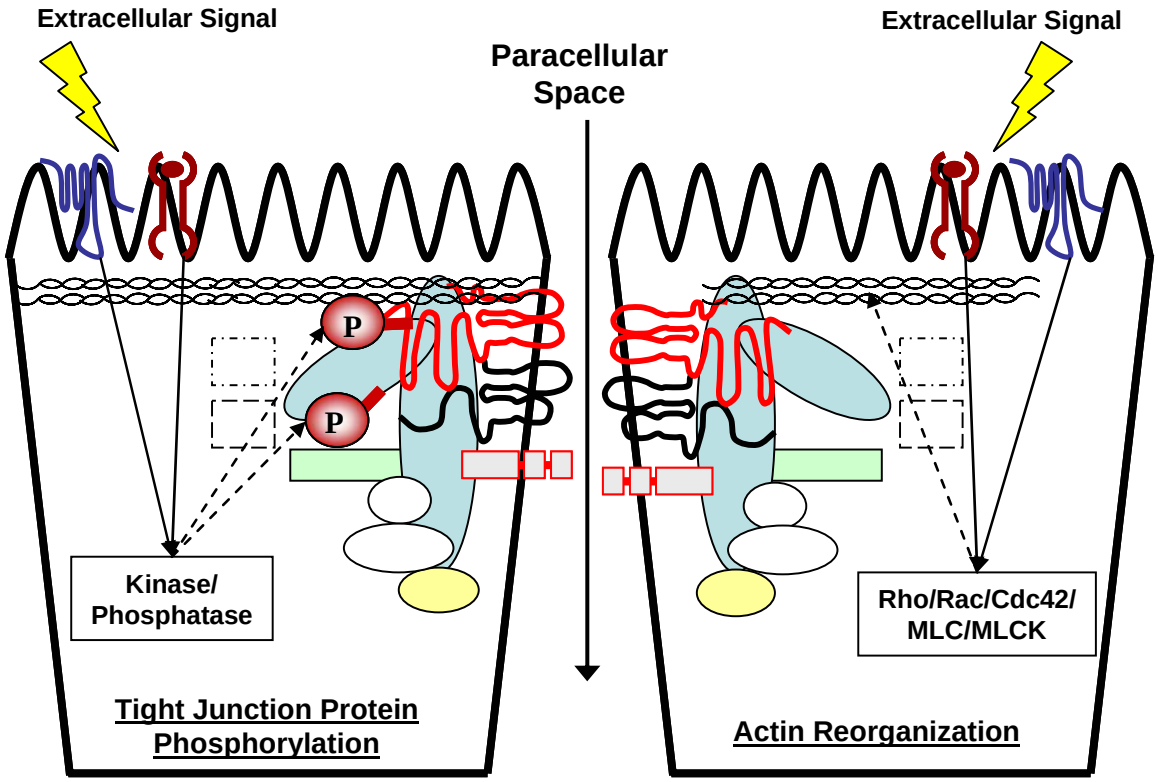

Figure 1.3. Schematic representation of the two major intracellular molecular mechanisms associated with the regulation of tight junction function leading to increased paracellular permeability through the intercellular space. Following an extracellular stimuli (or intracellular stimuli, not depicted), numerous signaling pathways may lead to changes to the phosphorylation state of specific tight junction proteins (left cell) or alterations to the organization of the perijunctional actin myosin ring (right cell) that lead to transient opening of the paracellular space. See Figure 1.2 (page 6) for identification of depicted proteins. Adapted from a figure provided by Dr. Alan Fanning (School of Medicine, Department of Cellular and Molecular Physiology, University of North Carolina at Chapel Hill). Not all known tight junction proteins are depicted.

recognized that disruption of the actin cytoskeleton, for example by actin-depolymerizing drugs such as cytochalasin and phalloidin, leads to extensive disruption of tight junction barrier properties[166, 167]. The regulation of tight junctions via reorganization of the actin cytoskeleton is known to be mediated via numerous signaling molecules such as the small GTPases Rac, Rho, and Cdc42. All three of these proteins have been shown to be important regulatory enzymes regulating barrier function through actin filaments. Expression of either dominant negative or constitutively active forms of Rac, Rho, and Cdc42 has been specifically demonstrated to alter actin organization by a number of laboratories[168-170], consistent with inactivation of these proteins by Clostridium difficile toxins that also leads to disorganization of actin filaments[171]. Indeed an abundance of studies have established the fundamental and critical requirement of filamentous actin organization in the maintenance of tight junction barrier function in epithelial cells, although additional studies are clearly necessary to determine the exact mechanisms and regulatory components involved in the multifaceted interaction between tight junctions and the perijunctional actin myosin ring.

In addition to alteration and disruption of the actin cytoskeleton, changes to the phosphorylation state of a number of tight junction proteins has been observed in conjunction with changes to tight junction barrier function. For example, phosphorylation of myosin light chain induces the perijunctional actin myosin ring to contract and has been associated with a loosening of the tight junction[172], a result that suggests these two molecular mechanisms are intimately related. Reversible phosphorylation of proteins is probably the most important regulatory modification of cellular components, involved in such crucial cellular functions as mitosis, metabolism, growth and differentiation, and gene expression. The primary components of the tight junction, ZO-1, ZO-2, ZO-3, occludin, and claudins, are all

phosphoproteins[38, 39, 173-176], and changes to the phosphorylation state of these proteins at tyrosine, serine, and threonine residues is believed to be an important step in the regulation of tight junction function. Further, a number of PKCs have been shown to directly phosphorylate tight junction proteins, *e.g.* ZO-1[177], ZO-2[174], occludin[178], and claudins[179]. Disruption of tight junction integrity by ATP depletion induced a decrease in phosphorylation of several tight junction regulatory proteins, followed by a subsequent increase in phosphorylation and re-establishment of tight junction integrity during ATP repletion[176]. The phosphorylation state of occludin correlates with occludin localization and function at the tight junction[180]; thus, the phosphorylation of occludin may be involved in tight junction assembly[39]. Interestingly, two strains of MDCK cells that differ markedly in TEER (thirty fold) have a similar number of tight junction strands and content of ZO-1[181]; however, the level of phosphorylation of ZO-1 in the low resistance strain is approximately twice that of the high resistance strain[182]. Differences in claudin expression have since been attributed to the difference in resistance between these two strains of MDCK cells, although a possible relationship between claudin expression and ZO-1 phosphorylation state has not been further explored. Increased tyrosine phosphorylation of both ZO-1 and ZO-2 following treatment of MDCK cells with a tyrosine phosphatase inhibitor correlates with increased tight junction permeability[183]. Additionally, dephosphorylation of ZO-1, occludin, and claudin-1 have been observed concomitantly with reduction in TEER and increase in mannitol and inulin flux across MDCK cells transfected with a protein phosphatase (PP)2A holoenzyme, ABαC[184]. More recently, PKCθ phosphorylation of claudin-1 and claudin-4 in Caco-2 cells[175] and PKCε phosphorylation of claudin-4 in ovarian cancer cells [179] have been demonstrated to be important factors in the regulation of tight junction barrier function in

these cell lines. Although alteration in the phosphorylation state of tight junction proteins has often been associated with altered tight junction permeability, a direct cause-effect relationship between the phosphorylation of a tight junction protein and increase paracellular permeability has not been established unequivocally.

D. Regulation of the Tight Junction by Phospholipase C Enzymes

1. Isozyme Families and Catalytic Function

Phosphoinositide-specific phospholipase C (PLC) isozymes comprise a group of related enzymes that specifically cleave the polar head group from inositol containing phospholipids, and play important roles in the regulation of many cellular functions. PLC isozymes are activated in response to both intracellular and extracellular stimuli, including hormones, growth factors, G-proteins, and neurotransmitters, leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form the classic intracellular second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Highly soluble IP_3 is released into the cytosol, and is known to bind to receptors at the endoplasmic reticulum where it induces calcium release, and thereby functions to regulate intracellular calcium levels[185, 186]. More recent reports have demonstrated sequential phosphorylation of IP_3 by novel kinases to generate additional inositol polyphosphates IP_4, IP_5, IP_6, and IP_7, which have been shown to be necessary for mRNA export, DNA repair, and proper regulation of gene expression[187-189]. Increased levels of DAG, either alone or in conjunction with increased calcium, activate the novel and conventional PKC isozymes, respectively[190-192].

In the late 1980's and early 1990's, three mammalian PLC isozymes (β, γ, and δ) were first isolated and their corresponding DNA sequences were determined[193, 194]. To date, six families of PLC (β, γ, δ, ε, ζ, and η) consisting of thirteen total isozymes have been

identified in humans, including PLCβ1, β2, β3, β4, PLCγ1, γ2, PLCδ1, δ3, δ4, PLCε, PLCζ, PLCη1, and η2[195-198] (Figure 1.4). It is noteworthy to point out that PLCδ2, originally identified in bovine tissue, has recently been recognized as the human homolog of PLCδ4, and therefore not a distinct human isozyme[199]. PLCs vary in molecular size from the approximately 70 kDa PLCδ to the much larger 250 kDa PLCε, and share little sequence similarity even within isozyme families. They do share a common core domain structure which includes the PH domain, EF domain, C2 domain, and the highly conserved catalytic core made up of two regions, known as X and Y domains[200, 201] (Figure 1.4). The catalytic domain contains alternating α-helices and β-strands to form an incomplete triose phosphate isomerase (TIM) barrel. Inositol-containing substrates are an absolute requirement for PLC activity, as the 2-position hydroxyl in the inositol ring participates in a unique intramolecular nucleophilic attack on the phosphorous to produce a cyclic intermediate. A PLC active site histidine then abstracts a proton from water promoting hydrolysis of the cyclic phosphodiester intermediate[197].

Outside of the highly conserved catalytic domains, the remaining domains have unique and important functions. Although EF domain of PLCs have interesting similarities to calcium saturated forms of calmodulin, they do not appear to bind this metal in PLCs, leaving the importance of this domain yet to be discovered[202]. Instead, the C2 domain is thought to bind calcium in a fashion that may stabilize the interaction of PLCs to the membrane. Most PLCs contain a pleckstrin homology domain (PH), conventionally believed to be specifically involved in membrane binding[203], have been recently established as mediators of protein-protein interactions as well[204, 205]. The lack of amino acid conservation in PH domains between isozyme families suggests that this domain may be responsible for

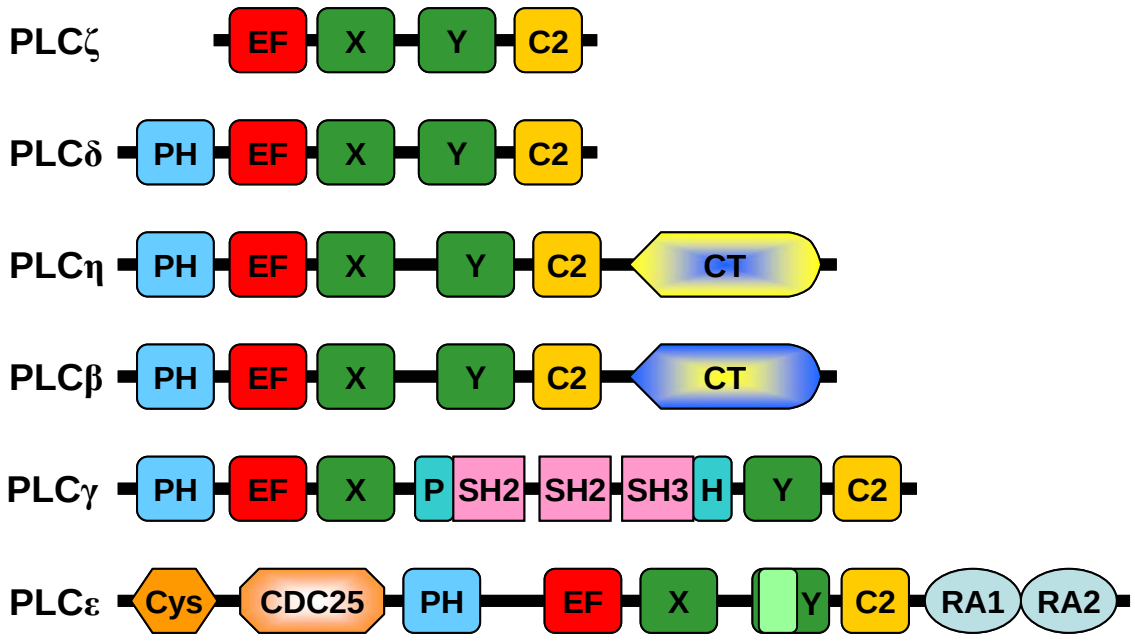

Figure 1.4. Domain organization and amino acid sequence comparisons among human PLC isozymes. Six families of PLC isozymes are distinguished by different structural domains. Figure was reproduced from published sources.[195, 211].

Legend: Cys (cysteine rich region), CDC25 (guanine nucleotide exchange domain, PH (pleckstrin homology domain), EF (EF-hand domain), X and Y (core catalytic domains, i.e. TIM barrel), C2 (calcium and lipid binding domain), CT (C-terminal domain), RA1 and RA2 (Ras-associating domains).

Note: CT domains contain PDZ binding motifs and are unique between PLCβ and PLCη. PLCη contains alternatively sliced variants lacking this motif.

isozyme specific regulation in distinct membrane regions[206]. PLCγ isozymes contain two PH domains, the second of which is split by a number of src homology domains (SH). SH2 domains are uniquely responsible for targeting of these isozymes to phospho-tyrosine residues and are involved in their activation by receptor tyrosine kinases (RTKs), while the SH3 domain binds with high affinity to proline rich sequences in other proteins, including actin[207]. PLCβ and PLCη isozymes contain a carboxy terminal (CT) region rich in basic residues. This unique domain has been found to be important for interaction with PDZ domains on membrane scaffolding proteins, for nuclear import and export, as well as for specific activation of PLCβ isozymes by GPCRs and G-protein subunits[208-210]. Unique to PLCε are two C-terminal Ras-associating domains and an N-terminal guanine nucleotide exchange domain, both believed to be important for the unique regulatory mechanisms governing the activity of this isozyme[211]. Despite the unique domains associated with different PLC isozyme families, there is a high degree of conservation among active site residues; therefore, it is not surprising that they all act predominantly on just two substrates, PIP and PIP_2, with some catalytic activity toward PI[193]. As a result, it is likely that PLC isozymes achieve selectivity in their physiological functions by differential regulatory mechanisms as well as by different tissue and cellular distribution.

2. Tissue Distribution and Subcellular Localization

Differences in tissue distribution provide a mechanism for diversity in the physiological functions of PLCs, some of which have been confirmed by transgenic studies in knockout mice (Reviewed in section 4). Of the PLCβ isozymes, β1 and β3 are the most widely expressed, at particularly high levels in specific regions of the brain[197, 212-216], with PLCβ3 also reported to be highly expressed in parotid gland and kidney[217, 218]. PLCβ2

appears to be expressed primarily in hematopoeitic cells of the immune system[217, 219-221], while PLCβ4 expression is restricted to specific regions of the brain, *e.g.* cerebellum and retina[222, 223]. Of the PLCγ isozymes, PLCγ1 is ubiquitously expressed, particularly high in the brain and lungs[212, 224], while PLCγ2, although expressed quite widely throughout the body as well, appears to be most abundant in cells of hematopoietic origin[212, 225, 226]. PLCε expression is highest in the heart, with expression also high in both the lung and kidney[227]. Of the PLCδ isozymes, δ1 is the most widely expressed, with highest expression in the heart, lung, skeletal muscle, brain, and testis[212, 228]; however, tissue levels of this isozyme appear to be relatively low in comparison to PLCβ and PLCγ[215]. Surprisingly, relatively little is known with respect to tissue distribution of PLCδ3 and PLCδ4 isozymes, although their expression has been reported in a number of rat tissues[229]. More recently identified PLCζ has been reported to be sperm specific[230, 231], while PLCη isozymes have thus far been exclusively found in neurons[195].

Differences in subcellular localization of PLC isozymes may also provide perspective with respect to diversity in individual functions of different isozyme families. Upon activation, every PLC isozyme generates two products, IP_3 and DAG. Conventional dogma suggests that cytosolic IP_3 leads to increased intracellular calcium levels via receptor binding at the endoplasmic reticulum, implying little possibility for downstream selectivity. More recently however, the identification of IP_3 receptors at other cellular locations[232, 233], as well as the sequential formation of inositol polyphosphates within the cytosol [187-189] implies a multitude of diversity for mediating numerous discrete downstream signaling events. DAG remains in the membrane and activates conventional and novel PKC isoforms. Activation of localized signaling complexes that includes a receptor, a PLC isozyme and an associated

PKC isozyme could also produce a mechanism for selective downstream signaling. Very recently, a signaling complex including EGF receptor, PLCγ1, and PKCε has been reported, providing direct evidence for such a hypothesis[234]. Therefore, differences in subcellular localization are likely a key component for selectivity between isozyme-dependent downstream signaling pathways, a hypothesis supported by sequence variability in PLC PH domains, which are thought to mediate membrane binding as well as protein-protein interactions. The cellular localization of most PLCs has typically been reported to be primarily cytosolic in the resting state, and following receptor activation, they are then transiently recruited to the cell membrane via isozyme specific interactions of their PH domains with lipid membranes[206, 235]. For example, in wild-type Caco-2 cells PLCγ1 is almost exclusively recovered in the cytosolic fraction; however, following EGF treatment and PLC activation, PLCγ1 is instead found entirely within the membrane fraction[236]. PLCγ also contains SH3 domains that bind proline-rich motifs on proteins; interestingly, ZO proteins contain proline-rich sequences that could bind and localize PLCγ to the tight junction. Although no reports exist that specifically colocalize PLCγ with junctional complexes, these isozymes have long been reported to colocalize with cortical actin filaments[207, 237], which line the cell periphery and link the cytoskeleton directly to the tight junction and neighboring cells. Recent studies further establish PLCγ interaction with cortical actin by demonstrating an SH2 mediated binding with the actin regulatory protein villin[238, 239], a protein expressed exclusively in epithelial tissues, including the intestine.

Evidence is emerging that PLCβ isozymes may be co-localized to the membrane with other signaling components. Most PLCβ isoforms are strongly associated with the membrane/particulate fraction, which is interesting since these enzymes bind phospholipids

weakly *in vitro*; therefore, other proteins[204] and/or the cytoskeleton[240] may organize these isozymes with other signaling proteins at the membrane. For example, recent reports suggest that scaffolding proteins containing PDZ domains play a significant role in the regulation of mammalian PLCβ isozymes. PDZ-containing proteins mediate the clustering of receptors and signaling molecules and thereby regulate agonist-induced signal transduction in polarized cells[241]. Interestingly, PDZ domains are found in ZO proteins of the tight junction, and a recent study has reported binding of PLCβ3 to ZO-1 in rat fibroblasts[209], providing direct evidence that PLCβ isozymes may be localized to junctional complexes. Consistent with this recent report, another reported component of PLCβ signaling, $G\alpha_{12}$, is co-localized with the tight junction in Caco-2 and MDCK cells[242], providing evidence for the likely presence of PLCβ signaling complexes localized in the vicinity of tight junctions.

An abundance of evidence has also clearly demonstrated localization of PLCβ isozymes to the nucleus, likely mediated via a nuclear localization signal sequence in their unique carboxy terminal extension[208], where they play a key role in cell proliferation and differentiation[243-246]; their presence at the nuclear membrane indicates diverse roles for these isozymes that may be mediated by differential regulation, as there is no evidence that G-protein subunits that regulate these isozymes at the cell membrane are expressed in the nuclear compartment. The role of PLCβ isozymes in nuclear signaling has been recently reviewed[247, 248]. While all four members of the PLCβ family have been localized both inside and outside the nucleus, the subcellular localization of PLCδ isozymes appears to be more specific. PLCδ4 is thought to be primarily nuclear[249], while PLCδ1 and PLCδ3 possess and nuclear export sequence within the EF-hand domain, implying they may shuttle between the nucleus and cytoplasm. Further, PLCδ1 has been reported to be mostly cytoplasmic, while

PLCδ3 is found in membrane fractions[250-253]. Although neither PLCγ isozyme contain a known nuclear signal sequence, they too have been reported to be localized to nuclei of certain cell types[243].

3. Differential and Overlapping Regulation of PLC Isozymes

The major differences in the core domain structure of PLC isozyme families are primarily reflected in their modes of regulation. A complete picture of how each isozyme family is controlled and regulated is far from complete; recent evidence has begun to emerge suggesting a higher level of regulation than previously believed, including cross-talk and regulation between distinct PLC isozyme families[196].

PLCβ isozymes are regulated via G-protein coupled receptors (GPCR) of the Gq family. Upon occupation of GPCR by an agonist, the α-subunit of the heterotrimeric G-protein exchanges GDP for GTP and dissociates from the βγ-dimer. The α-subunit, and for some isozymes the Gβγ dimer, selectively activate PLCβ isozymes without any effect on others, such as PLCγ[254, 255]. PLCβs contain a unique, highly basic CT domain which promotes membrane association and is required for activation by Gα subunits[210]. Interestingly, this family of PLCs may contribute to self-regulation of its activation via "feedback deactivation" of GPCRs. PLCβs associate with Gαq and function as GTPases themselves[256], thus controlling the amplitude and duration of their own signaling.

PLCγ isozymes are regulated through RTKs via binding of hormones such as EGF to their corresponding receptors[257]. Agonist binding leads to dimerization of receptor subunits, stimulation of intrinsic tyrosine kinase activity of the receptor, and phosphorylation of specific tyrosine residues that serve as high affinity binding sites for src homology 2 (SH2) domains of many effector molecules, including PLCγ isozymes. The association of PLCγ

isozymes with the activated receptor results in phosphorylation of specific tyrosine residues on the lipase itself[258], leading to activation of PLCγ and subsequent substrate hydrolysis. Conceivably, both the phosphorylation and the physical association to the activated receptors are required to target PLCγ isozymes to the membrane[198]. The complete activation of these isozymes require phosphatidylinositol 3,4,5-triphosphate (PIP_3) which contributes to membrane binding of PLCγ via its PH domain. Interestingly, PLCγ isozymes also appear to be directly activated by phosphatidic acid[259, 260] a product formed from DAG as a result of DAG kinase activity, as well as unsaturated fatty acids such as arachidonic acid[261].

PLCε is uniquely regulated by $G\alpha_{12/13}$ or Gβγ subunits of heterotrimeric G-proteins as well as by both Ras and Rho GTPases[262-265]. These findings have increased the awareness of potential cross talk between PLC isozymes mediated by heterotrimeric and small monomeric GTPase signaling pathways within the cell[211]. Additionally, stimulation of cells with EGF, which increases cellular concentration of GTP-Ras, induces translocation of PLCε from the cytosol to the cell membrane[264], and presumably stimulates its activity. Thus, receptor stimulated PLC activity may include activation of multiple downstream PLCs in discrete patterns leading to defined physiological responses via specific downstream effector proteins.

Regulation of other PLCs is not as well studied. A much higher sensitivity of PLCδ isozymes to intracellular calcium concentrations suggests that their activation may occur indirectly via increases in intracellular calcium, and they may therefore serve to prolong receptor initiated PLC signaling[198, 250, 266, 267]. In addition, α1-adernergic and oxytocin receptors appear to regulate PLCδ1 activity via a GTP binding protein, known as high molecular weight G protein (G_h), which was found to form a complex with PLCδ1[268]. Sperm specific PLCζ has also been recognized as highly sensitive to calcium concentrations, and

plays a crucial role during fertilization; the third EF hand of this isozyme appears responsible for its high sensitivity to calcium (*i.e.* resting cell concentrations are sufficient for activation)[230, 269]. Injection of this isozyme into the oocyte by sperm initiates calcium oscillations that serve as the trigger for initiation of embryonic development[230, 231]. The most recently identified PLCη isozymes are thought to be neuronal specific and serve an important role in postnatal brain development[195]. They have also been recognized as highly sensitive to calcium concentrations[270], although co-expression of PLCη2 with Gβγ leads to increased activity *in vitro*, implicating GPCRs in the regulation of PLCη as well[271]; a hypothesis that is consistent with the existence of a CT domain in these isozymes, which is thought to be the domain responsible for Gβγ-mediated regulation of PLCβ isozymes. Cleary, diverse and overlapping regulatory mechanisms provide the potential for diversification in function, as well as opportunity for crosstalk, among thirteen PLC isozymes with the same limited set of endogenous substrates.

4. Cellular and Physiological Functions of PLC Isozymes

Due to the lack of specific chemical inhibitors for the numerous PLC isozymes, studies in transgenic mice have provided the most convincing evidence surrounding the physiological functions of PLCs. A number of PLCβ1 knockout mice models have been reported[213, 272-274], each of which has highlighted the importance of this isozyme in the maintenance and development of signaling pathways in the cerebral cortex and hippocampus via muscarinic and glutamate receptor signaling pathways, with reported phenotypes consistent with behavioral abnormalities observed in schizophrenia. Interestingly, mutation of this isozyme in mouse sperm reduced both fertilization and embryonic development rate, implicating PLCβ1 in reproductive events as well[275]. Although PLCβ2 is highly expressed in

hematopoietic cells, knockout of this isozyme had no impact on hematopoiesis; however, studies have demonstrated that PLCβ2 may be important in chemoattractant-induced signal transduction responses[276]. Knockout of the gene coding for PLCβ3, a widely expressed isozyme, produced an embryonic lethal on day 2 in which the blastocoel failed to develop[277], although a second study reporting PLCβ3 deficiency did not lead to embryonic lethality[216]. Instead, these mice demonstrated altered sensitivity to opioids such as morphine, implicating these isozymes in opioid mediated responses in the brain, similar to reported effects in one study with PLCβ1 deficient mice[278]. Such dramatic differences in phenotype between these two PLCβ3 knockout mice may be related to the differences in constructs used between the two studies, *i.e.* Wang *et al.* deleted exons 11-17 of the PLCβ3 gene, while Xie *et al.* only partially deleted one exon; this discrepancy needs further evaluation. Similar to knockout of PLCβ1, disruption of the gene for PLCβ4 leads to disruption of inhibitory pathways in the brain, particularly in the cerebellum, leading to ataxia and severe visual deficiencies[213, 223, 279], consistent with its reported expression in the retina. The extensive body of literature regarding knockout studies for PLCβ isozymes implies a critical role for this family in the developing brain.

Knockout of PLCγ1 in mice leads to embryonic lethality by day 9, demonstrating an absolute requirement for this isozyme in embryonic development[280], in agreement with its reported ubiquitous expression[197, 212]. Consistent with reports that PLCγ2 expression is largely restricted to cells of hematopoietic lineage, knockout of this isozyme does not lead to embryonic lethality, but rather developmental abnormalities in B cells leading to severe immunodeficiency and platelet disfunction[226, 281]. Specific knockout of PLCδ1 led to significant abnormalities in the skin, including progressive hair loss, epidermal hyperplasia,

and cyst development, suggesting an important role for this isozyme in epithelial tissue[282, 283]. Combined knockout of PLCδ1 and PLCδ3 was embryonic lethal at day eleven with abnormal proliferation and apoptosis of trophoblasts[284]. Disruption of PLCδ4 indicated roles in calcium oscillations in sperm[285] as well as in liver regeneration after partial hepatectomy[286]. Consistent with the high reported expression of PLCε in the heart, disruption of this isozyme in mice leads to significant abnormalities in cardiac function, specifically related to β-adrenergic[287] and EGF receptor[288] stimulation. Interestingly, no mouse knockout models of PLC isozymes have reported significant intestinal malfunctions that may implicate these isozymes in the regulation of barrier function in this organ, although no investigators have specifically assessed changes to any phenotypes related to epithelial barrier function in the gut in any of these knockout studies.

More recent studies in cellular knockdown models using antisense oligonucleotides or RNA interference (RNAi) techniques have provided more discrete and specific functional detail on the cellular roles of individual isozymes. Early studies with inducible antisense oligonucleotides targeting entire PLCβ, PLCγ, or PLCδ isozyme families in rat hepatocytes demonstrated differences in the roles of each isozyme family in cell proliferation (*i.e.* γ > δ > β)[289]. An abundance of additional studies over the last ten years have supported an essential role for PLCγ1 in facilitating cell proliferation, adhesion, migration, and motility, in many cases via interactions with actin and its binding partners[236, 239, 290-294], and consistent with the embryonic lethality observed in knockout mice[280]. Interestingly, knockdown of PLCε in an epithelial breast cell line also abrogated cell spreading via Ras dependent mechanisms[295] supporting the hypothesized cross-talk between the regulation of PLCγ and PLCε isozymes [264]. Specific knockdown of PLCζ supports its proposed role as the physiological trigger of

calcium oscillations required for the activation of early embryonic development[296]. Recently, RNAi mediated depletion of PLCβ3 in rat fibroblasts revealed a novel function for this class of isozymes in cell-cell communication via gap junctions[209]; thus, knockdown of this isozyme abrogated GPCR stimulated closure of gap junctions. It is likely that the continued application of genetic knockdown techniques in cell lines derived from various tissues will continue to reveal novel and complex functions of each PLC isozyme in different organs.

5. Role of PLC in the Regulation of Tight Junction Barrier Function

The PLC-dependent pathway has long been postulated as a regulatory pathway involved in the regulation of cellular junctional complexes[297-299]. Evidence in the literature implicates this well known signal transduction cascade in the assembly and biogenesis of tight junctions. For example, inhibition of the PLC-dependent pathway attenuates the assembly of tight junctions in MDCK cells[300, 301], while activation of PLC by thyrotropin-1 releasing hormone stimulates its assembly in the same cell line[297]. Activation of the downstream effector PKC with 1,2-dioctanoylglycerol, an analog of DAG, also stimulates tight junction assembly, while inhibition of this enzyme prevents the assembly[297]. Furthermore, lowering intracellular calcium levels inhibits the assembly of the tight junction[302]. Developmental studies also suggest a role for PLC in the assembly of junctional complexes; knockout of both PLCβ3 and PLCγ in mice produces an embryonic lethal mutant where development is severely disorganized[277, 280]. In drosophila, PLCγ is expressed predominately in the blastoderm cell[303], and lack of this PLC isozyme causes a developmental disorder known as small wing[304]. In general, tight junction formation is essential for early embryonic development[305], and PLC may play a crucial role in

development processes, a hypothesis supported by the numerous developmental defects observed in the various knockout mice that have been generated for different PLC isozymes.

The majority of literature that supports a role for PLC in the regulation of established tight junction barrier function is indirect, and often based on modulation of downstream effectors of PLCs, *i.e.* IP_3 and ensuing changes to intracellular calcium levels, as well as DAG and subsequent activation of PKC isozymes. For example, increased intracellular calcium activates the calmodulin-dependent kinase. Both PKC and the calmodulin-dependent kinase phosphorylate and alter myosin light chain kinase activity, which subsequently phosphorylates myosin light chain and induces contraction of the actin myosin ring, a mechanism previously postulated to alter tight junction permeability[172, 306]. The increase in tight junction permeability caused by medium chain fatty acids, such as capric and lauric acid, has been attributed to this mechanism, as chelation of cytosolic calcium and inhibition of myosin light chain kinase attenuated the observed increase in tight junction permeability caused by these two fatty acids[298]. However, there is conflicting evidence with respect to the effect of increased intracellular calcium levels on the tight junction barrier. For example, although treatment of T84 cells with the calcium ionophore, A23187, increased the permeability of mannitol across the monolayer[307], treatment of MDCK cells with the calcium ionophore, ionomycin, had no effect on the permeability of mannitol[308]. Interestingly though, when PKC activators were added to MDCK cells in combination with ionomycin, mannitol permeability was significantly increased, implicating a synergistic effect of both increased calcium and activated PKC, both the end result of PLC activation, on the permeability of tight junctions in MDCK cells. In support of a role for downstream effectors of PLC on tight junction barrier function, the type 3 IP_3 receptor, generally believed to be

localized specifically to the endoplasmic reticulum, has recently been localized to tight junctions in polarized MDCK cells, directly implicating IP_3, an end product of PLC substrate hydrolysis, in tight junction function[232, 233].

The role of PKC in the regulation of tight junction function also seems somewhat controversial. Experimental evidence suggests that activated PKC isozymes promote the assembly of tight junctions. For example, PKC-specific inhibitors calphostin C[177] and GF109203X[178] prevent TJ formation, while PKC agonists such as 1,2-dioctanoylglycerol and phorbol-12-myristate-13-acetate (PMA) induce the formation of tight junction fibrils in MDCK cells maintained in low calcium media[178, 309]. On the other hand, a number of studies have suggested that activated PKCs actually impair barrier function, consistent with the effects of increased calcium. Prolonged treatment of epithelial cells with PMA results in breakdown of the barrier, which can be blocked by the overexpression of kinase-inactive mutants of PKCα[310]. A number of biologically active agents like clostridium difficile toxin A[311], thrombin[312], and tumor necrosis factor-α[313] also seem to impair barrier function via PKC dependent pathways. Thus, various PKC inhibitors are reported to both impair tight junction assembly and function[177, 178, 297] as well as to promote tight junction assembly and function[174]. More recent studies have begun to clarify this apparent contradiction. There are at least eleven PKC isozymes differing in structure, substrate requirement, and cellular localization that are involved in the regulation of numerous cellular functions. PKCs are divided into three subfamilies: (i) conventional PKCs (α, βI, βII, γ) which are calcium dependent and activated by both phosphatidylserine and diacylglycerol, (ii) novel PKCs (δ, ε, η, θ) which are activated by both phosphatidylserine and diacylglycerol, but are not dependent on calcium, and (iii) atypical PKCs (ζ, λ, PRK for PKC-related kinases), which

are insensitive to both co-factors. Recent evidence suggests that the assembly of tight junctions is regulated by opposing actions of novel and conventional PKC isoforms[314]. Conventional PKC isoforms seem to downregulate tight junction assembly and maintenance, whereas novel PKC isoforms seem to support tight junction assembly and maintenance. Another recent report is in full agreement with these findings, as the novel PKCθ isozyme was found to be crucial for the maintenance of a functional tight junction barrier in Caco-2 cells[315]. Compelling experimental evidence suggests that atypical PKCs may also directly regulate TJ assembly by binding to the proteins PAR3 and PAR6[316], which regulate late stages of tight junction biogenesis[317]. However, since atypical PKCs are not directly activated by diacylglycerols, it is less likely that they are downstream effectors of PLC action.

Upstream regulators of PLC activity have also been reported to have effects on tight junction barrier function. As described previously, the activity of PLCβ and PLCγ isozymes are regulated upstream via GPCRs and RTKs, respectively. A number of studies have demonstrated changes to the barrier function of tight junctions in response to a number of known agonists of these receptors. For example, treatment of cells with PDGF[154], EGF[155], and vascular endothelial growth factor (VEGF)[318-320] have all been shown to modulate tight junction proteins and barrier function. Each of these growth hormones are endogenous agonists of their respective RTKs, *i.e.* PDGFR, EGFR, and VEGFR, each of which have been previously reported to be coupled to PLCγ isozymes[321-324]. A recent study has revealed that EGFR, PLCγ1, and PKCε may form the basis for a signaling complex at the plasma membrane[234], consistent with the reported effects of these two effector proteins, both upstream and downstream of PLCγ, on tight junction permeability. Another recent study found that PKCε phosphorylation of the cytoplasmic tail of claudin-4 was associated

increased paracellular permeabililty in ovarian cancer cells[179], providing an indirect link between PLCγ1 and claudin-4 at junctional complexes. In addition to growth factor stimulated changes to tight junction function, another study reported that nucleotides, ATP and ADP, substrates of P2Y GPCRs that couple to PLCβ isozymes [325, 326], increase the paracellular permeability of excised rat intestine, indirectly implicating PLCβ signal transduction in the regulation of tight junction permeability[152]. Interestingly, two recent studies have directly implicated PIP_2, the preferred substrate of PLC isozymes, in regulation of cell-cell junctions[209, 327]. A role for PIP_2 itself in mediating regulatory signal transduction within the cell has begun to emerge and is a rapidly growing area of research. This lipid target of PLCs has been shown to be important in actin filament assembly and remodeling, including the uncapping of barbed ends and the severing and bundling of filaments[328, 329], as well as in the regulation of a number of ion channels[330-332]. Clearly, indirect experimental evidence over the last fifteen years suggests a role for PLC signaling in the regulation of both the assembly and maintenance of the barrier function of both endothelial and epithelial tight junctions.

Recently, more direct evidence has emerged supporting a role for PLC isozymes in regulation of tight junction barrier function[333, 334]. These studies demonstrated that the potency of previously reported PLC inhibitors, *i.e.* alkylphosphocholines[335, 336], U73122[337], and 3-nitrocoumarin[338], is strongly correlated to their potency as paracellular permeability enhancers in MDCK cells, implicating inhibition of PLC as a mechanism for increasing paracellular permeability. Significant alterations to the actin cytoskeleton were observed following specific inhibition of PLCβ by alkylphosphocholines and U73122[334]. On the other hand, increases in the phosphorylation of ZO-1, ZO-2, and occludin were observed

concomitantly with increased tight junction permeability following specific inhibition of PLCγ by 3-nitrocoumarin[333]. Significantly, the increase in ZO-2 phosphorylation could be reversed, along with an attenuation of the increase in tight junction permeability, by pre-treating cells with the general serine/threonine kinase inhibitor staurosporine, suggesting a potential cause-effect relationship between ZO-2 phosphorylation and changes in tight junction function. These results implicate discrete downstream signaling events associated with selective inhibition of different PLC isozyme families, and more importantly, provide direct evidence for a role for these signaling enzymes in the regulation of epithelial tight junction barrier function.

E. Application to Drug Delivery

While the intestinal epithelium provides a protective barrier to the entry of intestinal pathogens, it also regulates and restricts the absorption of orally administered drugs and macromolecules. The ability to dose therapeutics orally confers a number of advantages when compared to alternative routes, such as parenteral administration. These advantages include easier administration and the avoidance of pain and discomfort associated with injectable formulations (which typically leads to increased patient compliance), prevention of possible infections due to reuse of needles, as well as less complicated and less expensive development strategies for pharmaceutical companies due to the lack of necessary sterile formulations.

For a hydrophilic drug or macromolecule that is not recognized by a carrier, oral absorption is poor because the drug cannot partition into the hydrophobic membrane and traverse the epithelial barrier via the transcellular pathway, and transport via the paracellular pathway is severely restricted by the very low surface area available for transport, as well as

the presence of tight junctions (Figure 1.1, page 4)). For example, the broad spectrum antibiotic cefoxitin and the antiviral zanamivir, both hydrophilic drug molecules, have oral bioavailabilities less than 5%, precluding their use in conventional oral formulations and limiting their clinical utility. Strategies to develop oral formulations for hydrophilic drug candidates typically include chemical modifications that provide increased lipophilicity to promote increased transcellular permeation, or that provide increased affinity for transporters to promote carrier mediated absorption (*i.e.* prodrugs). Unfortunately, structural changes often lead to reduced potency towards the intended pharmacological target, necessitating complete release of the prodrug moiety from the parent drug, and complicating drug design and the drug development process. Furthermore, this strategy is not typically useful for macromolecules such as insulin, heparin, or salmon calcitonin, as their polar surface area is simply too large to mask with a small chemical modification. An alternative approach to improve the oral absorption of hydrophilic drugs is to transiently alter the physical barrier imposed by the intestine, through controlled and reversible opening of tight junctions. This approach has a distinct advantage over the development of prodrugs, as it would be universally applicable to all hydrophilic drugs and macromolecules; however, it has generally been met with significant resistance in the pharmaceutical industry. A major concern stems from the fact that many intestinal pathological disorders are characterized by leaky epithelia, including inflammatory bowel diseases such as Crohn's disease and ulcerative colitis[339], as well as intestinal cancers[340]; therefore, there is a general fear that tight junction modulation is an approach to increase paracellular permeability that may lead to pathological outcomes, and therefore, should not be considered as a pharmacological approach. This fear is partly substantiated by the fact that a number of bacterial enterotoxins have considerable effects on

tight junction function. For example, clostridium perfringens enterotoxin (CPE)[341] and zonula occludens toxin (ZOT)[342] cause food poisoning and diarrhea in humans, effects thought to be mediated by their specific modulation of tight junctions[343, 344]. Interestingly however, specific amino acid sequences of these two toxins have been shown to be responsible for the observed effects on tight junction function (*i.e.* c-CPE and ΔG respectively), and when administered as small peptides, lack the observed toxicity associated with each enterotoxin, suggesting that tight junctions can be modulated without significant adverse events. Currently, these two peptides represent the most promising PPEs identified to date[345, 346]. A second major concern regarding the use of PPEs in humans stems from a general lack of understanding about how mature epithelial tight junctions are formed and regulated; however, in recent years, significant advancement in our understanding of how the tight junction is organized and regulated physiologically have made this approach to increasing oral absorption an achievable reality, and have begun to ameliorate these safety concerns.

Unfortunately, there has not yet been a safe and effective PPE approved for clinical use in humans in the United States. Many of the known PPEs have been found to lack selectivity, *i.e.* there is little separation between their potency as permeability enhancers and their potency to cause local intestinal toxicity, suggesting that their enhancement effect may be the result of erosion of the intestinal epithelium. The PPEs with these characteristics are typically amphiphilic, and their physicochemical properties suggest a propensity to accumulate in cell membranes, a characteristic that likely contributes to the lack of separation between efficacy and toxicity (Liu et al., 1999). Bile acids[347], fatty acids[298], acylcarnitines[348], alkylphosphocholines[334], and glyceride analogs[349] all fall into this category of PPEs. Most of these molecules were identified empirically, and their discovery was not necessarily based on

a strategy of rationale drug design, likely due to a lack of validated targets known to regulate tight junction function. Recent advances surrounding the biochemistry and physiology of junctional complexes have led to renewed interest in these complex subcellular structures, and continued research in this area should soon provide a number of pharmacological targets to realize the potential for transient and reversible opening of intestinal tight junctions to allow increased absorption of orally administered therapeutics.

F. Rationale for Proposed Research

The underlying molecular mechanisms regulating the barrier function of epithelial tight junctions and paracellular permeability are poorly understood. There exists an abundance of literature over the past fifteen years implicating PLC signal transduction as a potential component of the regulatory mechanisms governing both the assembly and maintenance of tight junction function; however, it is unclear which of the identified thirteen PLC isozymes, if any, are important. The majority of evidence supporting a hypothesis for PLC involvement has been developed indirectly by modulating upstream receptors and/or downstream effectors of these signaling molecules. Further, conflicting evidence exists with respect to how PLCs may be involved in regulating cell-cell junctions, *i.e.* some studies suggest that increased activity leads to tight junction modulation, while others imply that inhibition of activity alters tight junction function. It may be that different PLC families function in concert but with opposing function, akin to the recently proposed action of PKCs[314], *i.e.* one family supports assembly of tight junctions while another supports disassembly. Nonetheless, additional studies are clearly necessary to further understand the importance of PLC in epithelial tight junction regulation.

The overall goal of this research was to develop a clearer understanding of the molecular mechanisms involved in the regulation of intestinal epithelial tight junctions via the PLC catalyzed signal transduction cascade. The proposed research was intended to test the following hypothesis:

Phospholipase C isozymes are involved in the regulation of intestinal epithelial paracellular permeability. Inhibition of phospholipase C activity causes an increase in paracellular permeability across epithelial tissues via specific modulation of the tight junction.

As little information has been reported with respect to the expression of PLCs in the human small intestine, studies were designed in the first aim of this research to address this deficiency by evaluating the longitudinal mRNA expression of PLCs in this organ. Additional studies were designed to establish a relationship between the catalytic activity of PLC and the specific function of intestinal tight junctions by using a well established *in vitro* model for the human intestine, Caco-2 cells. Initially, the expression of PLCs in these cells was compared to that in the human small intestine, with the goal of establishing this cell line as an appropriate model in which to study the proposed hypothesis. Following this assessment, the potency of a homologous series of alkylphosphocholines (APCs) as well as U73122, drug molecules previously utilized to implicate PLC in tight junction function in MDCK cells, to both inhibit PLC activity and increase paracellular permeability was evaluated. The purpose of these studies and intended contribution to the literature was to provide direct evidence for a role for PLC in tight junction regulation in the human intestine.

The rationale for these initial studies was based on recent reports in MDCK cells demonstrating a strong relationship between PLC activity and tight junction function using a

number of previously reported chemical inhibitors of these isozymes (*i.e.* APCs and U73122). There exists some evidence in the literature suggesting that these purported specific inhibitors may exert off-target effects on cultured cells, raising some concern with regard to the observed relationship between PLC inhibition and tight junction function. Therefore, a second aim of this research was to evaluate the direct interaction of these reported inhibitors with specific PLC isozymes in a simple, cell-free system. The purpose of these studies was two fold: (1) to confirm that these inhibitors inhibit the catalytic activity of PLC, and (2) to obtain a clear understanding of their interaction with PLC at the molecular level. These studies would clarify previous reports that have questioned the specificity of these compounds towards PLC, and build evidence to support a hypothesis for inhibition of PLC activity as a molecular mechanism to increase epithelial paracellular permeability.

In recent years, the advent of techniques such as RNAi have provided the opportunity to more directly assess the functions of cellular proteins with diminished concerns for the potential lack of specificity of small drug molecules. The use of a genetic knockdown approach was critical for assessing the existence of a direct cause-effect relationship between PLC inhibition and increased paracellular permeability. Therefore, the final aim of this research was to suppress the expression and activity of individual PLC isozymes alone and in combination using RNAi, and to assess the impact of this suppression on the barrier function of tight junctions. Studies were designed to independently assess the effect of knockdown on both the assembly of tight junctions, as well as on the barrier function of already formed tight junctions. This approach allowed a more direct analysis of the role specific PLC isozymes play in the regulation of epithelial tight junction function.

The overall goal of this dissertation research was to test the hypothesis that a direct cause-effect relationship exists between the inhibition of PLC activity and increased paracellular permeability via specific modulation of epithelial tight junctions. This goal is of considerable interest, as PLC isozymes have been postulated for many years as potential regulatory enzymes controlling numerous cellular functions, including tight junction function[297-299, 333, 334, 350]; however, convincing evidence to support such a role for any PLC isozyme has yet to be established unequivocally.

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CHAPTER 2

Phospholipase C Inhibitors U73122 and Alkylphosphocholines Increase Paracellular Permeability in a Human Intestinal Epithelial Cell Line

ABSTRACT

Phospholipase C (PLC) isozymes comprise a large family of key cell signaling proteins that hydrolyze inositol containing phospholipids to form two intracellular second messengers, diacylglycerol (DAG) and inositol triphosphate (IP_3). Recently, PLCβ isozymes have been implicated in the regulation of epithelial paracellular permeability in Madin-Darby canine kidney (MDCK) cells, identifying these enzymes as potential pharmacological targets for increasing the permeability of hydrophilic drugs and macromolecules across epithelial cell monolayers, such as those in the small intestine. The specific expression pattern of these enzymes in human intestine and their role in regulating cellular signaling processes in this organ however, remain largely undefined. The purpose of this study was to evaluate the gene expression of phospholipase C isozymes in Caco-2 cells, an *in vitro* cellular model for the human intestinal epithelium, and to compare the expression levels in this cell model to those observed in the human gastrointestinal (GI) tract. Further, studies intended to specifically implicate PLCβ isozymes in the regulation of human intestinal paracellular permeability were performed. mRNA was detected for six different PLCs in Caco-2 cells, including PLCβ1, β3, δ3, ε, γ1, and γ2. Western blots confirmed expression of PLCβ1, β3, γ1, and γ2 proteins in Caco-2 cells, but not of δ3 and ε. Transcripts for all six of these PLCs were detected in all regions of the human small intestine. Interestingly, mRNA levels were as much as 100-fold lower along the small intestine relative to Caco-2 cells. The potency of a series of homologous alkylphosphocholines (APCs) to inhibit PLCβ activity varied forty five fold and correlated significantly with potency to enhance paracellular permeability across Caco-2 cells, suggesting that inhibition of PLCβ activity is associated with increased paracellular permeability in epithelial cells. Further, structurally unrelated PLC inhibitor,

U73122, also increased paracellular permeability and inhibited PLCβ activity in Caco-2 cells. In summary, the data describe the expression pattern of PLC mRNA in human tissues as well as in Caco-2 cells, an *in vitro* cell line derived from human colon, and results suggest that Caco-2 cells represent a suitable model with which to evaluate the role of PLC in regulating tight junction barrier function in the human intestine. Further, they support the hypothesis that inhibition of PLCβ activity leads to increased paracellular permeability.

INTRODUCTION

Phospholipase C (PLC) enzymes comprise a family of key proteins involved in the cellular turnover of inositol containing phospholipids. These enzymes cleave the polar head group from membrane lipids such as phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), intracellular second messengers that mobilize intracellular calcium and activate protein kinase C enzymes, respectively. To date, thirteen human PLC isozymes have been identified comprising six distinct and differentially regulated families (β1-4, γ1-2, δ1,3-4, ε, η1-2, and ζ)[1-3]. They vary in molecular size from the ~70kDa PLCζ to the much larger ~250kDa PLCε, and share little sequence similarity even within isozyme families. PLC enzymes do share a common core domain structure which includes the EF domain, C2 domain, and the highly conserved catalytic core made up of two regions, commonly referred to as X and Y domains[4, 5]. Given the high conservation of active site residues within all PLCs, it is not surprising that they all act predominantly on just two substrates, PIP and PIP_2, with some catalytic activity towards PI[6]; therefore, PLC isozymes likely achieve selectivity in their physiological functions by differential regulatory mechanisms. PLC signaling has been implicated in a number of critical cellular functions such as motility[7], migration[8], growth and differentiation[9, 10], as well as in the assembly and regulation of cell-cell junctions[11-13].

Neighboring epithelial cells are fused to one another via multi-protein junctional complexes near the apical membrane, consisting of tight junctions, adherens junctions, and desmosomes. The tight junction, the most apical component of junctional complexes, is generally considered the major barrier to the passage of ions, solutes, and water through the space between adjacent epithelial cells, as it forms a charge and size selective barrier within

the paracellular space. In the human intestine, tight junctions function as a component of the barrier limiting the access of luminal contents, including orally administered drugs, to the systemic circulation. The ability to selectively and reversibly modulate human intestinal tight junctions and increase paracellular permeability would have significant implications in the pharmaceutical industry, providing the opportunity to develop oral formulations for hydrophilic drugs and macromolecules that are typically very poorly absorbed following an oral dose.

A number of recent studies directly link PLC to specific regulatory components of junctional complexes, as PLCβ1 or β3 isozymes bind to Par[14], calmodulin[15, 16], and ZO-1[17]. PLCs have also been implicated in the regulation of established epithelial tight junction function in Madin-Darby canine kidney (MDCK) cells, as chemical inhibition of PLCβ isozymes correlates with increased paracellular permeability, and with alterations to the structural integrity of the cortical actin ring[18]. Extrapolation of the MDCK data to intestinal absorption in humans cannot be assumed however, due to the canine kidney origin of MDCK cells. Caco-2 cells, a cell line derived directly from the human colon[19] and used extensively as an *in vitro* model for the human intestine[20-24], are a more appropriate model in which to evaluate pharmacologically based paracellular permeability enhancement for the purposes of increasing intestinal absorption. Thus, the purpose of the current study was to evaluate the gene and protein expression of phospholipase C isozymes (PLCβ1, PLCβ2, PLCβ3, PLCβ4, PLCγ1, PLCγ2, PLCδ1, PLCδ3, PLCδ4 and PLCε) in Caco-2 cells in a quantitative fashion, and compare the expression levels to those observed along the human gastrointestinal tract, as well as in other organs of drug disposition. Further studies were designed to implicate PLCβ isozymes in the regulation of human intestinal tight junction permeability by

determining the potency of previously reported PLCβ inhibitors, alkylphosphocholines (APCs) and U73122[18], to inhibit agonist induced PLCβ activity and increase paracellular permeability across Caco-2 cell monolayers.

MATERIALS AND METHODS

Materials. Normalized first-strand cDNA preparations, prepared from pooled poly A^+ RNA from various human tissues, were purchased from BD Clontech (Palo Alto, CA) as a part of the Human Digestive System MTC™ panel and Human MTC™ panel I. The tissue sources were free of disease and are presented in Table 2.1. PolyATtract® System 1000 for isolation of mRNA directly from cells was obtained from Promega (Madison WI). TaqMan® universal PCR master mix and high capacity cDNA archive kit were purchased from Applied Biosystems (Foster City, CA). Gene specific pre-designed primer/probes sets for individual PLC isozymes and endogenous control, actin, were purchased from Ambion (Foster City, CA). Antibodies for PLC isozymes and whole cell lysates used as positive controls were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). APCs with various alkyl chain lengths were synthesized previously[18]. N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES), Hank's Balanced Salt Solution (HBSS), and other cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Twelve well Transwells™ were obtained from Costar (Cambridge, MA). D-[1-^{14}C]-Mannitol (55 mCi/mmol) and [2-^{3}H(N)]-myo-inositol (20 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). AG1-X8 formate columns and nitrocellulose membranes were obtained from Bio-Rad Laboratories (Hercules, CA). All other compounds and reagents were obtained from Sigma (St. Louis, MO).

Cell Culture. The Caco-2 cell line, clone HTB-37, derived from human colorectal carcinoma cells[19], was obtained from American Type Tissue Culture at passage 20. Cells were cultured at 37°C in minimum essential medium, supplemented with 10% FBS, 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in

an atmosphere of 5% CO_2 and 90% relative humidity. Cells (passage number 25 to 35) were seeded at a density of 60,000 cells/cm^2 in twelve well Transwells™. Medium was changed the day after seeding, and every other day thereafter (apical volume 0.5 ml, basolateral volume 1.5 ml). Cells were grown to confluence for at least 21 days during which time they differentiated into epithelial cell monolayers as evidenced by the establishment of a stable transepithelial electrical resistance (TEER) between 200-500 $\Omega*cm^2$. Cells were used between 21-27 days after seeding.

mRNA Isolation and cDNA Synthesis. mRNA was isolated directly from Caco-2 cells using the PolyATtract® System 1000 (Promega, Madison, WI) according to the manufacturer's instructions, and was quantified using Quant-iT™ RiboGreen® RNA Assay Kit (Molecular Probes, Eugene, OR). First strand cDNA synthesis was performed with the High Capacity cDNA Archive Kit (Invitrogen, Carlsbad, CA) using 1 μg mRNA. cDNA was quantified using PicoGreen® dsDNA assay kit (Invitrogen, Carlsbad, CA).

Real-time RT-PCR. Pre-designed and validated gene-specific primers and 5'-(FAM)-(TAMRA)-3' labeled TaqMan® probes for PLC isozymes (Assays on Demand) were purchased from Ambion (Foster City, CA). Singleplex or multiplex amplification reactions were performed using TaqMan® universal PCR master mix on a 7500 RT-PCR System (Applied Biosystems). Amplification was performed at 95ºC for ten minutes, followed by forty cycles at 95ºC for fifteen seconds, and 60ºC for sixty seconds. Reactions were run in a volume of 50 μl and contained 5 ng of cDNA. To obtain standard plasmid DNA for PLCβ1, β3, γ1, γ2, δ3, and ε, a partial cDNA fragment of each gene, incorporating the region of primer and probe recognition sequences, was amplified by RT-PCR from Caco-2 cDNA and cloned into the pCR®4-TOPO vector (Invitrogen, Carlsbad, CA). Primers designed for these

amplifications are presented in Table 2.2. Standard curves were constructed by plotting C_t values obtained from real time PCR reactions versus the logarithm of the initial copy number. A 5'-VIC labeled TaqMan® probe and primers set for β-actin was used for quantification of β-actin gene expression in each sample, and served as an endogenous control where noted. Each reaction was measured in quadruplicate and data reported as mean ± ranges.

Absolute quantification of target genes was achieved by calculating copy numbers of individual PLC mRNA transcripts. Calibration curves for each target gene were constructed by serial dilution of known amounts of standard plasmid DNA, the concentration of which was determined using the PicoGreen® dsDNA assay kit (Invitrogen, Carlsbad, CA). Copy numbers were calculated from the mass of standard plasmid DNA and molecular weight of DNA as follows:

$$\text{nmol plasmid DNA} = \frac{\text{ng plasmid DNA}}{(330\ \text{ng/nmol} \bullet 2\ \text{nt/base pair}) \bullet \text{number of base pairs}} \qquad (1)$$

where 330 ng/nmol is the average molecular weight of one DNA base.

$$\text{number of copies} = \text{nmol plasmid DNA} \bullet \frac{6.02 \times 10^{23}\ \text{copies/mol}}{10^{-9}\ \text{nmol/mol}} \qquad (2)$$

Western Blotting. Whole cell lysates were collected by washing cultured Caco-2 cells (day 21-27) three times with PBS+ (supplemented with 1.8 mM calcium chloride), adding 200 µl SDS sample buffer at 55°C, and sonicating each lysate briefly. Lysates were stored at -20°C until analysis by immunoblot using standard protocols. Primary antibodies were rabbit anti-PLCγ1 (1:100), rabbit anti-PLCγ2 (1:100), rabbit anti-PLCβ1 (1:100), and rabbit anti-PLCβ3 (1:500). Secondary antibodies were Alexa Fluor IRDye 700-conjugated donkey anti-rabbit. Imaging of Western blots was performed using an Odyssey detector (LI-COR, Lincoln, NE).

PLCβ activity by cellular assay. The activity of PLC in Caco-2 cells was determined by an adaptation of previously published methods[18, 25]. Cell media was aspirated from both compartments of Transwell™ inserts and replaced with serum-free media containing [^{3}H]-myo-inositol at 2 μCi/ml (4 μCi/well; 0.5 ml apical, 1.5 ml basolateral of inositol-free media) for 12 hours at 37°C. Cell media was aspirated from both compartments of Transwell™ inserts, washed twice with HBSS, supplemented with 10 mM HEPES and 25 mM glucose, pH 7.1 (transport buffer), and pre-incubated at 37°C for 30 minutes. Integrity of the tight junctions was confirmed with the measurement of TEER prior to the experiment. Experiments were initiated by replacing the apical media with 0.5 ml of transport buffer containing a test compound or vehicle, and the cells were incubated for indicated times at 37°C. PLC assays were initiated by replacing transport buffer in both compartments with transport buffer containing 50 mM LiCl, with or without adenosine triphosphate (ATP) to specifically activate PLCβ activity. The cells were incubated at 37°C for 15 minutes to allow accumulation of [^{3}H]-inositol phosphates. Incubations were terminated by aspiration of the transport buffer from both compartments and addition of 0.5 ml boiling 10 mM EDTA (pH 8.0) to the apical compartment. Cell lysates were applied to AG1-X8 formate columns for chromatographic isolation of [^{3}H]-inositol phosphates [26]. The amount of [^{3}H]-inositol phosphates was measured by liquid scintillation counting in a Packard Tri Carb 4000 Series spectrophotometer. Data from each inhibition experiment were normalized to the response observed with 300 μM ATP and were reported as the mean ± standard deviation performed in triplicate. The $IC_{50\ (PLC\beta)}$, defined as the concentration of test compound that causes a 50% decrease in ATP-stimulated PLCβ activity (accumulation of [^{3}H]-inositol phosphates), was determined.

Measurement of paracellular permeability enhancement. TEER and mannitol transport across epithelial cell monolayers are indicators of the paracellular permeability of the monolayer[18, 27, 28]. Tight junctions cause a restriction in the ionic flux across cell monolayers, giving rise to an electrical potential difference, measured experimentally as electrical resistance. Mannitol, a hydrophilic molecule, cannot readily partition into phospholipid membranes and diffuse transcellularly through cells, therefore, it must move across cell monolayers via the paracellular pathway. A decrease in TEER and an increase in mannitol permeability across Caco-2 cell monolayers were used as parameters to measure the efficacy of test compounds as paracellular permeability enhancers (PPEs).

TEER. Cell media was aspirated from both compartments (apical and basolateral) of Transwell™ inserts and replaced with transport buffer. Cell monolayers were pre-incubated at 37°C for 30 minutes and TEER was measured with an EVOM Epithelial Tissue Voltohmeter (World Precision Instruments, Sarasota, FL) and an Endohm-12 electrode to confirm the integrity of the monolayer. Experiments were initiated by aspirating transport buffer from the apical compartment and replacing it with transport buffer (0.5 ml) containing desired test compound or vehicle. Cell monolayers were incubated at 37°C and TEER values were measured at indicated timepoints. Data from each experiment were normalized to the response from the vehicle and were reported as the mean ± standard deviation of three experiments performed in triplicate. The effect of a test compound on TEER was evaluated at several concentrations, and its EC_{50} value, defined as the concentration that caused a 50% decrease in TEER with respect to the untreated control, was determined[18, 27, 28].

Mannitol permeability. Cell media was aspirated from both compartments of Transwell™ inserts and replaced with transport buffer. Cell monolayers were pre-incubated at 37°C for

thirty minutes, and tight junction integrity was confirmed with the measurement of TEER prior to the experiment. Transport experiments were initiated by replacing the apical media with 0.5 ml of transport buffer containing a test compound or vehicle, and [^{14}C]-mannitol at 10 μM. Permeability was monitored by the appearance of [^{14}C]-mannitol in the basolateral side (1.5 ml) during two thirty minute intervals according to the following equation:

$$P_{app} = dQ/dt * 1/(A) * 1/(C_0)\ units = nm/\sec\ (1\ x\ 10^{-7}\ cm/\sec) \quad (3)$$

where dQ/dt is the amount, Q, of mannitol measured in the basolateral compartment at time T. A is the membrane surface area and C_0 is the initial concentration of mannitol in the apical compartment. The amount of [^{14}C]-mannitol transported was measured by liquid scintillation counting in a Packard Tri Carb 4000 Series spectrophotometer. All transport experiments were conducted under sink conditions (transport experiments were designed such that less than 10% of the total amount of [^{14}C]-mannitol was present on the basolateral side at any given time). The permeability of [^{14}C]-mannitol in the presence of a test compound was normalized to that in the vehicle-treated cells and was reported as the mean ± standard deviation performed in triplicate. The EC_{10x}, defined as the concentration of a test compound that causes a ten fold increase in mannitol permeability with respect to the vehicle-treated control[18, 27, 28], was determined for each enhancer of paracellular permeability.

Data analysis. Data are expressed as the mean ± standard deviation from three measurements unless indicated otherwise. Where indicated, statistical significance was assessed using a two sample Student's t tests. Samples were assumed to have an unequal variance; significant differences were assigned at $p < 0.05$. For determination of $EC_{50\ TEER}$ values, the relationship between TEER (% Control) and enhancer concentration was fit to a three parameter Hill equation by nonlinear least-squares regression (WinNonlin, version 4.1).

Estimates were also generated for the sigmoidicity factor (γ) and E_{max}. For determination of EC_{10X} values, the relationship between mannitol permeability and enhancer concentration was fit to a four parameter Hill equation by nonlinear least-squares regression. Estimates were generated for the sigmoidicity factor (γ), E_0, EC_{50}, and E_{max}. The model output was used to estimate the concentration of each enhancer that caused a ten fold increase in the permeability of mannitol. For determination of $IC_{50\ PLC\beta}$ values, the relationship between PLCβ activity (% Inhibition) and enhancer concentration was fit to a four parameter Hill equation by nonlinear least-squares regression. Estimates were also generated for the sigmoidicity factor (γ), un-stimulated PLC activity (E_0), and E_{max}. Relationships between parameters estimating paracellular permeability enhancement and PLCβ activity were assessed via linear regression analysis, and the correlation was expressed by the Pearson correlation coefficient (r).

RESULTS

PLC expression in Caco-2 cells.

mRNA for six PLC isozymes was detected in Caco-2 cells (Figure 2.1), including PLCβ1, β3, γ1, γ2, δ3, and ε, but not PLCβ2, β4, δ1, and δ4. PLCβ3, γ1, and δ3 levels were the highest, followed by PLCβ1 and ε, while PLCγ2 was of the lowest expression. Expression at the protein level was established by western blot for PLCβ1, β3, γ1, and γ2; however, PLCδ3 and ε were not detected (Fig. 2.2).

PLC mRNA expression in the gastrointestinal tract and other tissues of drug disposition.

PLCβ1 and β3 mRNA was detected throughout the gastrointestinal (GI) tract with highest expression in the ileum and descending colon (Figure 2.3). PLCβ3 levels were comparably high in the duodenum and jejunum as well. On the other hand, ileal PLCβ1 expression was approximately three and five fold higher than that observed in the jejunum and duodenum, respectively. Both of these PLCβ isozymes demonstrate broad tissue distribution as evidenced by detection of mRNA in all tissues examined. Expression was highest in the pancreas for both isozymes, approximately three and five fold higher than any other tissue for β1 and β3 respectively. Notable levels for PLCβ1 were also detected in kidney and brain.

PLCγ1 and γ2 mRNA was detected throughout the GI tract, with highest expression in the stomach, jejunum, and ileum (Figure 2.3). PLCγ isozymes demonstrate broad tissue distribution, as mRNA was detected in all tissues examined. Similar to PLCβ1 and β3, PLCγ1 mRNA was highest in the pancreas, approximately four fold higher than any other tissue. On the other hand, PLCγ2 mRNA was highest in the kidney and liver, although amount in the pancreas was also high compared to other tissues examined. PLCγ1 was also

identified in the heart and liver at relatively high levels while PLCγ2 was also highly expressed in the lung and placenta.

PLCδ3 and PLCε mRNA was detected throughout the GI tract with highest expression in the colon for both isozymes (Figure 2.3). PLCδ3 mRNA was detected in all tissues examined with highest expression in the heart, the only tissue with higher levels than the colon. PLCε mRNA was also detected in all tissues examined, with highest expression in the pancreas.

Of the six major PLC isozymes identified at the mRNA level in Caco-2 cells (*i.e.* PLCβ1, β3, δ3, ε, γ1, and γ2), each was also found throughout the human GI tract. Major differences were observed between Caco-2 cells and human intestine for all six isozymes, as Caco-2 mRNA levels were substantially greater than those observed in the human intestine. Differences between Caco-2 cells and *in vivo* small intestinal tissue (*i.e.* duodenum, jejunum, and ileum) ranged from approximately thirteen fold for PLCγ2 to approximately two hundred twenty fold for PLCβ3. This result may be related to the fact that the Caco-2 cell line is derived from colon carcinoma, as PLC over-expression has often been associated with cancer incidence; however, a direct comparison between expression levels is confounded by the original source of mRNA for each sample (isolated mRNA for Caco-2 versus purchased poly A^+ RNA for tissue sources).

ATP stimulates PLCβ activity in Caco-2 cells.

To confirm the presence of functionally active PLCβ isozymes in Caco-2 cells, the ability of adenosine triphosphate (ATP) to stimulate PLC activity was evaluated. ATP is a known ligand for the purinergic $P2Y_2$ receptor that couples to PLCβ isozymes[29, 30]. ATP increased the accumulation of inositol phosphates in a concentration dependent manner

confirming the presence of functionally active PLCβ isozymes in Caco-2 cells (Figure 2.4). Maximal activation was approximately three fold, and was achieved with concentrations greater than or equal to 300 μM; therefore, this concentration was utilized in studies intended to assess chemical inhibition of stimulated PLCβ activity by reported inhibitors, U73122 and APCs.

Hexadecylphosphocholine inhibits ATP-stimulated PLCβ activity in Caco-2 cells.

Hexadecylphosphocholine (HPC) (Figure 2.5, C16) has previously been reported as an inhibitor of PLC isozymes[18, 31, 32]. The ability of HPC to inhibit ATP-stimulated PLCβ activity was evaluated in Caco-2 cells as a model for the human intestine. As shown in Figure 2.6, HPC attenuates ATP-stimulated PLCβ activity in a concentration dependent manner when applied apically to Caco-2 cells. The concentration of HPC that inhibited PLCβ activity by 50% ($IC_{50\ PLC\beta}$) was estimated to be 40 ± 19 μM.

Hexadecylphosphocholine increases paracellular permeability in Caco-2 cells.

HPC has been shown to increase paracellular permeability across epithelial cell monolayers[18, 33]. When Caco-2 cell monolayers were treated with HPC at various concentrations on the apical side, TEER decreased as a function of time over a 90 minute period, indicating an increase in paracellular permeability (Figure 2.7.A). When measured at a fixed time, *i.e.* after 30 minutes, the decrease in resistance and the increase in mannitol permeability were dependent on concentration (Figure 2.7.B); the concentration that decreased TEER by 50% ($EC_{50\ TEER}$) was estimated to be 248 ± 52 μM, and the concentration that increased mannitol permeability ten-fold (EC_{10X}) was estimated to be 317 ± 26 μM (Table 2.3). HPC is significantly less potent as an enhancer of tight junction permeability in Caco-2 cells as compared to MDCK cells[18].

Alkylphosphocholines increase tight junction permeability and inhibit ATP-stimulated PLCβ activity in Caco-2 cells.

Previous studies have suggested that the ability of phosphocholine containing molecules to increase paracellular permeability is dependent on the length of the alkyl chain[18, 27, 28, 34]. When Caco-2 cell monolayers were treated with APCs varying in alkyl chain length from ten to twenty carbons (Figure 2.5) on the apical side for thirty minutes, the decrease in resistance and increase in mannitol permeability were dependent on concentration for all APCs (Figure 2.8). The concentrations that decreased TEER by 50% ($EC_{50\ TEER}$) and increased mannitol permeability by ten fold (EC_{10X}) were dependent on alkyl chain length and are presented in Table 2.3. The $EC_{50\ TEER}$ and EC_{10X} values were strongly correlated (data not shown; $r > 0.99$, $p < 0.001$), and varied forty six and thirty two fold respectively when the alkyl chain length increased from ten to twenty carbons, with the maximum potency occurring with fourteen carbons in the alkyl chain. There was a substantial drop in potency between twelve and ten carbons.

To determine if the ability of APCs to increase paracellular permeability across Caco-2 cells was associated with their ability to inhibit PLC activity in these cells, the effect of all APCs on ATP-stimulated PLCβ activity was assessed. All APCs inhibited ATP-stimulated PLCβ activity in a concentration dependent manner (Table 2.3); their potency ($IC_{50\ PLC\beta}$) varied approximately forty five fold and was dependent on chain length. The relationship between potency as PLCβ inhibitors and potency as PPEs is depicted in Figure 2.9. The $IC_{50\ (PLC)}$ is linearly related to both $EC_{50\ TEER}$ and EC_{10x} (($r > 0.98$, $p < 0.001$) and ($r > 0.97$, $p < 0.001$) respectively). Interestingly, their potency as PLCβ inhibitors was from five to nine fold greater than their potency as PPEs.

U73122 inhibits ATP-stimulated PLCβ activity in Caco-2 cells.

U73122 (Figure 2.5) is a previously reported inhibitor of PLC activity[18, 35-38] that is structurally distinct from APCs; therefore, its ability to inhibit ATP-stimulated PLCβ activity in Caco-2 cells was evaluated. As shown in Figure 2.10, U73122 attenuates ATP-stimulated PLCβ activity in a concentration dependent manner when applied apically to Caco-2 cells. The concentration of U73122 that inhibited PLCβ activity by 50% ($IC_{50\ PLC\beta}$) was not determined, as aqueous solubility limited studies to concentrations ≤ 30 μM. U73343, the inactive analog of U73122 (Figure 2.10), had no effect of ATP-stimulated PLC activity at 30 μM (Figure 2.10).

U73122 increases paracellular permeability in Caco-2 cells.

U73122 has been reported to increase paracellular permeability across MDCK cells[18]. When Caco-2 cell monolayers were treated with U73122 at various concentrations on the apical side, TEER decreased as a function of time over a ninety minute time period, indicating an increase in paracellular permeability (Figure 2.11.A). Importantly, the inactive analog, U73343, had no effect on paracellular permeability at any concentrations tested. When measured at a fixed time, *i.e.* after 90 minutes, the decrease in resistance and the increase in mannitol permeability were dependent on concentration (Figure 2.11.B). The concentration that decreased TEER by 50% ($EC_{50\ TEER}$), and the concentration that increased mannitol permeability ten-fold (EC_{10X}) were not determined, as solubility limitations precluded treatment greater than 30 μM. Similar to APCs, U73122 is less potent as an enhancer of paracellular permeability in Caco-2 cells as compared to MDCK cells[18].

DISCUSSION

The PLC-dependent pathway has long been postulated to be a regulatory pathway involved in the regulation of cellular junctional complexes[11, 13, 39-41]. Recently, PLCβ isozymes have been directly implicated in the modulation of epithelial paracellular permeability in MDCK cells[18], suggesting that PLC may play an important role in the regulation of mature epithelial tight junctions. Although MDCK cells are a useful model for evaluating permeability of drugs across an epithelial monolayer, their canine kidney origin makes them less than ideal as a model to investigate regulation of paracellular permeability for the purposes of improving the oral absorption of hydrophilic drugs and macromolecules in humans. Caco-2 cells, a human intestinal cell line, are a more appropriate model in which to evaluate the potential role of PLC in the regulation of human intestinal epithelial tight junctions.

In the present study, RT-PCR was initially utilized to determine the expression pattern of PLC isozymes in Caco-2 cells. As shown in Figure 2.1, six isozymes of PLC were detectable at the mRNA level, with at least one member of each isozyme family expressed. Western blot analysis confirmed expression at the protein level for PLCβ and γ isozymes (Figure 2.2), but not for PLCδ3 and PLCε. High levels of PLCβ1 in Caco-2 cells is notable, as most cell lines, including many of colon carcinoma origin, fail to express detectable levels of this isozyme[2, 42]. While the two PLCβ isozymes were quite similar in mRNA expression with respect to one another (*i.e.* approximately two fold difference), mRNA expression for PLCγ1 was more than six fold higher than that for PLCγ2, suggesting it is the dominant isozyme of this family in Caco-2 cells.

In order to establish Caco-2 cells as a suitable model in which to evaluate the PLC-dependent pathway as a potential regulator of intestinal permeability, it was critical to establish that PLC isozymes expressed in Caco-2 cells are also expressed in the human small intestine. Therefore, the expression profile of the six PLC isozymes found in Caco-2 cells was quantitatively compared to that in the human gastrointestinal tract, as well as in other organs of drug disposition. There are few reports in the literature evaluating expression of PLCs in any segments of the human small intestine. Because PLC over-expression has often been associated with cancer incidence, many studies have evaluated differences in expression between carcinomas of gastric or colon tissue and their corresponding healthy tissue[42-45]. These studies have provided evidence for several PLC isozymes in gastrointestinal tissues (PLCβ, γ, and δ), although none have reported expression in the small intestine. In the present study, RT-PCR data revealed that the human small intestine expresses all six PLC isozymes detected in Caco-2 cells, although no distinct expression pattern of these PLC isozymes in the small intestine was evident (Figure 2.3).

After confirmation that functionally active PLCβ isozymes are expressed in Caco-2 cells (Figure 2.4), the ability of known inhibitors to attenuate ATP-stimulated PLCβ activity and increase paracellular permeability was evaluated. Because an entire series of APCs was available (with variable alkyl chain lengths and variable reported potency towards PLCβ [18]), this series of inhibitors was evaluated first (Figure 2.5). A concentration dependent decrease in PLCβ activity by HPC was observed; the concentration of HPC that inhibited PLCβ activity by 50% ($IC_{50\ PLC\beta}$) was estimated to be 40 ± 19 μM (Figure 2.6). Within the series of APCs, the ability to inhibit PLCβ activity was dependent on alkyl chain length with a

maximum potency occurring with fourteen to sixteen carbons in the alkyl chain (Table 2.3), similar to their potency in MDCK cells[18].

After developing an understanding of the ability of APCs to inhibit ATP-stimulated PLC activity, their ability to increase paracellular permeability across Caco-2 cells was evaluated by monitoring the change in TEER, as well as the change in permeability of the paracellular marker, mannitol. A time and concentration dependent drop in TEER was observed following treatment of Caco-2 cells with varying concentrations of HPC (Figure 2.7), consistent with previously reported effects in MDCK cells[18] and Caco-2 cells[33]. However, several differences were noted between the two cell lines. The $EC_{50\ TEER}$ value was substantially greater than in MDCK cells. It was also noted that the kinetics of the observed effect appeared to be different in Caco-2 cells than in MDCK cells, as the effect in Caco-2 cells appeared to have two distinct phases, an immediate effect that resulted in a rapid drop in TEER (60% of control within ten minutes), followed by a slower phase that led to complete abolishment of TEER after approximately sixty minutes of treatment. Following assessment of the effect of HPC on TEER, its ability to increase the permeability of mannitol was determined. This value was consistent with effects on TEER, and was also less potent than the observed effect in MDCK cells[18].

Following studies with HPC, the effects of the APC series (C10 - C20) on both TEER and mannitol permeability were evaluated in Caco-2 cells (Figure 2.8, Table 2.3). Within the series, $EC_{50\ TEER}$ values were similar to EC_{10X} values, and a strong relationship between these two parameters was evident (data not shown); therefore, it was possible to utilize an $EC_{50\ TEER}$ value as a marker for increased paracellular permeability of Caco-2 cells to APCs. Their potency as PPEs was dependent on chain length, with C14 being the most potent of the

series, followed by C16, C18, C20, C12, and C10. Consistent with the reduced efficacy of HPC, $EC_{50\ TEER}$ and EC_{10X} values were significantly grater than those in MDCK cells for most of the series. Interestingly, the effect of C10 and C12 on paracellular permeability was similar between the two cell lines; further, the potency of C12 for increasing paracellular permeability across Caco-2 cells was similar to that previously reported[27].

Within the series of APCs, the chain length dependence for inhibition of PLCβ activity was identical to that for both decreasing TEER and increasing mannitol permeability; thus, a relationship between mannitol permeability and PLCβ inhibition was evident in Caco-2 cells (Figure 2.9). However, the potency of APCs to inhibit PLCβ was significantly greater than their potency to increase paracellular permeability; EC_{10X} values were from five to nine fold greater than $IC_{50\ PLC\beta}$ values, obscuring the relationship between these two parameters. For example, the concentrations that inhibit PLCβ activity by 50% (*i.e.* $IC_{50\ PLC\beta}$) have little to no effect on mannitol permeability; increases in mannitol permeability are only evident when PLCβ activity is completed inhibited. Together, these observations suggest that the effects of APCs on the two cell lines may be different, and that different permeability enhancement mechanisms may be governing the observed effects of APCs on paracellular permeability in Caco-2 cells as compared to MDCK cells. Given the amphiphilic nature of this series of compounds, direct interaction with the apical cell membrane leading to changes in membrane fluidity and lipid composition is a plausible alternative mechanism of paracellular permeability enhancement that has been previously postulated[34, 46]. Additional data support this hypothesis[47], as a thirty minute treatment of HPC at concentrations close to $EC_{50\ TEER}$ caused significant redistribution of a fluorescent paracellular probe molecule from an almost exclusively paracellular localization to one suggesting ready access to the cytosol

of Caco-2 cells. Therefore, differences in the apical membrane phospholipid composition between cells lines could explain the differences in the potency of APCs as PPEs between MDCK and Caco-2 cells. More studies are warranted to further evaluate the direct impact APCs have on apical membranes of Caco-2 cells at concentrations relevant to their potency as both PLC inhibitors and PPEs.

Consistent with the reduced potency of APCs on paracellular permeability in Caco-2 cells as compared to MDCK cells, the potency of another reported PLC inhibitor, U73122 (Figure 2.11), as a PPE was also reduced. After a 30 minute treatment of 30 μM U73122, PLCβ activity was inhibited approximately 50% (Figure 2.10) and TEER dropped to 80% of control (Figure 2.11); however, after an extended treatment of 90 minutes, TEER continued to decline to 40% of control cells. After the same 90 minute treatment, mannitol permeability was increased approximately ten fold. The inactive analog of U73122, U73343, was unable to inhibit PLCβ activity or increase paracellular permeability across Caco-2 cells at similar concentrations, implicating the maleimide moiety in U73122 in the observed effects on both PLC inhibition and paracellular permeability. Recent studies have suggested that nucleophiles present in any experimental test system can have a direct impact on the observed effects of U73122 on cellular phenotypic responses[48]. Therefore, variable expression of accessible nucleophiles between MDCK and Caco-2 cell systems may contribute to the observed differences in potency of U73122 as a PLC inhibitor and PPE in these studies. Further studies are necessary to confirm this hypothesis.

Results generated in this study support a role for PLC isozymes in the regulation of epithelial paracellular permeability, although notable differences are apparent between the effects of tested PLC inhibitors on both MDCK and Caco-2 cell lines from both chemical

classes. Further studies are necessary to confirm the role of PLC isozymes in the regulation of epithelial paracellular permeability, and to establish PLC as a viable pharmacological target for the purposes of increasing the oral absorption of hydrophilic drugs, nutrients, and macromolecules. More specific approaches that could selectively inhibit the expression and activity of specific PLC isozymes would be beneficial to assess the role of these signal transduction enzymes in regulation of cellular functions such as tight junction barrier formation and maintenance of paracellular permeability.

ACKNOWLEDGEMENTS

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Table 2.1. Tissue sources for poly A^+ RNA Isolation

Tissue	Source *
esophagus	39 M/F Caucasians, ages 17-72
stomach	7 M/F Caucasians, ages 20-55
duodenum	30 M/F Caucasians, ages 17-75
jejunum	6 M/F Caucasians, ages 20-57
ileum	8 M/F Caucasians, ages 18-57
ileocecum	19 M/F Caucasians, ages 24-58
cecum	29 M/F Caucasians, ages 18-63
colon, ascending	5 M/F Caucasians, ages 18-50
colon, descending	7 M/F Caucasians, ages 31-61
colon, transverse	19 M/F Caucasians, ages 24-59
rectum	6 M/F Caucasians, ages 24-52
liver	4 M/F Caucasians, ages 44-50
heart	3 M Caucasians, ages 33, 55, and 55
brain (whole)	2 M Caucasians, ages 43 and 55
placenta	17 Caucasians, ages 19-33
lung	2 F Caucasians, ages 24 and 32
skeletal muscle	8 M/F Caucasians, ages 29-60
kidney	6 M/F Caucasians, ages 28-52
pancreas	15 M/F Caucasians, ages 22-69

* Abbreviations: M, male; F, female

Table 2.2. Primers used to generate standard plasmid DNA for quantification of mRNA in human tissues.

Protein	Forward Primer (base pair position)	Reverse Primer (base pair position)	Accession #
hPLCβ1*	5'-atgatcacagtggtgtatgggcct (307-330)	5'-aacagagggatcctcatggcaagt (3480-3457)	NM182734
hPLCβ3	5'-ggcgcgggagtaagttcatcaaa (68-90)	5'-ctcaaaggctgcaatgcgaagtga (2400-2377)	NM000932
hPLCγ1	5'-gggcaccgtcatgactttgttcta (214-237)	5'-tcttgcggtatagcgggtgtttct (2369-2346)	NM002660 NM182811
hPLCγ2	5'-tctgaacaaagtccgtgagcggat (935-958)	5'-gcagaaatgccaggtttgggtcat (3549-3526)	NM002661
hPLCδ3	5'-acatcttcttcgtgcagcacatcg (434-457)	5'-atgcggatttggatgaagagcgtg (2471-2448)	NM133373
hPLCε	5'-caaaggcggcatgaagggatttca (4222-4245)	5'-aagtgaacgcggaacagaaactgc (6423-6400)	NM016341

* Reverse primer for PLCβ1 not present in variant with accession number NM015192.

Table 2.3. Potency of alkylphosphocholines (APCs) as paracellular permeability enhancers ($EC_{50\ TEER}$ and EC_{10X}) and as inhibitors of ATP-stimulated PLCβ activity ($IC_{50\ PLC\beta}$).

APC Chain Length	$EC_{50\ TEER}$ (μM)	EC_{10X} (μM)	$IC_{50\ PLC\beta}$ (μM)
C10	9800 ± 1375	8333 ± 1320	1304 ± 837
C12	735 ± 62	576 ± 160	123 ± 28
C14	213 ± 23	261 ± 90	29 ± 13
C16	248 ± 52	317 ± 26	40 ± 19
C18	299 ± 19	384 ± 94	70 ± 38
C20	429 ± 77	481 ± 45	104 ± 8

See Figure 2.5 for chemical structures of compounds in the APC series.

Results are reported as the mean ± standard deviation of three experiments performed in triplicate.

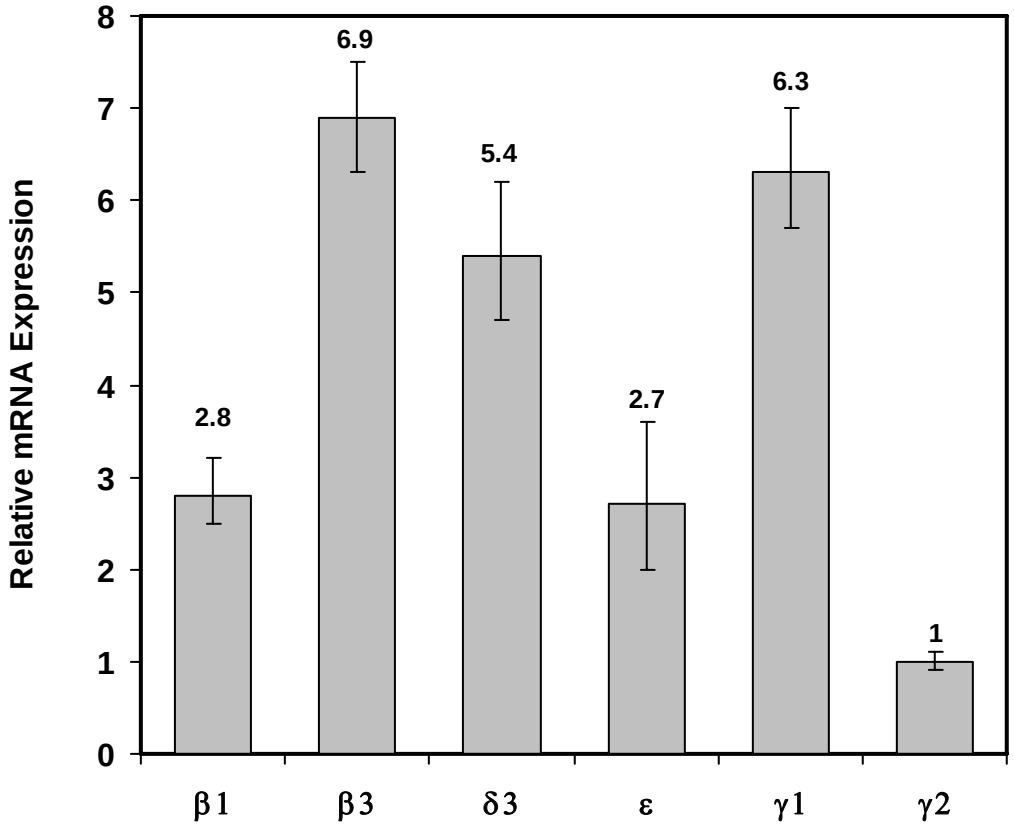

Figure 2.1. PLC mRNA expression in Caco-2 cells. Expression levels of PLC mRNA in Caco-2 cells were determined by real-time RT-PCR. All data were normalized to the β-actin expression level within each sample, and the level of each isozyme is expressed relative to PLCγ2, the isozyme with the lowest expression. Data represent mean ± range from triplicate determinations.

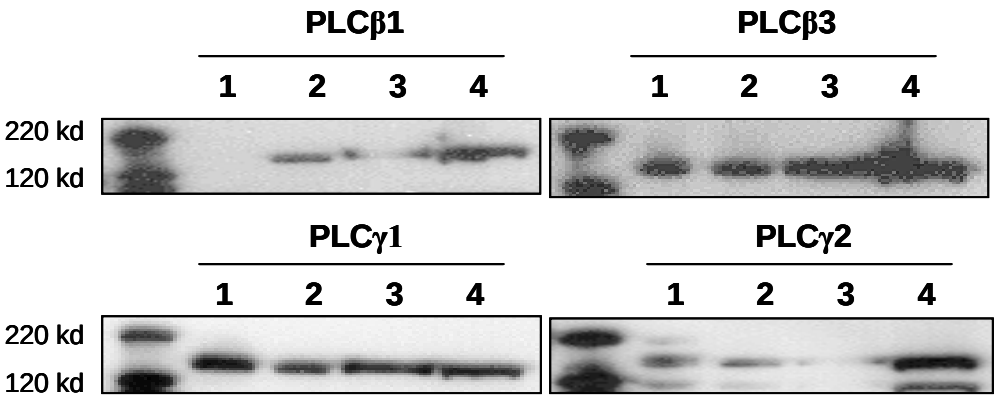

Figure 2.2. PLC protein expression in Caco-2 cells. Expression of PLCβ1, PLCβ3, PLCγ1, and PLCγ2 isozymes in Caco-2 cells determined via Western blot. Lanes 1 and 2 represent positive controls, *i.e.* A431 and MCF7 whole cell lysates respectively. Lanes 3 and 4 represent 1.5 and 15 μg of a Caco-2 whole cell lysate.

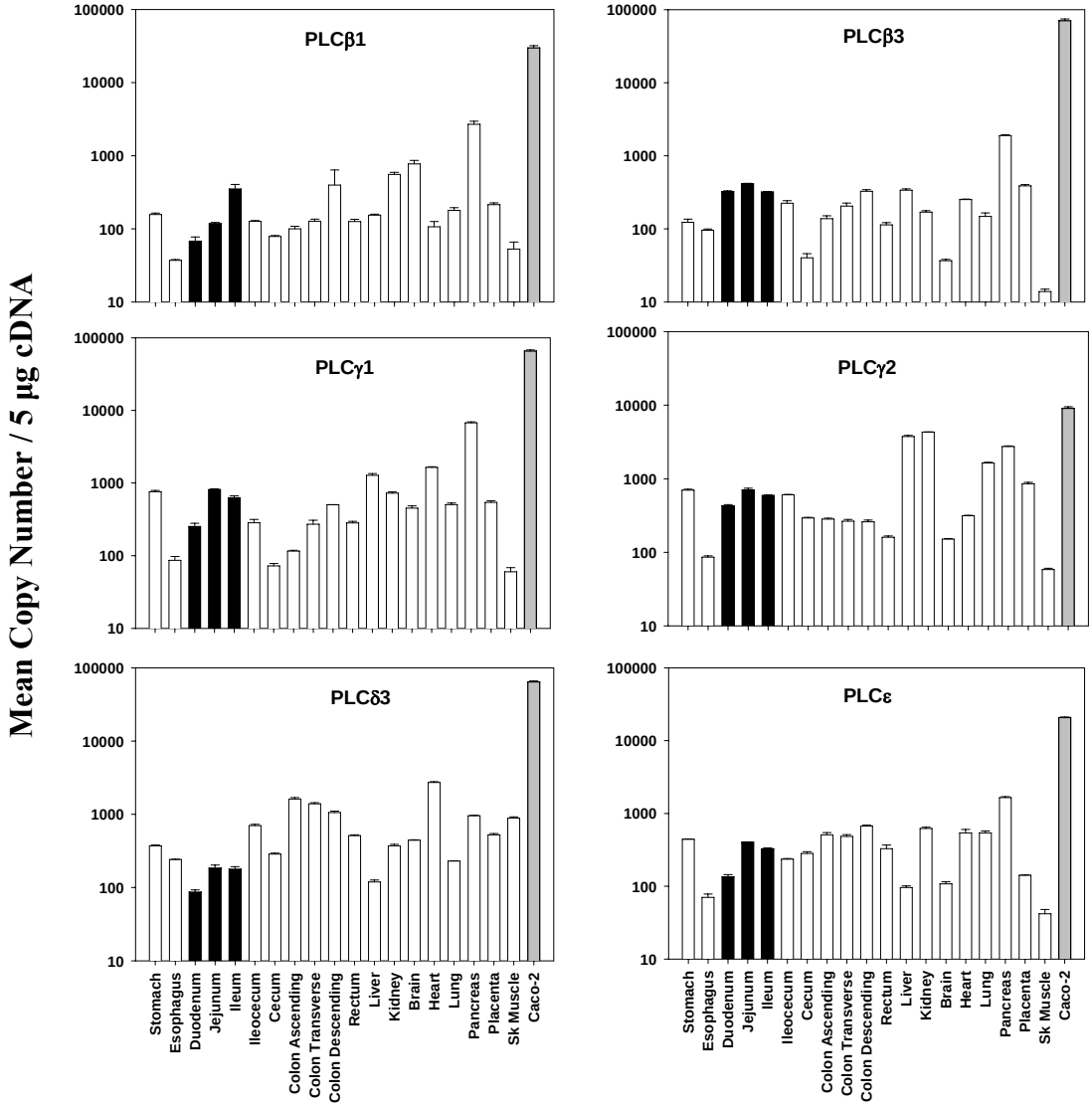

Figure 2.3. Human tissue distribution of PLC mRNA. Expression levels of PLC mRNA in multiple human tissues and Caco-2 cells were determined by quantitative real-time RT-PCR. Expression in Caco-2 cells (gray bar), along the small intestine (solid bars), and in other organs of drug disposition (open bars) is indicated. Tissue expression data were obtained using purchased poly A^+ RNA. Caco-2 expression data was obtained from laboratory isolated mRNA. Data represent mean ± standard deviation from triplicate determinations.

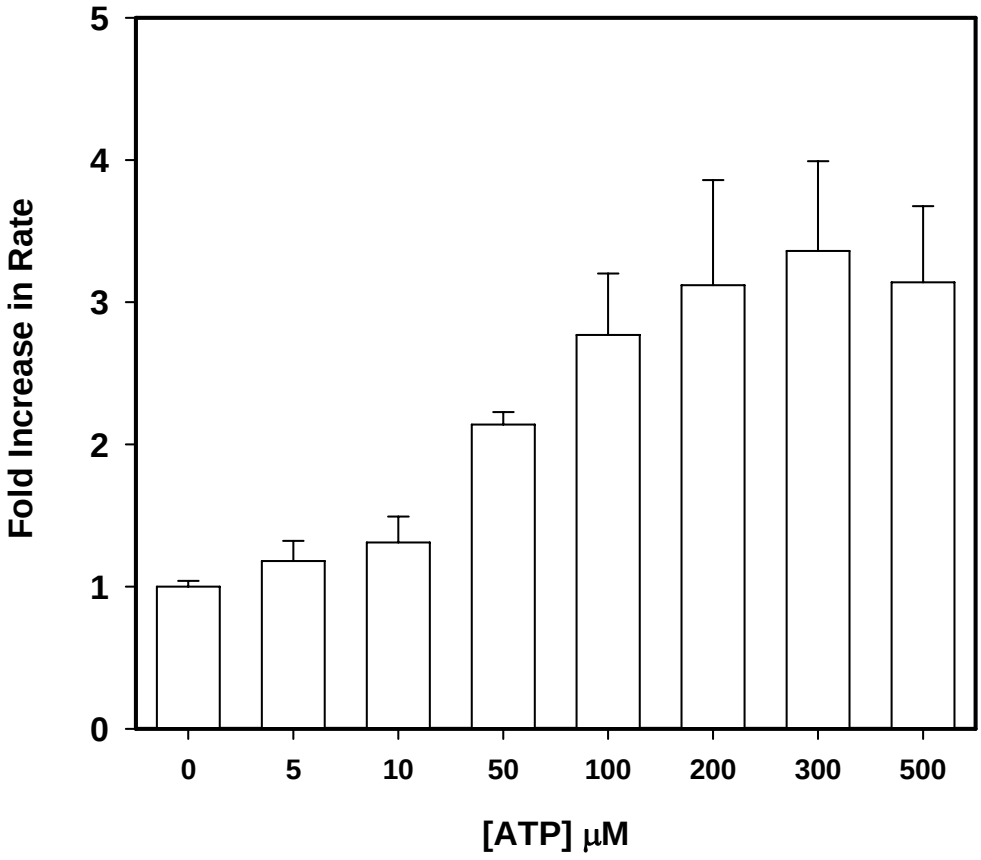

Figure 2.4. Stimulation of PLCβ activity in Caco-2 cells by ATP. Accumulation of inositol phosphates was determined by stimulating PLCβ activity with ATP for fifteen minutes. [^{3}H]-inositol phosphates were isolated by ion exchange chromatography and quantified via liquid scintillation counting. Data represent mean ± standard deviation from triplicate determinations.

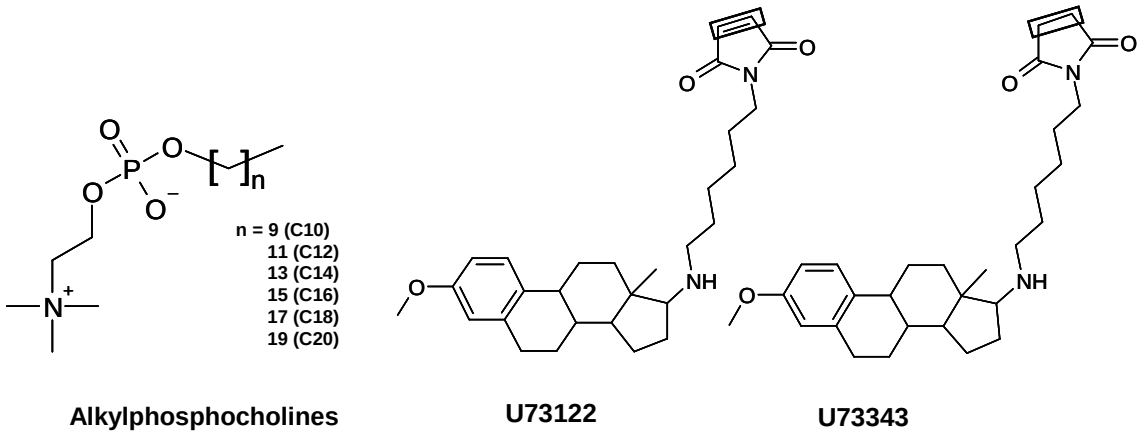

Figure 2.5. Structures of alkylphosphocholines, U73122, and U73343.

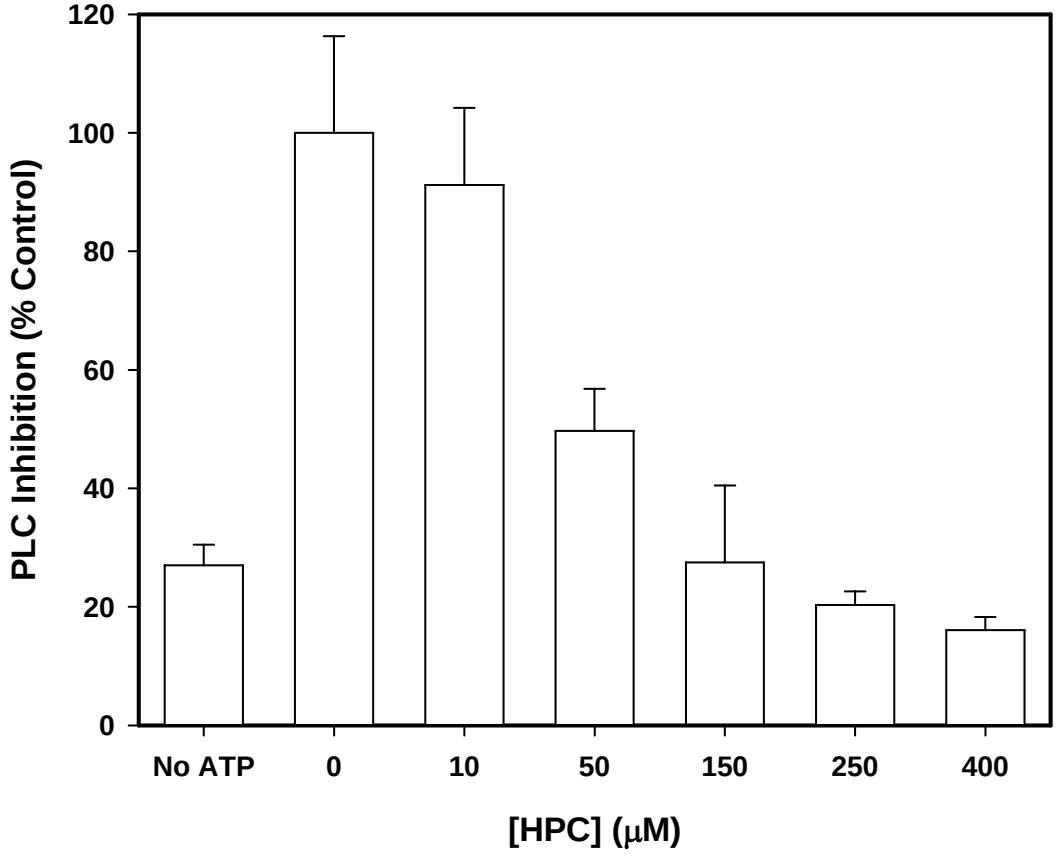

Figure 2.6. The effect of HPC on ATP-stimulated PLCβ activity in Caco-2 cells. Cells were labeled with [^{3}H]-myo-inositol overnight at 37°C, and then treated apically with HPC for thirty minutes. The effect of HPC on the accumulation of inositol phosphates was determined by stimulating PLCβ activity with 300 μM ATP for fifteen minutes. [^{3}H]-inositol phosphates were isolated by ion exchange chromatography and quantified via liquid scintillation counting. Data represent mean ± standard deviation from triplicate determinations from one representative experiment.

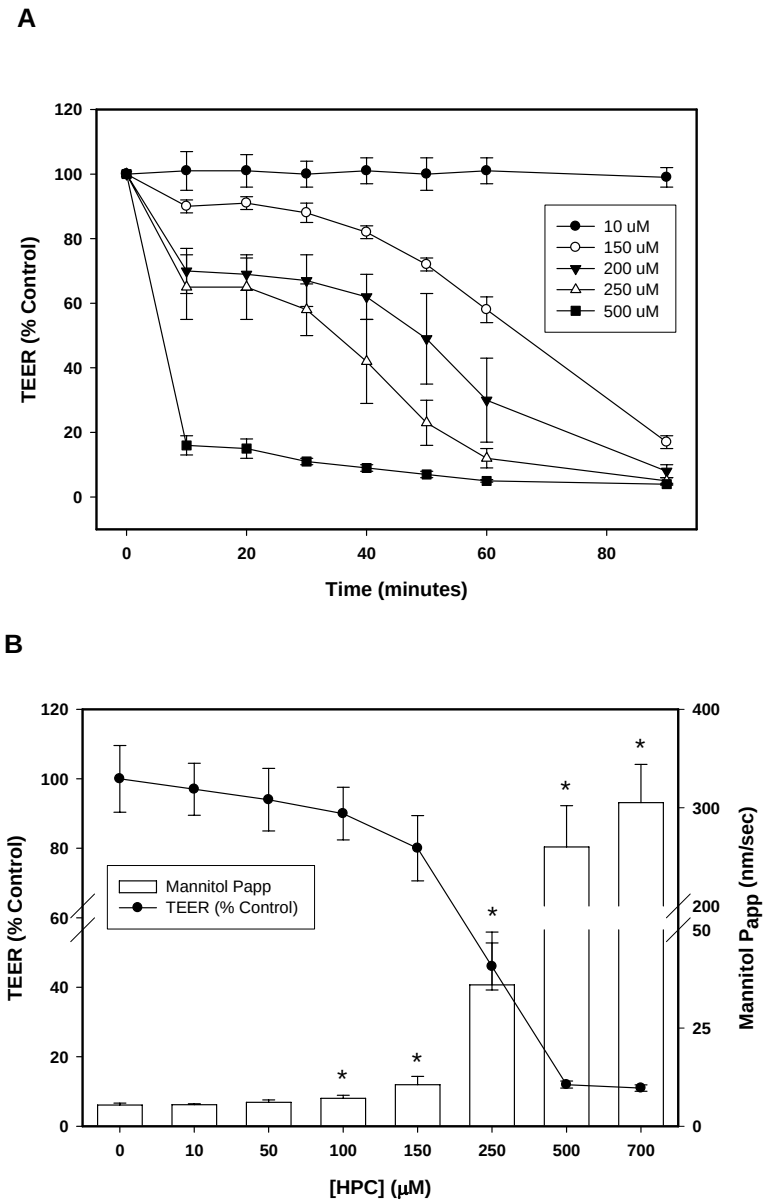

Figure 2.7. The effect of HPC on paracellular permeability across Caco-2 cell monolayers. (A) Cells were treated apically with HPC at indicated concentrations and TEER was measured at ten minute intervals for ninety minutes. (B) Cells were treated apically with HPC and TEER was measured after thirty minutes. To evaluate HPC induced changes in paracellular permeability, [^{14}C]-mannitol was added to the apical compartment, and the amount appearing in the basolateral compartment during the 30-60 minute period post-treatment was measured. Data represent mean ± standard deviation from triplicate determinations from one representative experiment. Asterisks indicate significant difference ($p < 0.05$) for mannitol permeability.

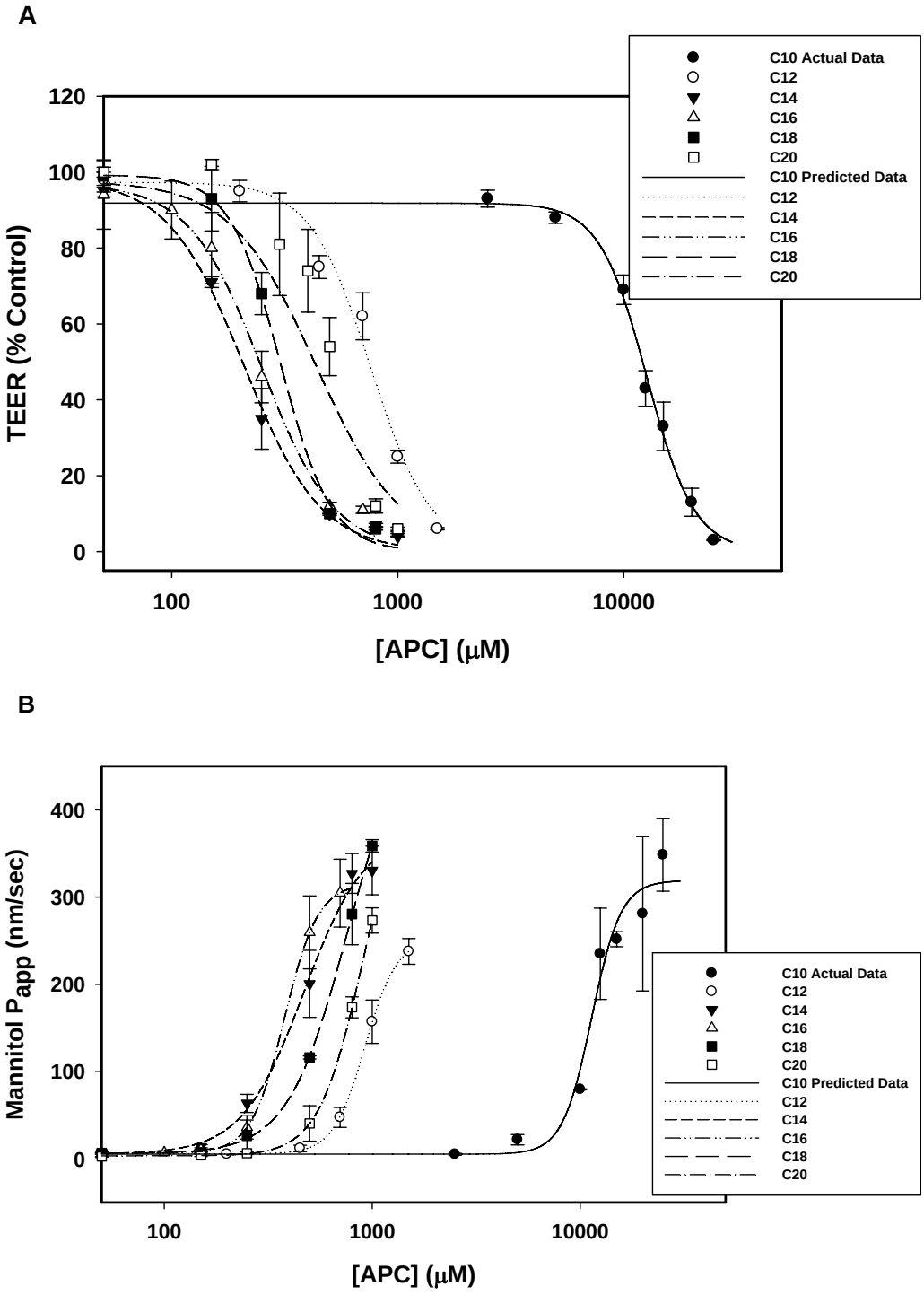

Figure 2.8. The effect of alkylphosphocholines (APC) on paracellular permeability across Caco-2 cell monolayers. Cells were treated apically with indicated APC at 37°C for thirty minutes. (A) TEER (B) The amount of [^{14}C]-mannitol appearing in the basolateral compartment during the 30-60 minute period post-treatment. Data represent mean ± standard deviation from triplicate determinations from one representative experiment for each APC.

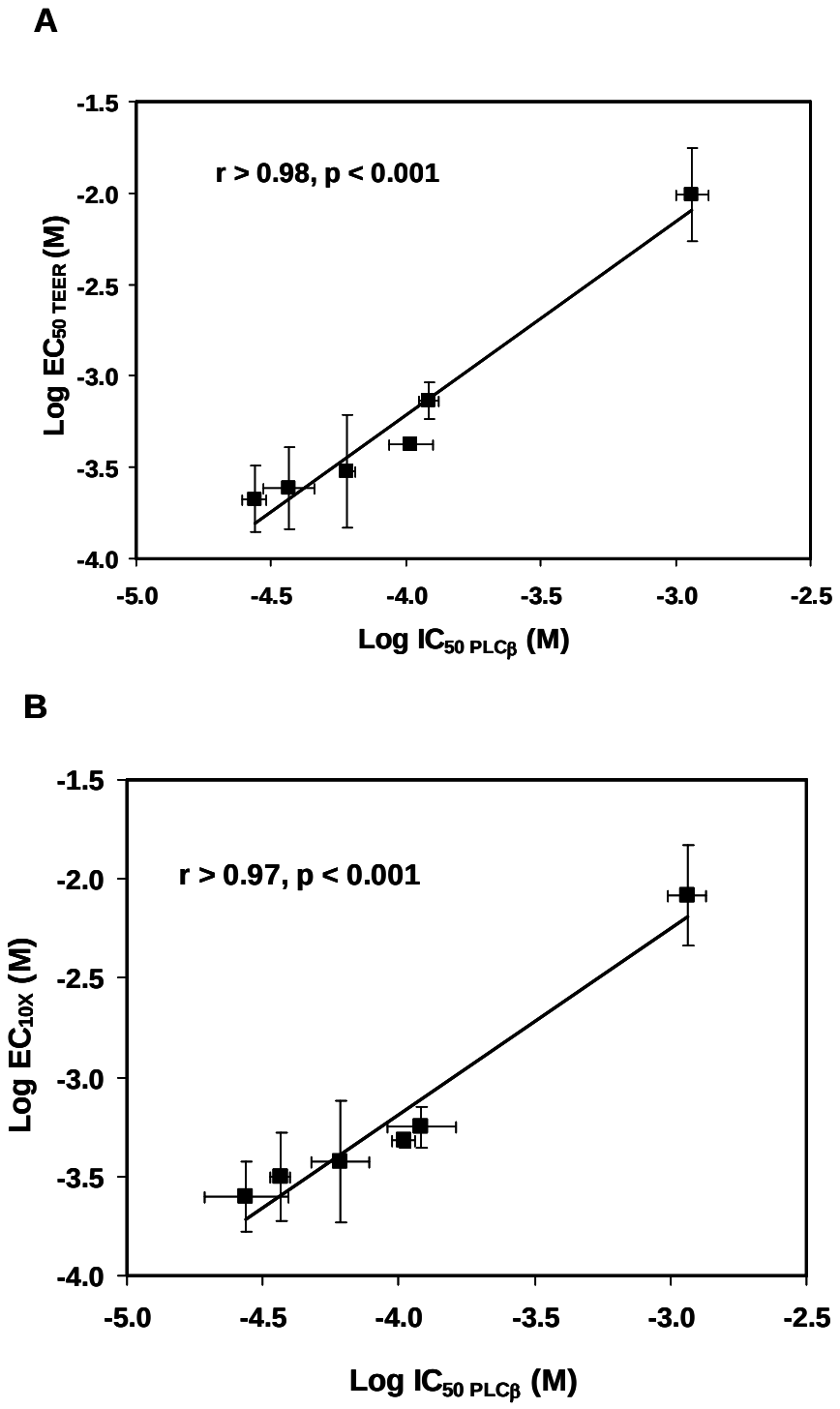

Figure 2.9. The relationship between inhibition of PLCβ activity and increase in paracellular permeability by APCs in Caco-2 cell monolayers. (A) The relationship between the $IC_{50\ PLC\beta}$ and $EC_{50\ TEER}$ (B) the relationship between the $IC_{50\ PLC\beta}$ and EC_{10X}.

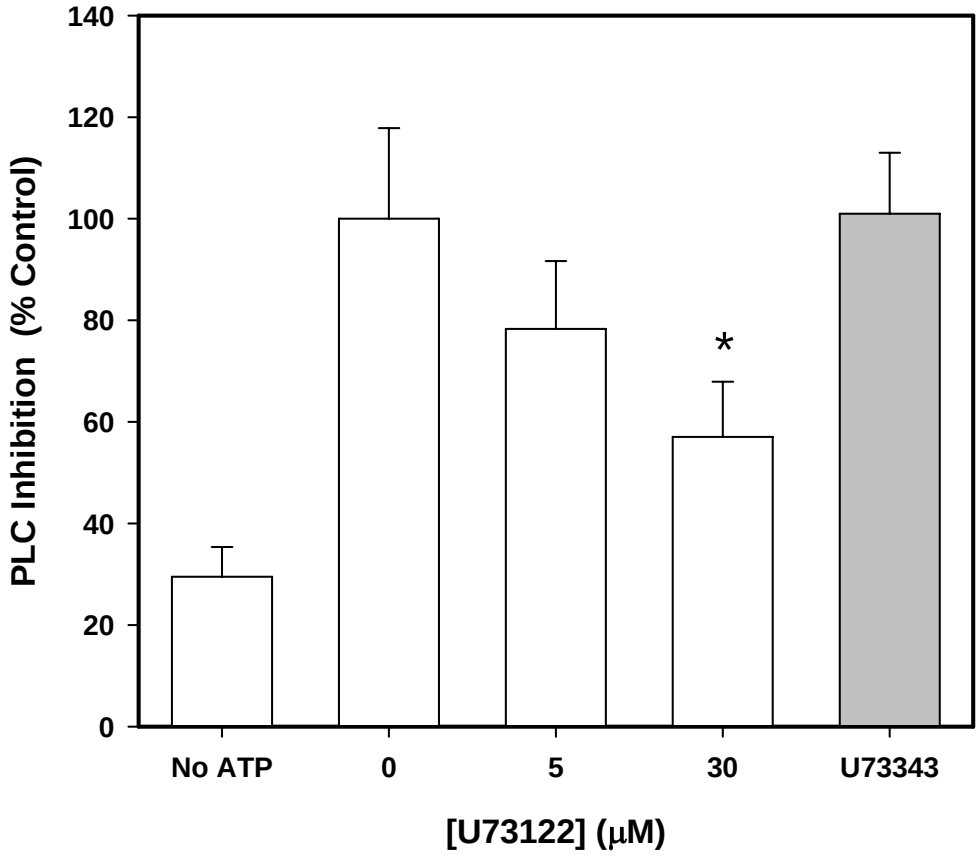

Figure 2.10. The effect of U73122 on ATP stimulated PLCβ activity in Caco-2 cells. Cells were labeled with [^{3}H]-myo-inositol overnight at 37°C, then treated apically with U73122 at indicated concentrations, or with U73343 (30 μM), for thirty minutes. Accumulation of inositol phosphates was determined by stimulating PLCβ activity with 300 μM ATP for fifteen minutes. [^{3}H]-inositol phosphates were isolated by ion exchange chromatography and quantified via liquid scintillation counting. Data represent mean ± standard deviation from triplicate determinations from one representative experiment. Asterisks indicate significant difference ($p < 0.05$).

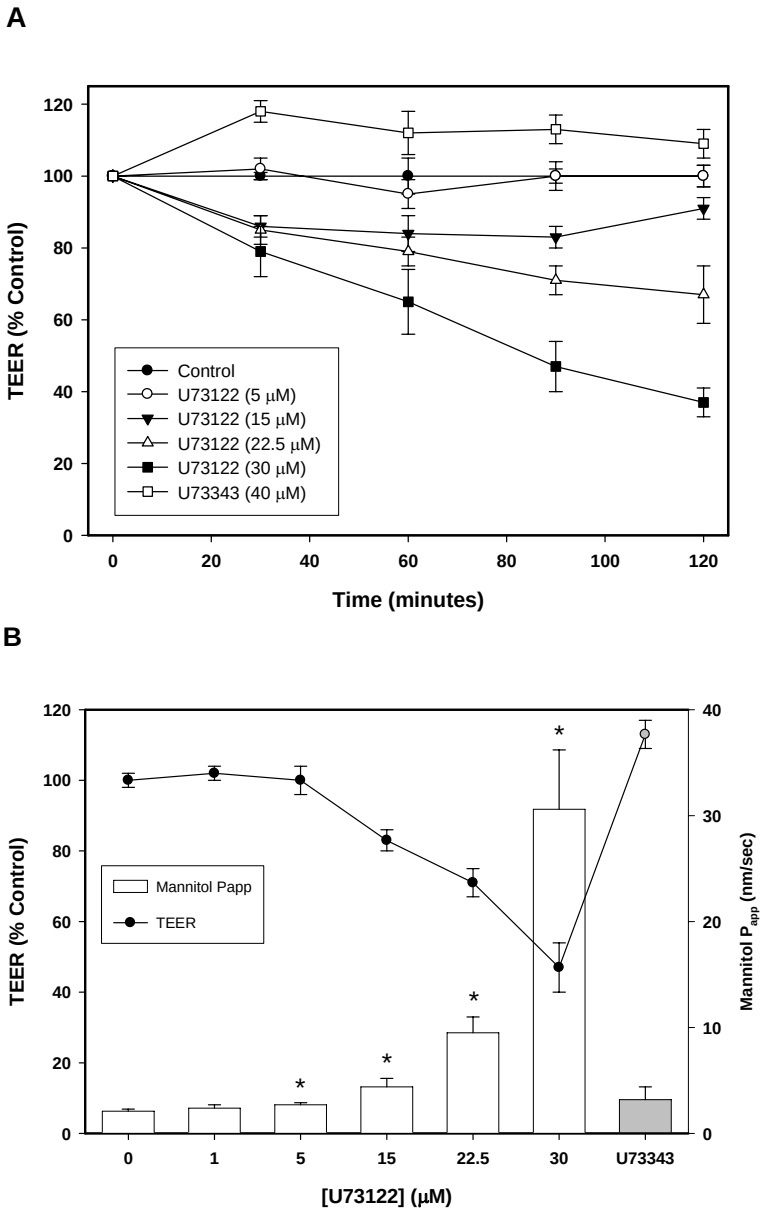

Figure 2.11. The effect of U73122 on paracellular permeability across Caco-2 cell monolayers. (A) Cells were treated apically with U73122 at indicated concentrations and TEER was measured every thirty minutes for two hours. (B) Cells were treated apically with U73122 at indicated concentrations, or with U73343 (30 μM), and TEER was measured after ninety minutes. To evaluate U73122 induced changes in paracellular permeability, [^{14}C]-mannitol was added to the apical compartment, and the amount appearing in the basolateral compartment during the 90-120 minute period post-treatment was measured. Data represent mean ± standard deviation from triplicate determinations from one representative experiment. Asterisks indicate significant difference ($p < 0.05$) for mannitol permeability.

CHAPTER 3

RNAi KNOCKDOWN OF PHOSPHOLIPASE C ISOZYMES IN MDCK II CELLS HAS NO IMPACT ON ESTABLISHMENT OR MAINTENANCE OF TIGHT JUNCTION BARRIER FUNCTION

ABSTRACT

Phospholipase C (PLC) isozymes comprise a large family of key cell signaling proteins that hydrolyze inositol containing phospholipids to form two cellular second messengers, diacylglycerol (DAG) and inositol triphosphate (IP_3). Recently, PLCβ and PLCγ isozymes have been implicated in the regulation of epithelial paracellular permeability in both Madin-Darby canine kidney (MDCK) and Caco-2 cells using previously reported PLC inhibitors, implicating this important class of signaling enzymes as potential regulators of epithelial tight junction function. To confirm the potential role of these enzymes in the barrier function of epithelial tight junctions, RNA interference was used to suppress the expression of specific PLC isozymes in MDCK cells, and the effect on tight junction function and structure was evaluated. mRNA was detected for two isozymes of these PLC families in MDCK cells, PLCβ3 and PLCγ1. Surprisingly, depletion of both enzymes, alone or in combination, had no impact on paracellular permeability across cell monolayers, and no clearly detectable effect on the subcellular localization of tight junction proteins ZO-1, ZO-2, or occludin. Further, RNAi-mediated suppression of these isozymes in combination had no effect on the potency of previously reported PLC inhibitors, U73122 and hexadecylphosphocholine, to increase paracellular permeability across MDCK cell monolayers, implying that these compounds may increase permeability via alternative mechanisms. In addition to the lack of effect on mature tight junction function, depletion of both PLC isozymes, alone or in combination, had no effect on the assembly of tight junctions that were disrupted by removal of extracellular calcium. The present results indicate that PLCβ3 and PLCγ1 are not crucial for the formation or maintenance of tight junction barrier function in epithelial cells, and that reported PLC

inhibitors, U73122 and alkylphosphocholines, increase the paracellular permeability of epithelial cell monolayers via mechanisms independent of their effects on PLC enzymes.

INTRODUCTION

Phospholipase C (PLC) enzymes have previously been postulated to be involved in the regulation of tight junction function in epithelial cell monolayers[1-4], although there is little direct evidence to support this hypothesis. Reports that have specifically implicated PLC in the regulation of tight junction barrier function have demonstrated that chemical agents that inhibited PLC in both Madin-Darby canine kidney (MDCK) and Caco-2 cell monolayers also increased paracellular permeability (Chapter 2)[5-8]. Specific downstream events have been attributed to this PLC inhibition-mediated increase in paracellular permeability, including reorganization of the cortical actin ring[8], redistribution of tight junction proteins ZO-1[5] and claudin-1[6], as well as hyperphosphorylation of tight junction protein ZO-2[7]. On the other hand, a number of unrelated reports with these same inhibitors indicate they may be non-specific in nature, and exert "off-target" effects on cultured cells[9-12], raising speculation regarding the proposed hypothesis that inhibition of PLC activity directly leads to changes in the barrier function of epithelial tight junctions.

In order to establish a cause-effect relationship between inhibition of PLC activity and increased paracellular permeability across epithelial cells, a more specific approach to inhibition of individual PLC isozymes was required, as evidence is lacking for chemical agents that can specifically inhibit PLC isozymes in cellular systems without affecting other cellular functions. RNA interference (RNAi), a process by which exogenous double stranded RNA silences homologous genes in whole cells, offers an alternative to the use of chemical inhibition for achieving highly specific inhibition of functional proteins in cells and tissues. Short interfering RNA (siRNA) molecules homologous to a variety of target genes have been transiently transfected with high efficiency into numerous mammalian epithelial cell lines,

including both Caco-2[13, 14] and MDCK cells[15], to effectively silence genes with greater than 80% knockdown efficiency. Thus, in addition to the advantage of increased target specificity over chemical inhibitors of PLC, siRNAs provide the opportunity to specifically address the role of individual PLC isozymes on epithelial tight junction function independently, and in combination.

Previous reports have specifically implicated isozymes of the PLCβ and PLCγ families in the regulation of paracellular permeability (Chapter 2)[7, 8]; therefore, the purpose of the present study was to design oligonucleotides to suppress the expression of individual PLCβ and PLCγ isozymes, and examine the subsequent effects on the maintenance of tight junction barrier function. This approach should overcome the issues of potential "off-target" effects associated with reported PLC inhibitors such as alkylphosphocholines (APCs) and U73122. As established in Chapter 2, Caco-2 cells are a suitable, and preferred, *in vitro* model for assessing the role of PLC in epithelial tight junction regulation for the purpose of increasing oral absorption in humans; however, they are notoriously difficult to transfect with high efficiency, particularly in culture after the establishment of a confluent monolayer. MDCK cells on the other hand, although of canine kidney origin, are more amenable to a number of well established transfection approaches, and have been widely used as a model cell line to evaluate the underlying molecular mechanisms regulating epithelial tight junction function. Further, their expeditious growth rate and tendency to rapidly establish confluent monolayers, particularly when seeded at high density, offers the unique advantage of transfecting these cells in suspension to knockdown targeted proteins, and preserving the capability of assessing the effect on the maintenance of an established tight junction barrier[15, 16]. Therefore, transient transfection of MDCK cells to specifically knockdown the expression

of PLCβ and γ isozymes was used to determine if a cause-effect relationship exists between inhibition of PLC and increased paracellular permeability.

MATERIALS AND METHODS

Materials. Dulbecco's modified eagle media (DMEM), Hanks Balanced Salt Solution (HBSS), 4-12% Bis-Tris gels, MOPS running and transfer buffers, antioxidant, as well as all other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Gene specific primers were designed using Primer3 and purchased from Fisher. Oligonucleotides targeting PLC isozymes, as well as non-sense oligos, were purchased from Dharmacon Inc. (Lafayette, CO). Antibodies for PLC isozymes were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and GAPDH antibody was purchased from Imgenex (San Diego, CA). Antibody for ZO-1 was generously provided by the laboratory of Dr. James Anderson (Department of Cellular and Molecular Physiology, School of Medicine, The University of North Carolina at Chapel Hill). Antibodies for ZO-2 and occludin were from Zymed (San Francisco, CA). Alexa Fluor IRDye secondary antibodies for western blotting were purchased from Rockland (Gilbertsville, PA), while secondary antibodies for confocal microscopy were from Jackson ImmunoResearch Inc. (West Grove, PA). RNeasy Mini Kit and QIAamp RNA Blood Mini Kit were obtained from Qiagen (Valencia, CA). Six and twelve well Transwells™ and twelve and twenty-four well culture dishes were obtained from Costar (Cambridge, MA). D-[2-^{3}H]-mannitol (20 Ci/mmol) and [2-^{3}H(N)]-myo-inositol (20 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). AG1-X8 formate columns were obtained from Bio-Rad Laboratories (Hercules, CA). Hexadecylphosphocholine (HPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Nucleofector kit L was purchased from Amaxa Biosystems (Gaithersburg, MD). U73122 and all other chemical and reagents were purchased from Sigma.

Cell Culture and Transfection. MDCK II tet off cells (passage 11) were generously provided by the laboratory of Dr. James Anderson (Department of Cellular and Molecular Physiology, School of Medicine, The University of North Carolina at Chapel Hill), and were cultured at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity in DMEM with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 1% penicillin-streptomycin. Subconfluent MDCK cells (passage 11-20) were nucleofected in suspension (Amaxa Biosystems, Gaithersburg, MD) using nucleofector kit L according to the manufacturer's protocol and seeded at indicated seeding densities. Media was changed daily. Each transfection was performed with 1.0 x 10^6 cells, indicated amounts of free oligonucleotides, and 1 μg of pmaxGFP as a transfection marker. Experiments were typically performed three days post-transfection unless indicated otherwise.

siRNA oligonucleotides. Free oligonucleotide smartpools targeting specific PLC isozymes were designed in collaboration with Dharmacon Inc. Four different siRNAs were designed and combined as pools to maximize the knockdown efficiency of each targeted protein. A pre-designed non-sense oligonucleotide smartpool was also purchased from Dharmacon Inc. and served as a negative control where indicated.

RT-PCR. Tissue collection was performed in accordance with the Institutional Animal Care and Use Committee of GlaxoSmithKline, Inc. Following collection, tissues for positive controls were stored at -80°C until use. Total RNA was isolated from cells and tissues using RNeasy Mini Kit or QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. RNA was treated with TURBO DNase (TURBO DNA-free kit, Ambion Inc., Austin, TX) to remove genomic DNA contamination. RNA was quantified by UV absorbance at 260 nm on a UV-Vis spectrophotometer (Nanodrop Technologies,

Wilmington, DE), and its integrity was verified by an OD_{260}/OD_{280} ratio greater than 1.8. cDNA was synthesized from 5 μg of RNA for each tissue sample or cell lysate using Superscript III reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) with an equivalent amount of RNA included in a no-RT control for each separate RNA sample. Real-time PCR was performed with 1:50 dilutions of cDNA as well as no-RT and no template reaction controls. Reactions consisted of SYBR Green JumpStartTaq ReadyMix (Sigma-Aldrich Co., St. Louis, MO), primers at 0.3 μM, and 5 μL of sample (cDNA or control). Amplification was performed in a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) thermal cycler at 94°C for 2 minutes followed by 40 cycles at 94°C for 15 seconds, 54°C for 20 seconds, and 72°C for 25 seconds. Following amplification, a melting curve analysis was performed by heating the reactions from 50 to 99°C in 0.2°C intervals while monitoring fluorescence. All primer pairs are listed in Table 3.1 and produced amplicons of the predicted size on an 8% TBE-PAGE gel.

Western Blotting. Following transfection, MDCK cells were seeded in twenty four well culture dishes with 2.0 x 10^5 cells per well and cultured for indicated times. Media was changed daily. Whole cell lysates were collected at indicated timepoints by washing three times with PBS+ (supplemented with 1.8 mM calcium chloride), adding 200 μl SDS sample buffer at 55°C, and sonicating each lysate briefly. Lysates were stored at -20°C until analysis by immunoblot using standard protocols. Primary antibodies were rabbit anti-PLCγ1 (1:100), rabbit anti-PLCβ3 (1:500), and goat anti-GAPDH (1:2500). Secondary antibodies were Alexa Fluor IRDye 700-conjugated donkey anti-rabbit and Alexa Fluor IRDye 800-conjugated donkey anti-goat. Quantitative analysis of Western blots was performed using Odyssey infrared imaging system (LI-COR Inc., Lincoln, NE).

PLC activity by cellular assay. The activity of PLC isozymes was determined by an adaptation of previously published methods[7, 8, 17]. Following transfection MDCK cells were seeded in twenty four well culture dishes with 4.0 x 10^5 cells per well and cultured for indicated times. Medium was changed daily. On day 2, cells were labeled with [^{3}H]-myo-inositol (2 μCi/well) in 0.5 ml of serum free media for twelve hours at 37°C prior to assay. Assays were initiated by incubating cells with 100 mM LiCl for forty five minutes in either HBSS supplemented with 10 mM Hepes and 25 mM glucose or inositol and serum free media, to assess adenosine triphosphate (ATP)-stimulated and epidermal growth factor (EGF)-stimulated activity, respectively. After forty five minutes, treatment cells were supplemented with either ATP (100 μM) or EGF (100 ng/ml) for fifteen minutes to stimulate PLCβ and PLCγ activity respectively, and allow accumulation of [^{3}H]-inositol phosphates. Assays were terminated by aspirating the media and adding 1.0 ml of boiling 10 mM EDTA (pH 8.0) to lyse cells. The lysate was then applied to AG1-X8 formate columns for chromatographic isolation of [^{3}H]-inositol phosphates[18]. The amount of [^{3}H]-inositol phosphates was measured by liquid scintillation counting in a Packard Tri Carb 4000 Series spectrophotometer. Data from each experiment were normalized to the amount of accumulated [^{3}H]-inositol phosphates in MDCK cells in the absence of agonist, were compared to both non-transfected and non-sense transfected cells, and were reported as the mean ± standard deviation of three replicates.

Measurement of paracellular permeability. Following transfection, MDCK cells were seeded in twelve well Transwells™ with 4.0 x 10^5 cells per well, and cultured for indicated times. Medium was changed daily. Transepithelial electrical resistance (TEER) and permeability of hydrophilic paracellular marker mannitol were used as indicators of the

barrier function of epithelial tight junctions. As such, decreases in TEER or increases in paracellular permeability were perceived to indicate loss of tight junction barrier function. TEER measurements were performed at indicated times using an EVOM Epithelial Tissue Voltohmeter and an Endohm-12 electrode (World Precision Instruments, Sarasota, FL). For permeability measurements, cell media was first aspirated and cells were rinsed two times with pre-warmed transport buffer (HBSS supplemented with 25 mM glucose and 10 mM Hepes) and incubated at 37°C for thirty minutes. TEER measurements were performed prior to each experiment to confirm tight junction integrity. Permeability experiments were initiated by aspirating buffer from the apical compartment and replacing with buffer containing [^{3}H]-mannitol, and desired concentrations of PLC inhibitors, U73122 or HPC, where indicated, and incubating at 37°C for sixty minutes. Permeability was monitored by the appearance of compound in the basolateral side (1.5 ml) during the second 30-minute interval (*i.e.* 30-60 minutes), and was calculated according to the following equation:

$$P_{app} = dQ/dt * 1/(A) * 1/(C_0)\ units = nm/\sec\ (1\ x\ 10^{-7}\ cm/\sec) \quad (1)$$

where dQ/dt is the amount, Q, of mannitol measured in the basolateral compartment at time T. A is the membrane surface area and C_0 is the initial concentration of mannitol in the apical compartment. The amount of compound transported was measured by liquid scintillation counting in a Packard Tri Carb 4000 Series spectrophotometer. All transport experiments were conducted under sink conditions (experiments were designed such that less than 10% of the total amount of compound was present on the basolateral side at any given time). The permeability of [^{3}H]-mannitol in knockdown cells was compared to that in non-transfected and non-sense transfected cells, and was reported as the mean ± standard deviation of three replicates.

Calcium Switch Assay. Calcium switch assay was performed by an adaptation of previously published reports[19, 20]. Following transfection, MDCK cells were seeded in twelve well Transwells™ with 4.0 x 10^5 cells per well, and cultured for indicated times. Medium was changed daily and TEER was measured prior to the addition of low calcium (LC) medium on day two. LC medium was prepared with minimum essential medium containing spinner salts (SMEM, Invitrogen, Carlsbad, CA), 5% dialyzed FBS, 10 mM Hepes pH 7.2, glutamine, and 5 μM $CaCl_2$. FBS was dialyzed at 5°C against a buffer containing 150 mM NaCl and 50 mM Na_2HPO_4 pH 7.2 for twenty four hours, against the same buffer with 0.2 mM EDTA added for 24 hours, then again against the same buffer without EDTA two more times for twenty four hours each. Dialysis was performed using 32 mM diameter dialysis tubing (molecular weight cutoff of 12-14 kD) (Spectrum Laboratories, Irving, TX) against a forty fold volume of each buffer. Cells were incubated at 37°C in an atmosphere of 5% CO2 and 90% relative humidity for approximately twelve hours in LC media. TEER was measured again to confirm loss of barrier function, and normal calcium (NC) medium was then returned to both the apical and basolateral compartments. NC medium was prepared by slowly adding an appropriate amount of a 0.18 mM $CaCl_2$ stock solution to LC media, with stirring, to reach a final concentration of 1.8 mM $CaCl_2$. Formation of tight junction barrier function was evaluated by measurement of TEER at indicated times for the next twenty four hours, or until resistance had returned to initial levels.

Confocal Microscopy. Following transfection, MDCK cells were seeded in twenty four well culture dishes on top of ethanol sterilized glass coverslips (12 mm diameter) with 2.0 x 10^5 cells per well and cultured for indicated times. Media was changed daily. On day three following transfection, MDCK cells were rinsed three times with PBS^+ prior to fixation with

1% paraformaldehyde in PBS^+ for 30 minutes. Cells were then washed again three times with PBS^+, and paraformaldehyde fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 15 minutes, followed by three additional washes with PBS. All cells were then blocked for one hour at room temperature in 5% normal donkey serum (Jackson ImmunoResearch) with 1% BSA to block nonspecific binding, followed by three washes with PBS. Staining with appropriate primary and secondary antibodies was performed for one hour at room temperature in 1% BSA with three ten minute PBS washes following incubation with each antibody. Primary antibodies used were rat anti-ZO-1 (1:25; hybridoma supernatant (R40.76) provided by laboratory of Dr. J. Anderson, Cellular & Molecular Physiology, The UNC School of Medicine, Chapel Hill, NC), mouse anti-ZO-2 (1:250; Zymed Laboratories), and mouse anti-occludin (1:300; Zymed Laboratories). Secondary antibodies were goat anti-rat Cy3 and donkey anti-mouse Cy3 (1:1000; Jackson ImmunoResearch Inc.). Cells were mounted in Mowiol (Calbiochem, San Diego, CA) with 1.0% n-propyl gallate.

Confocal microscopy images were collected on a laser scanning Zeiss510 Meta Confocal System (Thornwood, NY) with a 63x 1.4NA plan-Panchromatic oil immersion lens using an argon (488 nm) and a Hone (543 nm) laser for detection of GFP and Cy3 labeled proteins, respectively. The system was operated with a pinhole size of one Airy unit. A series of XY images were collected along the Z-axis with a 0.36 am step size. Image projections were generated with Zeus LSM Image Browser version 3.2. Contrast adjustment was generated using Adobe Photoshop for consistent presentation of morphology (version 6.0; San Jose, CA).

Data analysis. Data are expressed as the mean ± standard deviation (SD) from three measurements unless indicated otherwise. Where indicated, statistical significance was

assessed using a two sample Student's t tests. Samples were assumed to have an unequal variance; significant differences were assigned at $p < 0.05$.

RESULTS

Knockdown of PLCβ3 and PLCγ1 expression in MDCK cells

In order to unambiguously establish which PLC isozymes are expressed at significant levels in the MDCK II cells used for these studies, primers were designed for each PLCβ and PLCγ isozyme (Table 3.1), excluding PLCβ4, because the expression of this isozyme has been reported to be restricted to specific regions of the brain and retina[21, 22], and thus, would be unlikely to be expressed in a canine kidney epithelial cell line. Other isozyme families were not evaluated in this study, because there is no direct evidence to suggest that they are involved in the regulation of epithelial tight junctions. mRNA for PLCβ3 and PLCγ1 isozymes was clearly detected in MDCK cells via RT-PCR, while mRNA for PLCβ1, PLCβ2 and PLCγ2 was not (Figure 3.1), suggesting that PLCβ3 and PLCγ1 are the dominant receptor regulated PLCs expressed in these cells. Western blots further confirmed the protein expression of these two isozymes in MDCK cells (Figures 3.2.B and 3.3.B).

In order to directly investigate the role of individual PLC isozymes in the regulation of epithelial tight junction function, the expression of both PLCβ3 and PLCγ1 was suppressed using RNAi. MDCK cells were transiently transfected with free oligonucleotides using Amaxa nucleofection to knockdown expression of both proteins individually. Four siRNAs targeting either PLCβ3 or PLCγ1 were combined and used as pools in order to maximize knockdown efficiency. Efficiency of knockdown was quantified from immunoblots of cell lysates for up to five days post-transfection (Figures 3.2.A and 3.3.A). Both pools efficiently depleted targeted PLC isozymes in MDCK cells without affecting the expression levels of the non-targeted PLC. Depletion of PLCβ3 was more efficient than PLCγ1 with respect to both time and siRNA concentration (Figure 3.2 and Figure 3.3).

Maximum knockdown of PLCβ3 was achieved by day 3 (approximately 80%), was similar for all siRNA concentrations tested, and was maintained out to five days (Figure 3.2). Maximum knockdown of PLCγ1 was achieved by day 2 (approximately 70%), was more efficient with siRNA concentrations ≥ 5 μM, but interestingly, was not maintained throughout the five day test-period. Instead, protein expression returned to near control levels by day 5 (approximately 30% knockdown for all tested concentrations) (Figure 3.3). Subsequent experiments intended to evaluate the effect of PLC isozyme depletion on tight junction barrier function were performed on day 3 post-transfection using 2 and 5 μM siRNA for PLCβ3 and PLCγ1, respectively. One μg of pmaxGFP was co-transfected in all experiments to assess transfection efficiency. Notably, the transfection efficiency was determined to be approximately 70-80% (data not shown but consistent with reported transfection efficiency for MDCK II cells by Amaxa Inc. (Gaithersburg, MD)), suggesting that silencing in transfected cells approached 100% for each isozyme.

Knockdown of PLCβ3 and PLCγ1 expression in MDCK cells attenuates agonist induced PLC activation.

To confirm that specific knockdown of both PLCs leads to an isozyme specific reduction in PLC catalytic activity, the ability of known agonists for both isozymes (*i.e.* ATP for PLCβ and EGF for PLCγ) to stimulate PLC activity was assessed in knockdown cells, and compared to non-transfected and non-sense transfected cells. Specific knockdown of each isozyme resulted in almost complete abolishment of PLC activation by their respective agonists (Figure 3.4), confirming that the observed reduction in protein expression had functional consequences on the ability of exogenous agonists to stimulate the activity of the respective isozyme.

Maintenance of tight junction barrier function following PLC isozyme knockdown.

Chemical inhibition of PLC activity has previously been shown to increase paracellular permeability across both MDCK and Caco-2 cell monolayers (Chapter 2)[7, 8]. To address the possibility that either PLCβ3 or PLCγ1 is specifically involved in regulating epithelial tight junction function in MDCK cells, paracellular permeability was evaluated in knockdown cells and compared to non-transfected and non-sense transfected cells. Sub-confluent MDCK cells were transfected in suspension and over-seeded in order to rapidly establish a confluent monolayer[16]. This seeding density and subsequent rapid tight junction formation provides the opportunity to assess the importance of each PLC isozyme on the maintenance of barrier function. On each day following transfection and seeding, TEER was measured to assess barrier function. Depletion of either PLCβ3 or PLCγ1 had no effect on the resistance across MDCK cell monolayers, suggesting that epithelial tight junction function is unaffected by knockdown of either enzyme (Figure 3.5.A). To further confirm that tight junction barrier function is maintained in knockdown cells, the permeability of paracellular marker mannitol was determined on day three, or the point of maximal knockdown. Consistent with TEER data, the permeability of [^{3}H]-mannitol was unchanged in knockdown cells (Figure 3.5.B), confirming the lack of effect of PLC isozyme knockdown on tight junction barrier function in MDCK cells.

Establishment of tight junction barrier function following PLC isozyme knockdown.

To evaluate the effects of PLC isozyme depletion on the assembly of tight junctions, knockdown cells were subjected to a calcium switch assay (see Materials and Methods) and compared to non-transfected and non-sense transfected cells (Figure 3.5.C). Following the re-addition of normal calcium medium to cells, both control cells and knockdown cells

established tight junctions within two hours, as indicated by the initial increase in TEER. Further, the TEER profile over the following twenty four hour period was identical in all conditions, and returned to initial levels by twenty four hours. This result suggests that neither PLCβ3 nor PLCγ1 is necessary for the formation of epithelial tight junctions in MDCK cells.

Maintenance and establishment of tight junction barrier function following simultaneous knockdown of both PLCβ3 and PLCγ1.

The individual knockdown of PLCβ3 and PLCγ1 isozymes had no impact on either the maintenance of already established tight junctions, or on the biogenesis of tight junctions following calcium switch. To assess the possibility that the function of these two isozymes is redundant in MDCK cells, and that one isozyme can compensate for the reduced or eliminated function of the other, the expression of both isozymes was depleted simultaneously using RNAi for up to five days post-transfection (Figure 3.6). Consistent with individual knockdown studies, the knockdown of PLCβ3 was more efficient than the knockdown of PLCγ1. Maximum knockdown of PLCγ1 was achieved by day one (approximately 60%), but again was not maintained throughout the five day test-period, as protein expression returned to near control levels by day 5. Knockdown of PLCβ3 was again maximized at day three (approximately 80%); interestingly though, the knockdown of PLCβ3 was not maintained throughout the five day test-period as it was when depleted alone. On the other hand, expression increased at day four compared to day three and had returned to 50% of control by day five. Subsequent experiments intended to evaluate the effect of combined knockdown on tight junction barrier function were performed on day 3 post-transfection using 2 and 5 μM siRNA oligonucleotides for PLCβ3 and PLCγ1, respectively.

Consistent with individual knockdown studies, simultaneous depletion of both PLCβ3 and PLCγ1 had no effect on TEER (Figure 3.7.A), or on the permeability of paracellular marker mannitol across MDCK cell monolayers (Figure 3.7.B), suggesting that epithelial paracellular permeability is unaffected by simultaneous depletion of both enzymes. In addition, the combined knockdown had no impact on the establishment of TEER following calcium switch (Figure 3.7.C). These results further suggest that neither PLCβ3 nor PLCγ1 isozymes are required for the maintenance or formation of stable epithelial tight junctions; in addition, the results exclude the possibility that PLC isozyme function is redundant.

Subcellular localization of tight junction proteins following knockdown of PLCβ3 and PLCγ1.

Despite the absence of functional consequences to tight junction barrier function in MDCK cells following RNAi-mediated depletion of specific PLC isozymes, the effects of knockdown on the subcellular localization of tight junction proteins was examined. As previously indicated, specific downstream events have been associated with the PLC inhibition-mediated increase in paracellular permeability, including redistribution of tight junction proteins ZO-1[5] and claudin-1[6], as well as hyperphosphorylation of ZO-1, ZO-2, and occludin[7]. To examine the possibility that these observed events are a result of PLC inhibition, but not related to changes in tight junction barrier function, the subcellular localization of ZO-1 and ZO-2, as well as transmembrane protein occludin, was assessed in combination knockdown cells and compared to non-sense transfected cells. As previously reported, both ZO-1 and occludin were expressed exclusively at cell-cell junctions in control cells[23-27], while ZO-2 demonstrated junctional staining, as well as some speckled cytosolic and nuclear staining[28-31]. As shown in Figures 3.8, 3.9, and 3.10, the subcellular localization

of these three tight junction associated proteins was unaffected when PLCβ3 and PLCγ1 were depleted in combination.

The potency of hexadecylphosphocholine and U73122 as paracellular permeability enhancers in PLCβ3 and PLCγ1 depleted MDCK cells.

Because the specific knockdown of PLCβ3 and PLCγ1 in MDCK cells did not cause changes to the barrier function of tight junctions, the effects of previously established PLC inhibitors and paracellular permeability enhancers, U73122 and HPC (Figure 3.11), on paracellular permeability in knockdown cells was explored. The rationale for this study is as follows: if U73122 and HPC increase paracellular permeability via direct inhibition of PLC, their potency as paracellular permeability enhancers should be significantly reduced in knockdown cells due to the absence of their proposed pharmacological target. As shown in Figures 3.12 and 3.13, the ability of both HPC and U73122 to increase paracellular permeability (*i.e.* decrease TEER and increase mannitol permeability) was unchanged in knockdown cells as compared to non-transfected and non-sense transfected cells. This result is consistent with the effect of RNAi-mediated PLC depletion on paracellular permeability, and further suggests that inhibition of PLC activity does not lead to an increase in paracellular permeability across MDCK cell monolayers.

DISCUSSION

The tight junction forms a charge and size-selective barrier between adjacent epithelial cells restricting the passage of ions, water, and non-charged solutes through the intercellular space. This intricate protein complex consists of both transmembrane proteins that combine to form the physical barrier of the tight junction by spanning the intercellular space and interacting with transmembrane proteins on neighboring cells (*e.g.* claudins and occludin), as well as membrane scaffolding proteins that link the transmembrane proteins to the actin cytoskeleton and arrange them adjacent to important cytosolic regulatory proteins (*e.g.* ZO-1, ZO-2, ZO-3). An abundance of data has emerged in recent years implying that the function of these intercellular structures is influenced by numerous physiological and pathophysiological stimuli[32, 33], suggesting that their barrier properties can be manipulated by pharmacological intervention of regulatory signal transduction pathways[34-36]. One such pathway implicated in the regulation of tight junction barrier function is the PLC-mediated signal transduction cascade. Chemical inhibition of PLC enzymes has been strongly associated with increased tight junction permeability in two models of epithelia, MDCK and Caco-2 cells (Chapter 2)[7, 8]. In the present study, RNA interference was employed to specifically inhibit the functional activity of PLC isozymes in MDCK cells, in an attempt to provide direct evidence for a cause-effect relationship between reduced PLC activity and loss of tight junction barrier function.

The expression profile of specific PLCβ and PLCγ isozymes in MDCK cells has not been clearly defined. One report suggests that PLCβ1, β2, and β3 isozymes are all expressed at the protein level[37], and although β1 and β3 tissue expression is reported to be widespread, the expression of β2 appears restricted to cells of hematopoietic origin[38], making their

presence in kidney cells a surprise. A recent report has clearly demonstrated the expression of PLCγ1 in MDCK cells[39], although little evidence supporting the expression of PLCγ2 in these cells has surfaced. Therefore, RT-PCR was initially utilized to establish the presence of individual PLCβ and PLCγ isozymes in MDCK cells. As shown in Figure 3.1, two isozymes were highly expressed at the mRNA level, one from each family (*i.e.* PLCβ3 and PLCγ1), while no PLCβ1, PLCβ2 or PLCγ2 were detected, implying that PLCβ3 and PLCγ1 are the only isozymes of their respective families expressed in MDCK II cells. Western blot analysis confirmed expression at the protein level for both isozymes (Figures 3.2.B and 3.3.B).

After establishing which individual isozymes of these two PLC families are viable targets in MDCK II cells, siRNA oligonucleotide pools were used to specifically deplete the expression of each isozyme alone, and in combination. Complete suppression of PLC expression was not achieved for either isozyme, nor was it expected given the transient transfection methodology utilized to deliver siRNA to cells; therefore, it is conceivable that residual protein would be sufficient to maintain their respective functional roles and responsibilities within the entire population of cells. Because transfection efficiency was determined to be approximately 70%, silencing in transfected cells was assumed to approach 100%. Complete suppression of each isozyme in such a high percentage of cells seems sufficient to expect global changes to the functional behavior of each with respect to the hypothesized changes to paracellular permeability across a monolayer of transfected cells. Hence, the ability of known agonists for each isozyme to stimulate PLC catalytic activity in knockdown cells was evaluated. Exogenously administered ATP and EGF have been previously reported to increase the production of inositol phosphates in epithelial cells, end-products of PLC catalytic action, via activation of cell surface receptors specifically linked to

PLCβ and PLCγ isozymes, respectively (Chapter 2)[7, 8, 40-42]. As shown in Figure 3.4, the ability of ATP and EGF to stimulate the activity of their respective isozymes was almost completely abolished in knockdown cells, implying that the achieved knockdown efficiency via siRNA is substantial enough to expect global changes to the functional consequences of PLC action within the entire population of cells. In further support of this hypothesis, it was noted that the suppression of PLCγ1 was not maintained throughout the five day test period as it was for PLCβ3; instead, expression returned to near control levels by day five (Figure 3.3.A). A recent study in MDCK cells reports that RNAi mediated suppression of PLCγ1 leads to reduced cell migration[39]; therefore, the inability to maintain stable knockdown of PLCγ1 for extended periods in the present study may be due to the following scenario: the knockdown cells were unable to grow and migrate at typical rates following transfection; however, the small percentage of non-transfected cells maintaining normal growth properties continued to grow normally, and (increasingly) overcame the suppressed growth function of the initial population of transfected cells as time in culture progressed, leading to an apparent recovery of expression levels for PLCγ1. This observation implies that a known functional consequence of PLCγ1 suppression (*i.e.* cell migration) was reduced in transfected cells in the present study. If so, it could be argued that other functions of this isozyme should also be adversely affected, including regulation of paracellular permeability. Interestingly, when both PLC isozymes were depleted simultaneously, the knockdown of PLCβ3 was not maintained throughout the five day period as it was when this isozyme was suppressed alone. Instead, its expression returned to 50% of control by day five, suggesting that the PLCγ1 phenotype (*i.e.* reduced cell migration) affects the knockdown efficiency of other suppressed proteins. To unequivocally reach this conclusion however, cells lines stably expressing siRNA targeting

each PLC isozyme would need to be generated in order to achieve complete knockdown of each isozyme, and unambiguously assess functional consequences.

Once sufficient knockdown of each isozyme was achieved, the impact of PLC depletion on tight junction barrier properties in MDCK cells was evaluated. When plated at sufficiently high density, MDCK cells begin to form junctions within a few hours, and are completely polarized within twenty four hours[16]. On the other hand, maximal suppression of PLCs was not achieved until day two, after mature tight junctions have already been formed. This temporal separation allows the opportunity to address the hypothesis that PLC isozymes are required for the maintenance of an established tight junction barrier and are involved in regulating paracellular permeability across cell monolayers. Data presented in Figure 3.5 and 3.7 suggest that depletion of both PLC isozymes, either alone or in combination, has no impact on either the maintenance or assembly of tight junctions in MDCK cells, as assessed by the permeability of paracellular marker mannitol and by TEER formation following calcium switch. These results contradict previous reports using chemical inhibitors of PLC that have demonstrated significant increases in paracellular permeability across cell monolayers following treatment with these inhibitors (Chapter 2)[5-8].

Because specific changes in either the subcellular localization or phosphorylation status of ZO-1, ZO-2, and occludin have all been previously associated with increased paracellular permeability, presumably resulting from chemical inhibition of PLC activity[5, 7], the subcellular localization of each of these tight junction associated proteins was evaluated in control and knockdown cells. Despite the fact that PLC knockdown had no apparent effect on the maintenance of barrier function, it was possible that depletion of PLC enzymes could lead to altered localization and function of tight junction proteins without measurable

changes to paracellular permeability, as there is no definitive evidence that the observed changes to these proteins in previous studies was the ultimate cause of the increased permeability. More recently, evidence for direct binding of PLCβ3 to the PDZ3 domain of ZO-1 has been reported[43], confirming a direct interaction of PLC with tight junction scaffolding proteins, and supporting a hypothesis that this isozyme regulates the function of ZO-1. As shown in Figures 3.8, 3.9, and 3.10, depletion of PLCβ3 and PLCγ1 alone or in combination had no visibly significant impact on the subcellular localization of ZO-1, ZO-2, or occludin. These results imply that previously observed changes to specific tight junction proteins is not likely related to inhibition of PLC activity, although the phosphorylation status of each protein was not evaluated in the current study. Further, these results imply that neither PLCβ3 nor PLCγ1 play a role in maintaining the subcellular localization of these proteins once mature tight junctions have been established. However, as indicated earlier, in order to unequivocally reach this conclusion, cells lines stably expressing siRNA targeting each PLC isozyme should be used to achieve complete knockdown of each isozyme before completely ruling out a role for PLC isozymes in the subcellular localization of these proteins.

One explanation for the apparent contradiction between the present study and previous studies with chemical inhibitors could be that purported specificity of reported PLC inhibitors was overestimated, and that their effects on paracellular permeability are mediated by effects unrelated to PLC. As indicated earlier, some literature reports with these compounds have suggested that they may be non-specific in nature, and exert "off-target" effects on cultured cells[9-12], consistent with a hypothesis that their ability to inhibit PLC activity is not related to the observed effects on tight junction barrier properties. To provide

additional evidence to support the current findings (*i.e.* PLC inhibition has no impact on tight junction barrier properties), the effect of HPC and U73122 (Figure 3.11) on paracellular permeability was evaluated in cells depleted of both PLCβ3 and PLCγ1, and directly compared to non-transfected and non-sense transfected cells. If the effects of these two PLC inhibitors on tight junctions are mediated via their interaction with PLC, their potency as permeability enhancers should be reduced or eliminated in cells lacking PLC isozymes. As shown in Figure 3.12 and Figure 3.13, the potency of both U73122 and HPC as paracellular permeability enhancers was unchanged in knockdown cells, providing further evidence that inhibition of PLC does not lead to increase paracellular permeability in epithelial cells. In addition, this result implies that U73122 and HPC cause increased paracellular permeability by mechanisms other than inhibition of PLC activity; although additional studies are required to confirm this hypothesis.

The results presented in this study provide evidence to suggest that neither PLCβ3 nor PLCγ1 isozymes are required for the maintenance, or formation, of tight junction barrier function in MDCK cells. However, as isozymes from other PLC families that may also be expressed in MDCK cells were not suppressed, these results do not rule out the possibility that inhibition of other PLCs leads to changes in paracellular permeability; a report that HPC inhibits the activity of PLCδ1 provides support for such a hypothesis[44]. Further, these results do not completely rule out a potential role for PLCβ and PLCγ isozymes in the regulation of tight junction function. A number of studies have indirectly suggested that PLC activation may lead to changes in the function of junctional complexes. For example, a number of agonists of receptors known to couple to PLCs have been reported to affect the functional integrity of tight junctions[45-48]. Further, increase in intracellular calcium levels[49, 50], activation

of specific PKC isozymes[51, 52], as well as non-PLC-mediated depletion of membrane PIP_2 content[43, 53], all downstream effects of PLC activation, have been demonstrated to increase paracellular permeability or alter the barrier function of junctional complexes in a number of systems. Clearly, further study is warranted to probe this hypothesis.

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I would like to acknowledge the laboratory of Dr. James Anderson, including Dr. Alan Fanning, Dr. Christina Van Italia, and Jenny Holmes (Department of Cellular and Molecular Physiology, School of Medicine, University of North Carolina, Chapel Hill, NC) for helpful discussions and assistance with these studies. I would also like to acknowledge the important contributions of Wendy Salmon and Dr. Michael Chua of the Michael Hooker Microscopy Facility at UNC Chapel Hill for advice and assistance with confocal microscopy imaging.

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Table 3.1. Designed primer sequences for canine PLCs.

Protein	Forward Primer (base pair position)	Reverse Primer (base pair position)	Accession #	Product Size (bp)
PLCβ1	5'-tcggtcaccggatcttgccagt (2252-2273)	5'-agctggaagcatcagaggctggt (2346-2324)	XM542896	95
PLCβ2	5'-cggtggatcgcattgacgtggt (2015-2036)	5'-aggtgcgcacactgcgttca (2104-2085)	XM544615	90
PLCβ3*	5'-ggccattgcagaaaccgcct (1292-1311)	5'-ttggcctgctgcttcgctga (1381-1362)	XM533243	90
PLCγ1	5'-atgcgcctttcagagccggt (1831-1850)	5'-acgcatcagcatgtgctcgg (1932-1913)	XM542998	102
PLCγ2	5'-cgacgtcgtgcaggccatcaaa (1156-1177)	5'-tgctgctccacgcaacagtg (1248-1229)	XM546812	93

All primers were designed using Primer3 software [54].

PLCβ4 has been reported to be specifically localized to regions of the brain and retina[21, 22]; therefore, its expression in MDCK cells was not assessed.

* Eleven splice variants are reported in Pubmed for canine PLCβ3. The designed forward primer sequence is present in all eleven, however, the amino acid position is different in variant 3 (XM861950). The designed reverse primer sequence is present in only nine of the eleven reported variants, excluding variant 4 (XM861961) and 5 (XM861973); in addition, the amino acid position is different in variant 3.

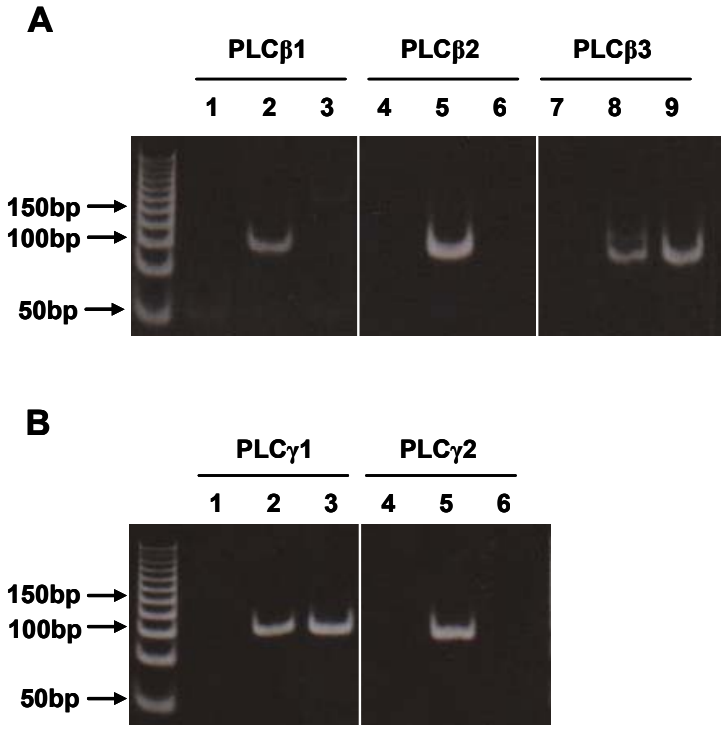


Figure 3.1. PLC mRNA expression in MDCK cells by qualitative RT-PCR. Products of RT-PCR amplification reactions were run on an 8% TBE-PAGE gel and visualized via Sybr-green fluorescence. (A) Expression of PLCβ isozymes in MDCK cells. Lanes 1, 4, and 7, represent negative controls, *i.e.* non-reverse-transcribed mRNA from MDCK cells. Lanes 2, 5, and 8 represent positive control tissues, *i.e.* reverse transcribed mRNA isolated from canine intestine, whole blood, and canine intestine respectively. Lanes 3, 6, and 9 represent MDCK cells, *i.e.* reverse transcribed mRNA isolated from MDCK cells. (B) Expression of PLCγ isozymes in MDCK cells. Lanes 1 and 4 represent negative controls, *i.e.* non-reverse-transcribed mRNA from MDCK cells. Lanes 2 and 5 represent positive control tissues, *i.e.* reverse transcribed mRNA isolated from canine intestine and whole blood, respectively. Lanes 3 and 6 represent MDCK cells, *i.e.* reverse transcribed mRNA isolated from MDCK cells.

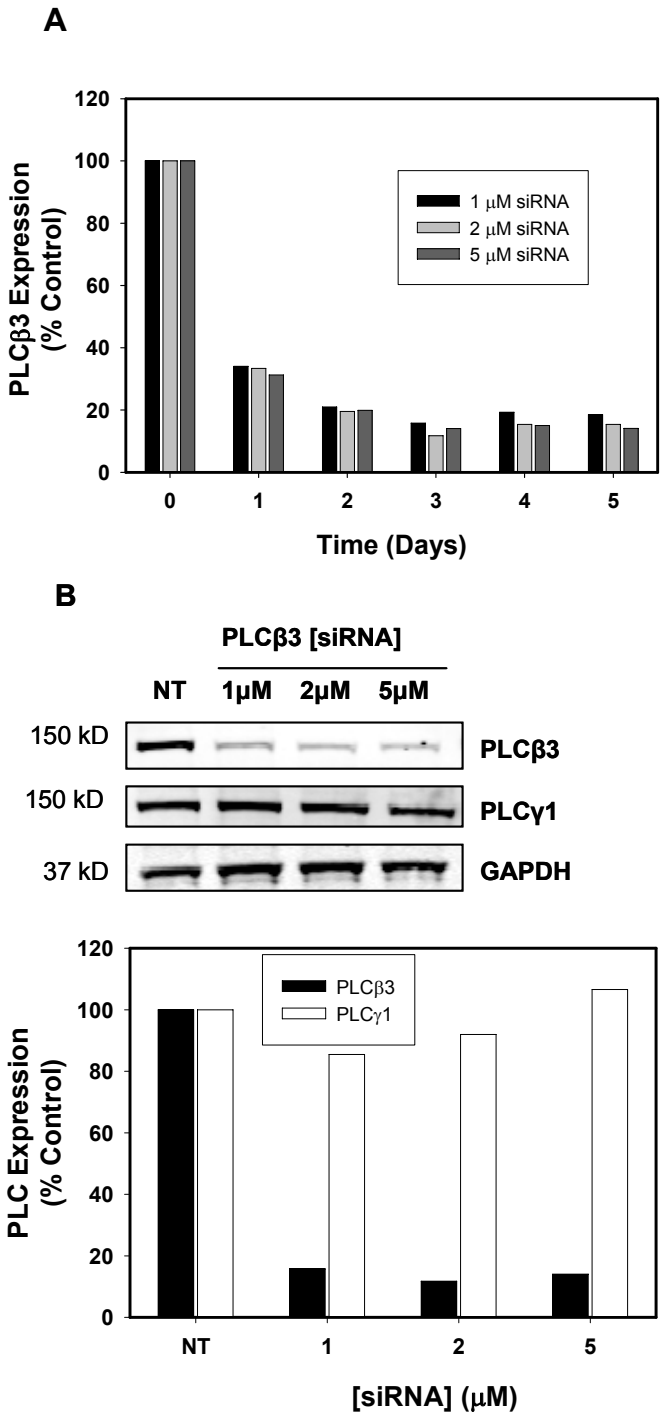


Figure 3.2. RNAi-mediated knockdown of PLCβ3 in MDCK cells. MDCK cells were transfected with siRNA oligonucleotides targeting PLCβ3 and expression was compared to non-transfected cells (NT). GAPDH served as loading control. (A) Expression of PLCβ3 with respect to days in culture and siRNA concentration. (B) Expression of PLCβ3and PLCγ1 on day three post-transfection with respect to siRNA concentration.

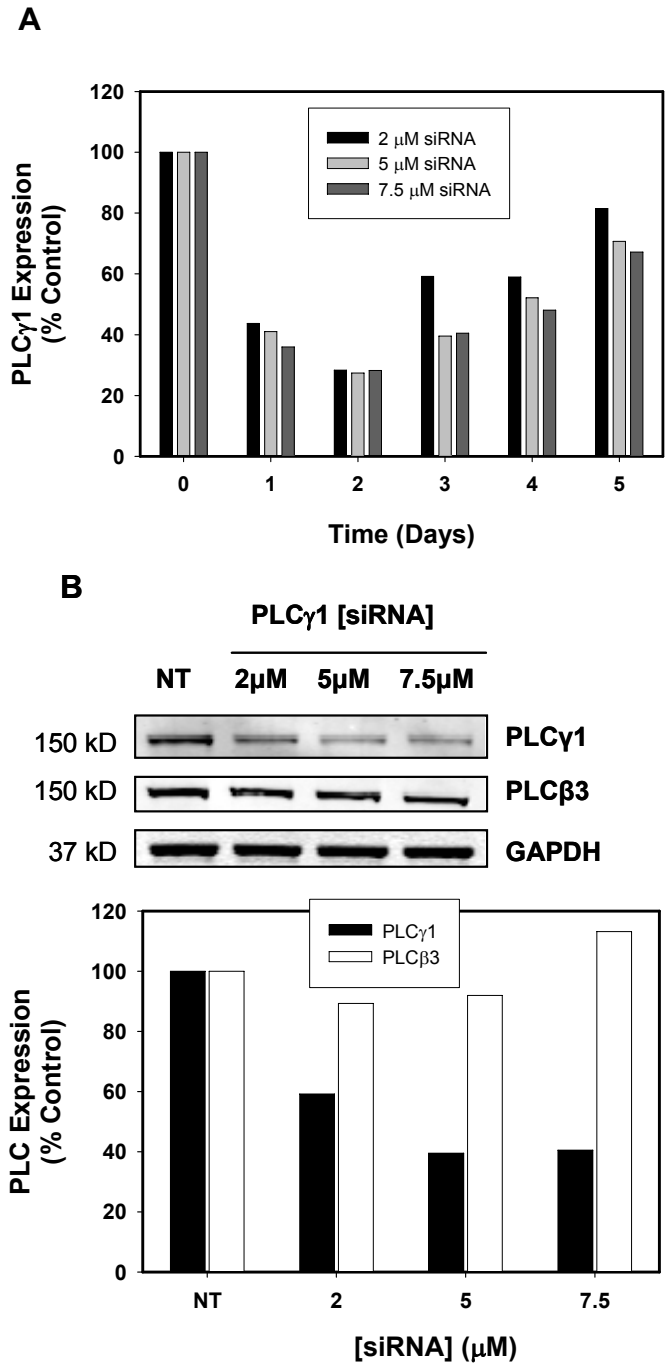


Figure 3.3. RNAi-mediated knockdown of PLCγ1 in MDCK cells. MDCK cells were transfected with siRNA oligonucleotides targeting PLCγ1 and expression was compared to non-transfected cells (NT). GAPDH served as loading control. (A) Expression of PLCγ1 with respect to days in culture and siRNA concentration. (B) Expression of PLCγ1and PLCβ3 on day three post-transfection with respect to siRNA concentration.

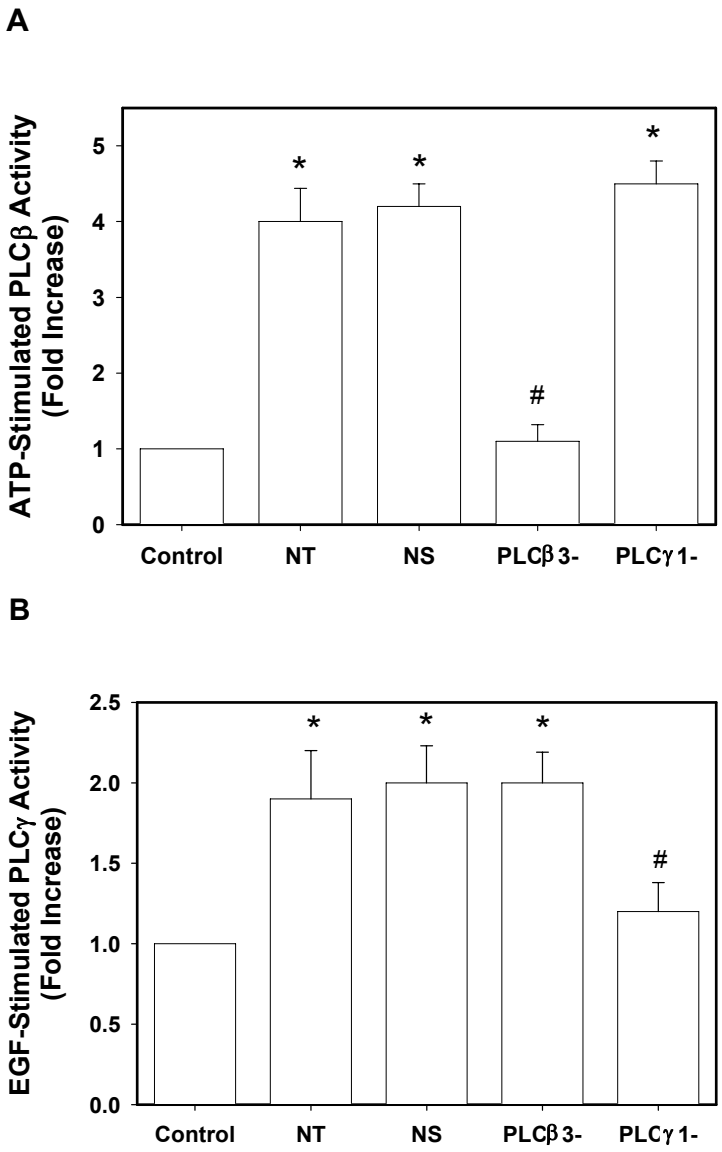


Figure 3.4. The effect of RNAi-mediated knockdown of PLCβ3 and PLCγ1 on agonist induced PLC activation in MDCK cells. Cellular accumulation of [^{3}H]-inositol phosphates was determined during a fifteen minute treatment period with either (A) 100 μM ATP or (B) 100 ng/ml EGF, to stimulate PLCβ and PLCγ activity respectively, and was compared to non-transfected (NT) and non-sense transfected (NS) cells. Data represent mean ± standard deviation from triplicate determinations. Error bars are omitted from control cells in both figures to simplify data presentation, as each tested condition was compared to its own set of un-stimulated cells that served as control. Asterisks (*) indicate significant difference ($p < 0.05$) compared to control. Number sign (#) indicates significant difference ($p < 0.05$) compared to NT.

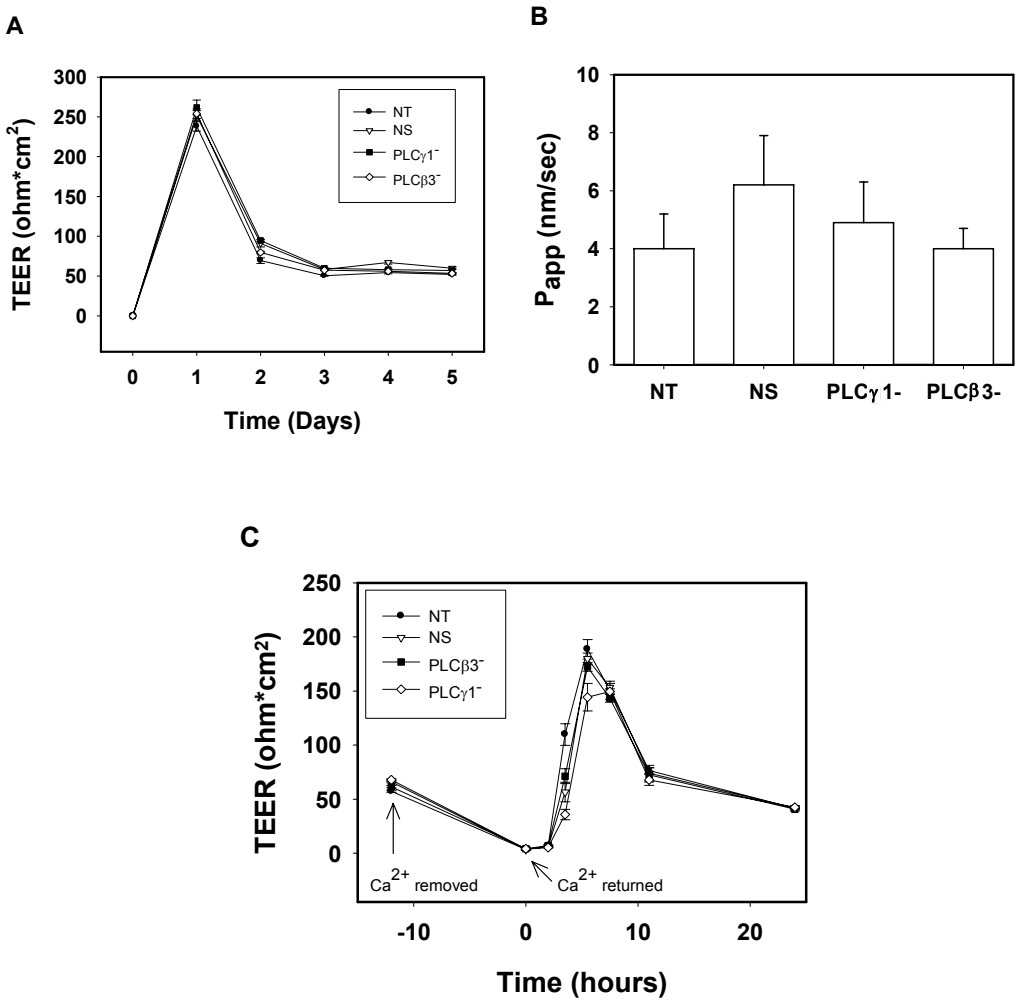


Figure 3.5. The effect of RNAi-mediated knockdown of PLCβ3 and PLCγ1 on tight junction barrier function in MDCK cells. Knockdown cells were compared to both non-transfected (NT) and non-sense transfected (NS) cells. (A) TEER was measured every twenty four hours for five days following transfection. (B) On day three, paracellular marker mannitol was added to the apical compartment and absorptive permeability during the 30-60 minute interval post-dose was determined. (C) On day two following transfection, low calcium media was applied to cells for twelve hours. Following the twelve hour treatment period, normal calcium media was returned to cells and TEER was measured for twenty for hours to assess tight junction formation. Data represent mean ± standard deviation from triplicate determinations.

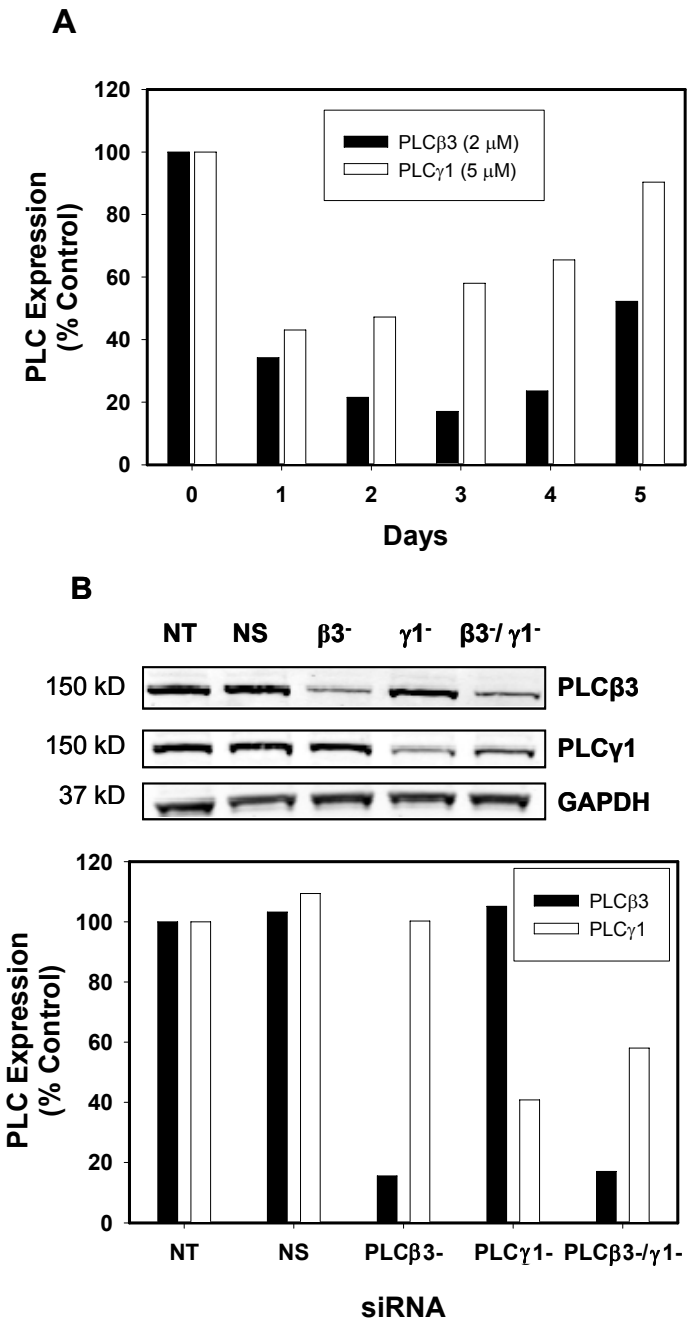


Figure 3.6. RNAi-mediated knockdown of PLCβ3 and PLCγ1 alone and together in MDCK cells. MDCK cells were transfected with siRNA oligonucleotides targeting both cPLCβ3 (2 μM) and cPLCγ1 (5 μM) and expression was compared to non-transfected (NT) cells. Non-sense transfected cells (NS) served as an additional control. GAPDH served as loading control. (A) Expression of both isozymes with respect to days in culture during simultaneous knockdown. (B) Expression of both isozymes on day three post transfection alone and in combination.

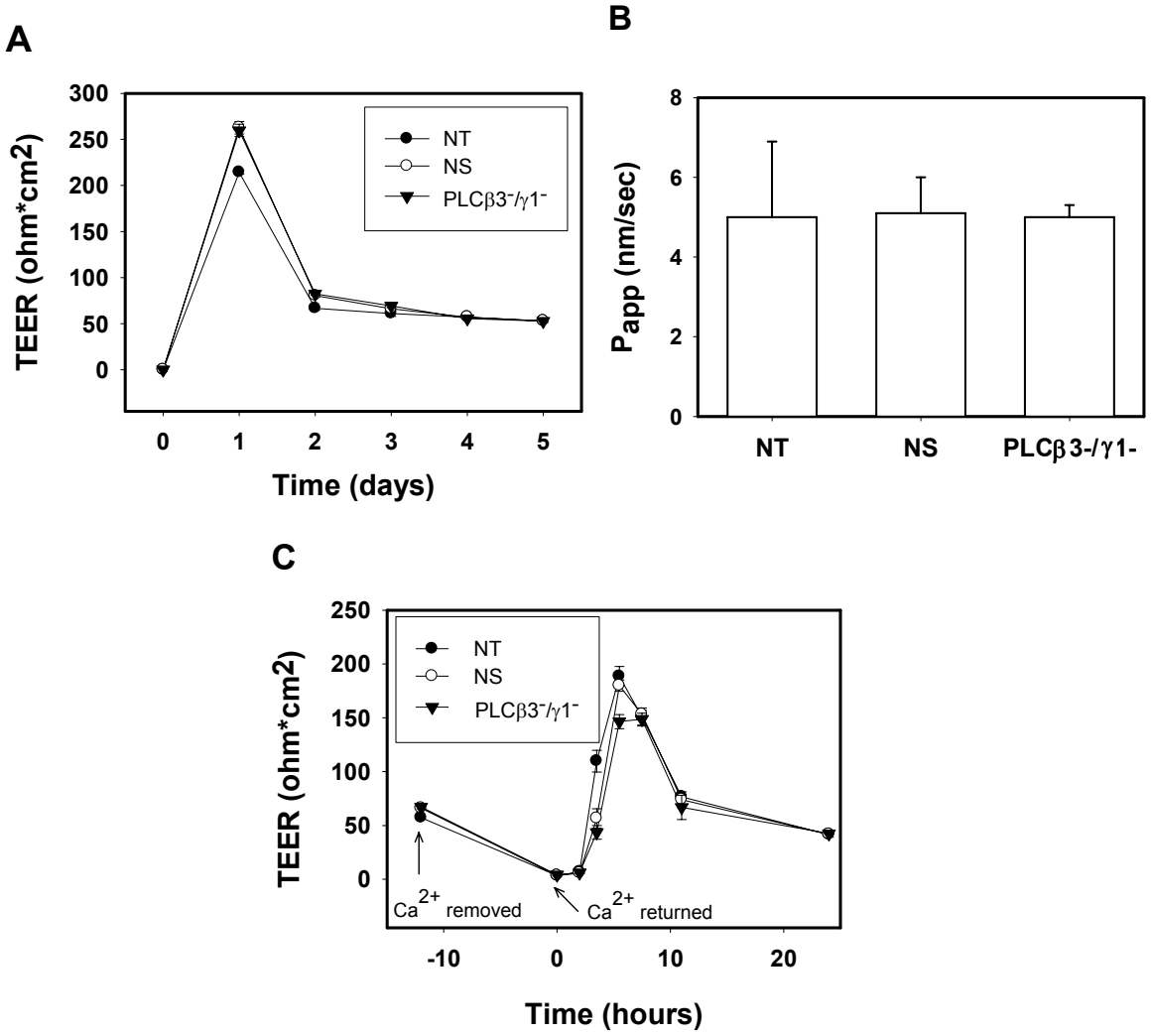


Figure 3.7. The effect of simultaneous RNAi-mediated knockdown of PLCβ3 and PLCγ1 on tight junction barrier function in MDCK cells. Knockdown cells were compared to non-transfected (NT) and non-sense transfected (NS) cells. (A) TEER was measured every twenty four hours for five days following transfection. (B) On day three, paracellular marker mannitol was added to the apical compartment and absorptive permeability during the 30-60 minute interval post-dose was determined. (C) On day two following transfection, low calcium media was applied to cells for twelve hours. Following the twelve hour treatment period, normal calcium media was returned to cells and TEER was measured for twenty for hours to assess tight junction formation. Data represent mean ± standard deviation from triplicate determinations.

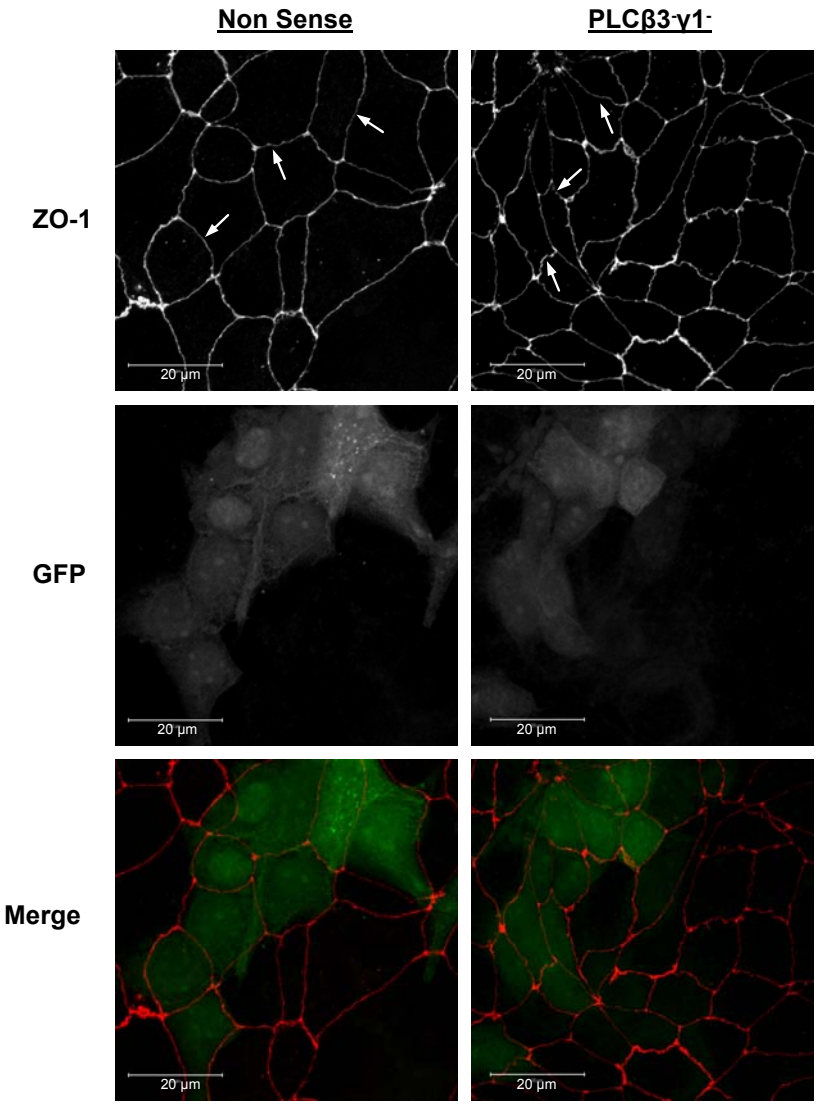


Figure 3.8. The effect of simultaneous RNAi-mediated knockdown of PLCβ3 and PLCγ1 on the subcellular localization of tight junction protein ZO-1. Knockdown cells were compared to non-sense transfected cells (NS). Cells were fixed with 1% paraformaldehyde and stained with appropriate antibodies on day three post-transfection. GFP was used to identify transfected cells. Cell populations were selected that contained a mixed population of transfected and non-transfected cells. Arrows indicate cell borders of transfected cells. Images were collected using a Zeiss510 Meta confocal microscope with a 63x plan-Apochromat oil immersion lens. Maximum XY projections were generated with Zeiss LSM Image Browser version 3.2 and processed using Adobe Photoshop. Individual staining of ZO-1 and GFP are presented in black and white to provide increased contrast and clarity while merged image is presented in color.

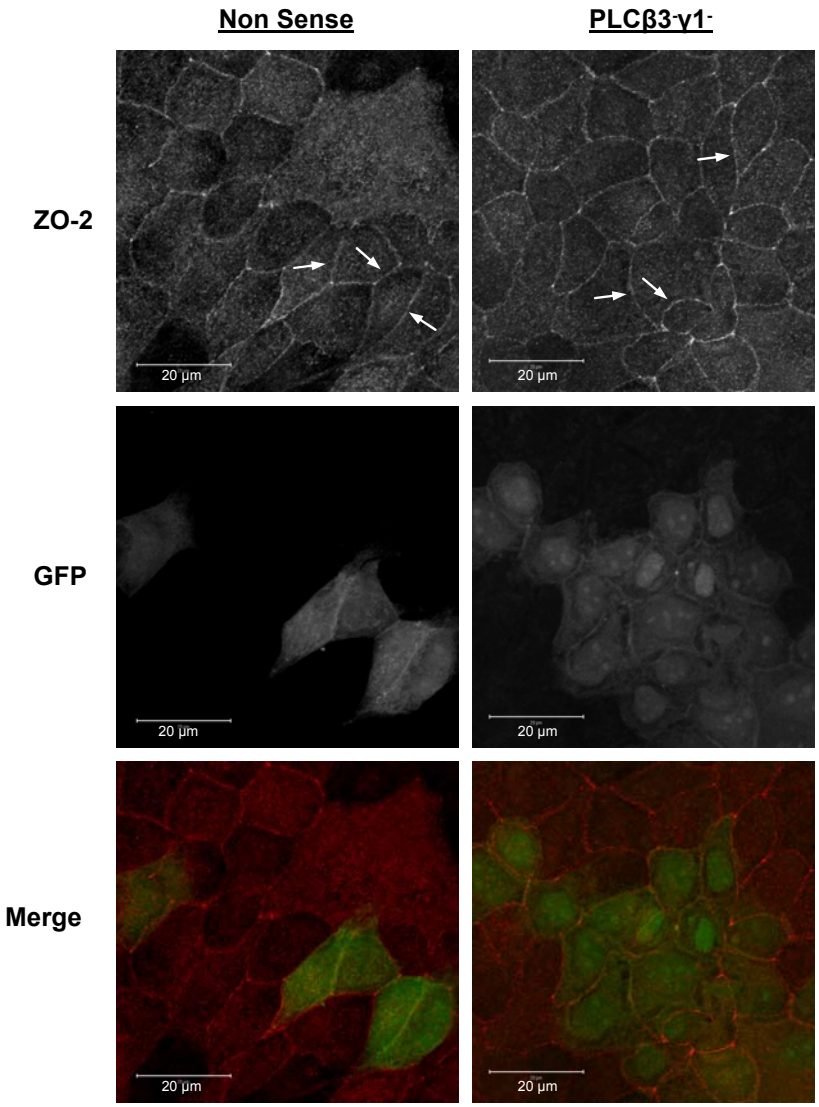


Figure 3.9. The effect of simultaneous RNAi-mediated knockdown of PLCβ3 and PLCγ1 on the subcellular localization of tight junction protein ZO-2. Knockdown cells were compared to non-sense transfected cells (NS). Cells were fixed with 1% paraformaldehyde and stained with appropriate antibodies on day three post-transfection. GFP was used to identify transfected cells. Cell populations were selected that contained a mixed population of transfected and non-transfected cells. Arrows indicate cell borders of transfected cells. Images were collected using a Zeiss510 Meta confocal microscope with a 63x plan-Apochromat oil immersion lens. Maximum XY projections were generated with Zeiss LSM Image Browser version 3.2 and processed using Adobe Photoshop. Individual staining of ZO-2 and GFP are presented in black and white to provide increased contrast and clarity while merged image is presented in color.

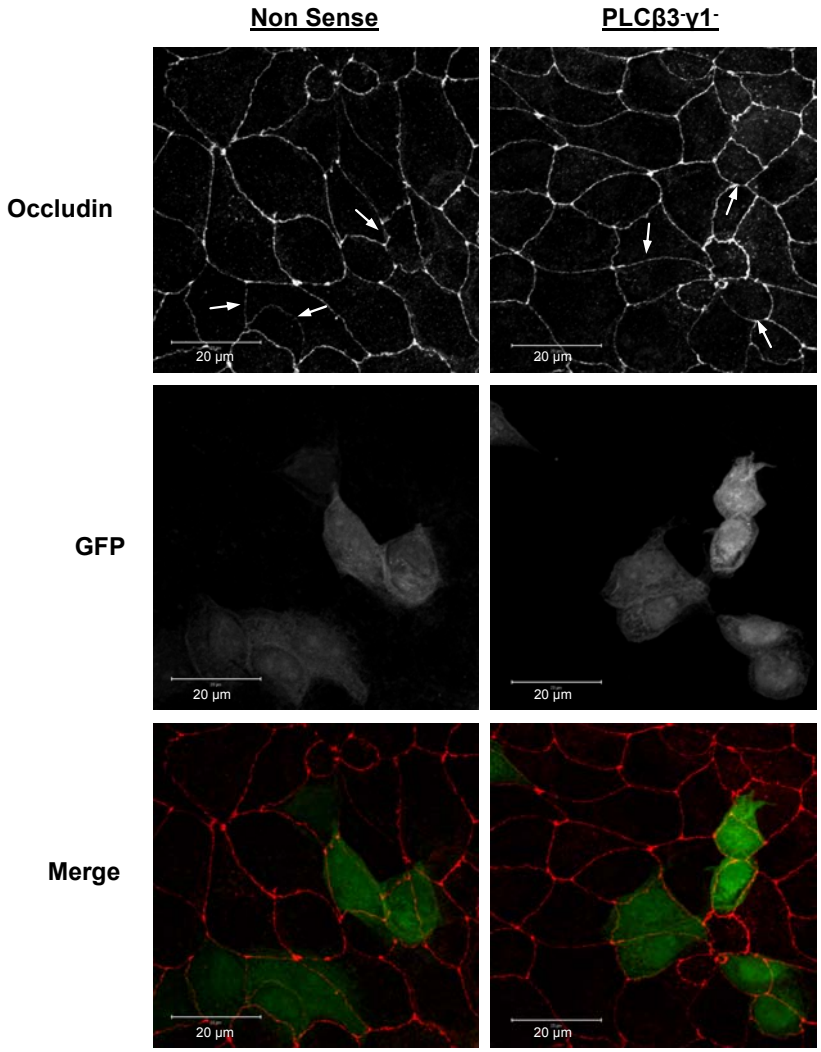
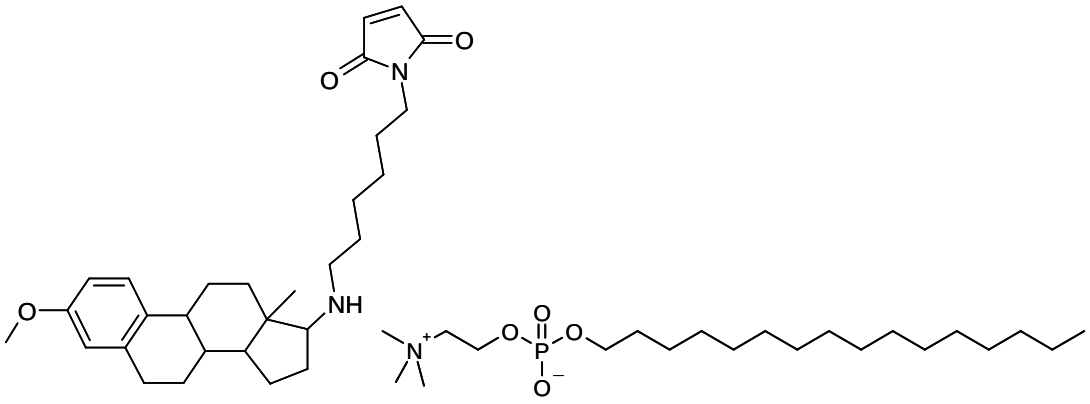


Figure 3.10. The effect of simultaneous RNAi-mediated knockdown of PLCβ3 and PLCγ1 on the subcellular localization of tight junction protein occludin. Knockdown cells were compared to non-sense transfected cells (NS). Cells were fixed with 1% paraformaldehyde and stained with appropriate antibodies on day three post-transfection. GFP was used to identify transfected cells. Cell populations were selected that contained a mixed population of transfected and non-transfected cells. Arrows indicate cell borders of transfected cells. Images were collected using a Zeiss510 Meta confocal microscope with a 63x plan-Apochromat oil immersion lens. Maximum projections were generated with Zeiss LSM Image Browser version 3.2 and processed using Adobe Photoshop. Individual staining of occludin and GFP are presented in black and white to provide increased contrast and clarity while merged image is presented in color.



U73122 **HPC**

Figure 3.11. Structures of U73122 and hexadecylphosphocholine (HPC).

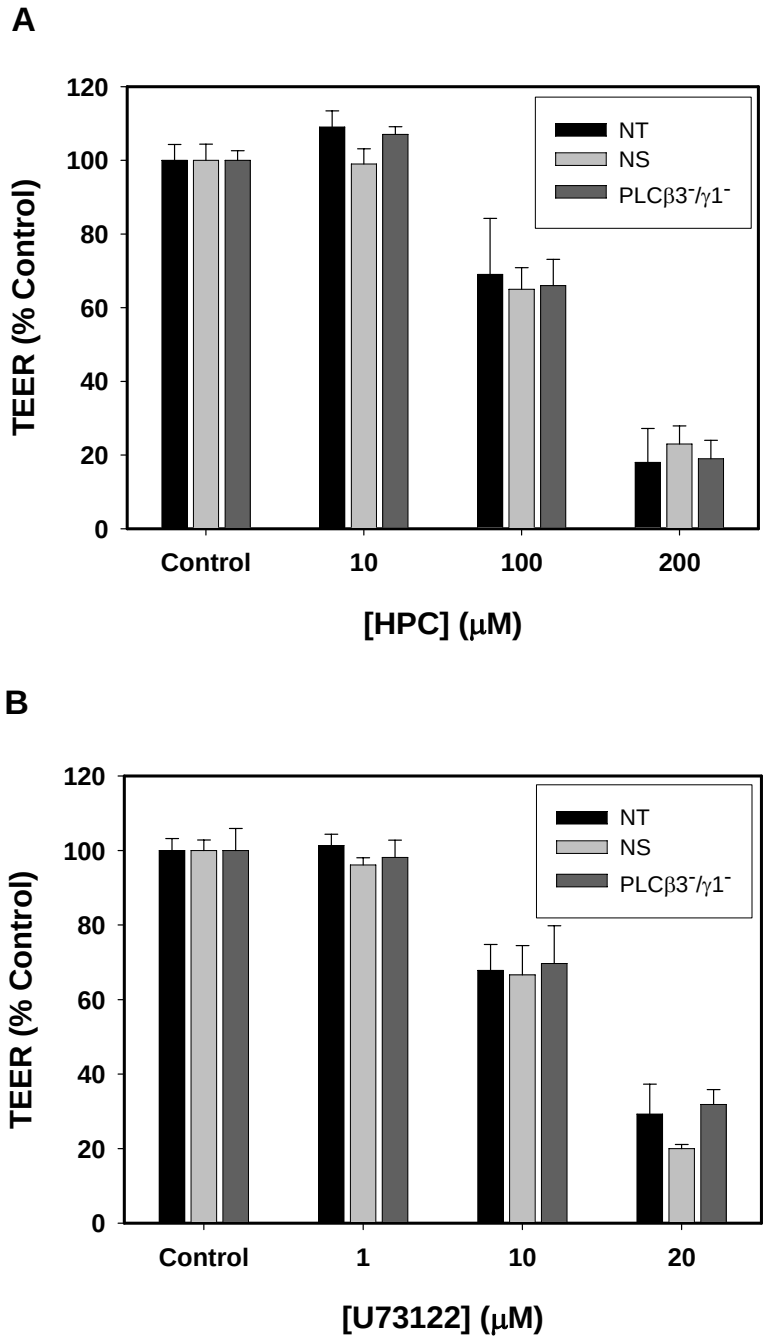


Figure 3.12. The effect of simultaneous RNAi-mediated knockdown of PLCβ3 and PLCγ1 on the potency of HPC and U73122 as paracellular permeability enhancers in MDCK cells. Cells were treated apically with either (A) HPC or (B) U73122 at indicated concentrations for thirty minutes and TEER was measured. Data represent mean ± standard deviation from triplicate determinations from one representative experiment.

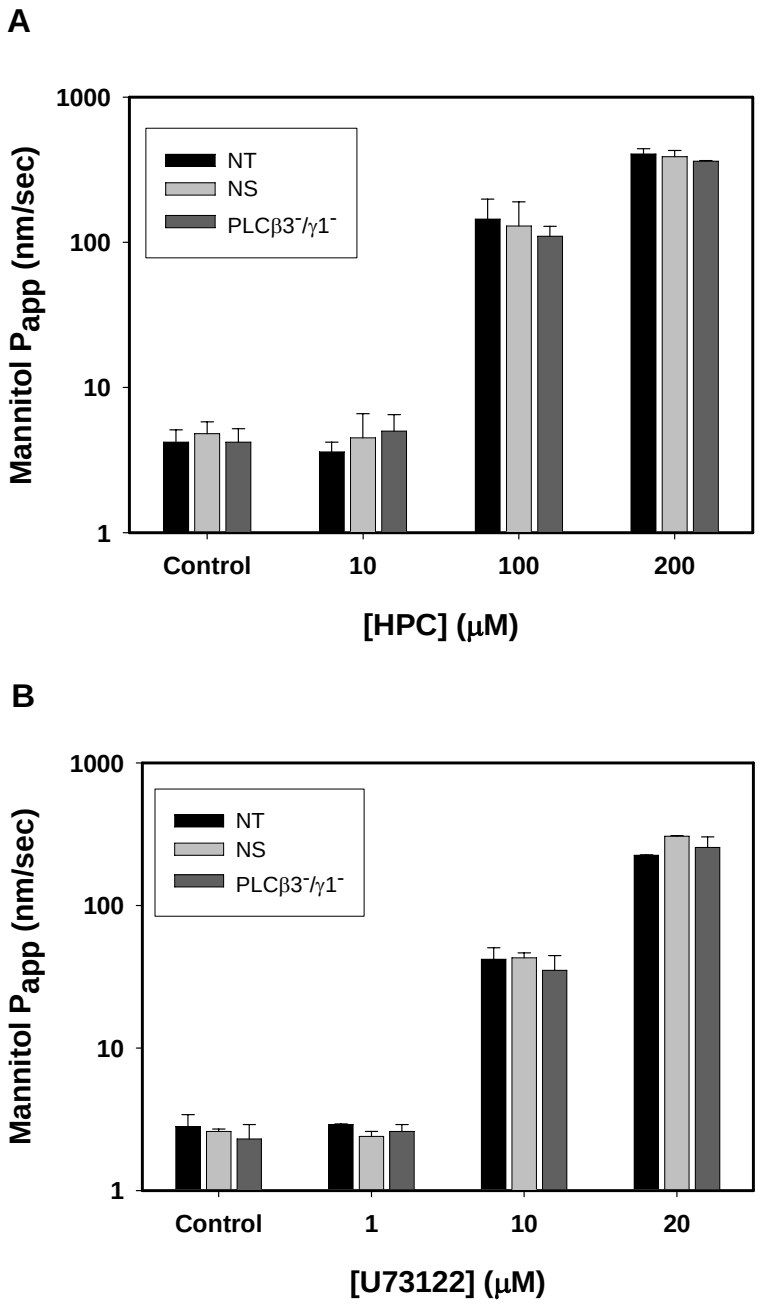


Figure 3.13. The effect of simultaneous RNAi-mediated knockdown of PLCβ3 and PLCγ1 on the potency of HPC and U73122 as paracellular permeability enhancers in MDCK cells. Cells were treated apically with either (A) HPC or (B) U73122 at indicated concentrations for thirty minutes and the absorptive permeability of mannitol was determined during the 30-60 minute period post-treatment. Data represent mean ± standard deviation from triplicate determinations from one representative experiment.

CHAPTER 4

DIRECT ACTIVATION OF HUMAN PHOSPHOLIPASE Cβ3 BY U73122 IN DODECYLMALTOSIDE MIXED MICELLES VIA ALKYLATION AT CYSTEINE RESIDUES

ABSTRACT

Phospholipase C (PLC) enzymes hydrolyze the polar head group from inositol-containing membrane phospholipids, and are a key family of regulatory proteins involved in numerous cellular functions. U73122 is one of only a few small molecules reported to inhibit the activity of these enzymes in cellular and non-cellular systems, and has been broadly applied as a pharmacological tool to implicate PLC in observed experimental phenotypes. The purpose of the present study was to develop a better understanding of the molecular interaction between U73122 and PLC by evaluating the effects of U73122 on human PLCβ3 (hPLCβ3) in a simple, cell free system. PLC activity was assessed by measuring changes in [^{3}H]-inositol phosphate formation following incubation of hPLCβ3 with [^{3}H]-phosphatidylinositol-4,5-bisphosphate (PIP_2) in a dodecylmaltoside micellar system. It was hypothesized that the maleimide-containing U73122 would irreversibly inhibit the activity of PLC via covalent binding to cysteine residues. Surprisingly, U73122 was found to increase the activity of hPLCβ3 in DDM mixed micelles in a concentration and time dependent manner (EC_{50} = 13.6 ± 5 μM). Activation was attenuated by thiol containing nucleophiles, L-cysteine and glutathione, suggesting that covalent modification of cysteine residues on the enzyme is essential for the activation. Mass spectrometric analysis of U73122-activated hPLCβ3 confirmed alkylation at up to eight cysteine residues, specifically identified by LC/MS/MS peptide sequencing. This study is the first to report direct activation of PLC via a mechanism involving cysteine alkylation.

INTRODUCTION

U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) (Figure 4.1) is an aminosteroid first reported as an inhibitor of PLC dependent processes in 1989[1]. Many reports have since demonstrated that several isozymes of PLC are inhibited by U73122, but not by U73343, a close structural analog of U73122 containing N-alkylsuccinimide instead of N-alkylmaleimide moiety[2-4], establishing U73122 as the prototypical inhibitor of PLC enzymes. These reports have been previously substantiated in both MDCK and Caco-2 cells as U73122, but not U73343, inhibits ATP-stimulated PLCβ activity in both cells lines in a concentration dependent manner, while U73343 does not (Chapter 2)[5]. In addition to its effects on PLC, U73122 at similar concentrations has been found to increase the permeability of epithelial cell monolayers to the paracellular marker mannitol, implicating PLC isozymes in the modulation of paracellular permeability (Chapter 2)[5]. However, recent studies (Chapter 3) contradict this hypothesis, as RNAi-mediated knockdown of both PLCβ3 and PLCγ1 in MDCK cells has no effect on the assembly or maintenance of tight junction barrier function. Therefore, the observed increase in paracellular permeability following treatment of epithelial cells with U73122 likely occurs via an alternative mechanism. Several laboratories have demonstrated that U73122 is not selective for PLC, as effects on a variety of other proteins have been reported including telomerase[6], 5-lipoxygenase[7], histamine H1 receptor[8], calcium channels[9], and phosphatidylinositol (PI)-dependent as well as PI-independent exocytic processes[10]. These studies provide examples of alternative cellular targets that may be affected by U73122 treatment; however, none have been directly linked to regulation of tight junction function. Interestingly, although U73122 clearly inhibits receptor-mediated activation of PLC, indirect

evidence for activation of PLC activity has also been reported[1, 11], implying complex effects of this compound on the activity of these enzymes in whole cell systems.

Consistent with the numerous reports identifying alternative cellular targets for U73122, recent studies have questioned the use of this compound as a truly selective modulator of PLC, specifically addressing the presence of the electrophilic maleimide moiety that is replaced by a succinimide moiety in the inactive analog, U73343 (Figure 4.1)[11, 12]. Maleimides are inherently reactive and readily react with cellular thiols and amines, providing a likely mechanism for the observed off-target effects. Despite the numerous studies reporting U73122 as an inhibitor of PLC, as well as it's widespread use as a probe molecule to implicate the involvement of PLC in signaling pathways and phenotypic cellular response, little work has been done to understand the interaction of this molecule with PLC at the molecular level. In the present study, the ability of U73122 to inhibit the catalytic activity of hPLCβ3 in a simple, cell free, mixed micellar system was investigated. Surprisingly, these studies revealed that U73122 activates, not inhibits, the activity of hPLCβ3 in a concentration dependent manner.

MATERIALS AND METHODS

Materials. Dodecylmaltoside (DDM) was purchased from Fluka. [^{3}H]-PIP_2 (phosphatidylinositol 4,5-bisphosphate, [myo-inositol-2-^{3}H(N)]) (20 Ci/mmol) was purchased from American Radiolabeled Chemicals. PIP_2 and hexadecylphosphocholine were purchased from Avanti Polar Lipids. 3-nitrocoumarin was synthesized as described previously[13]. U73122, U73343, N-ethylmaleimide, fatty acid free bovine serum albumin (BSA), and all other reagents were purchased from Sigma unless otherwise indicated. Purified hPLCβ3 was generously provided by the laboratory of Dr. T. K. Harden (Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill). Trypsin, thermolysin, and endoproteinase GluC for digestion of hPLCβ3 were from Promega, Fluka, and Roche respectively.

Mixed Micellar PLC Assay. The activity of hPLCβ3 in a cell free system was evaluated by an adaptation of previously published methods[14, 15]. Briefly, [^{3}H]-labeled and unlabeled PIP_2 (5 nmol), was reconstituted in a 1 mM DDM solution and mixed with assay buffer containing 40 mM Hepes (pH 7.4), 480 mM KCl, 40 mM NaCl, 8 mM EGTA, 4 mM $MgCl_4$, and 7.6 mM $CaCl_2$. Compounds at desired concentrations, and purified hPLCβ3 in 1% fatty acid-free BSA and 10 mM Hepes (pH 7.0), were subsequently added to the mixture. To initiate assays, samples were moved to a 37°C water bath and incubated for 2-10 minutes such that less than fifteen percent total substrate was hydrolyzed. At designated times, reactions were stopped by addition of 750 µl $CHCl_3$:MeOH:HCl (40:80:1), followed by the addition of 100 µl water, 250 µl $CHCl_3$, and 250 µl 0.1 M HCl. Samples were vortexed and centrifuged at 3000 rpm for 10 minutes at 4°C. The amount of [^{3}H]-inositol phosphates formed was measured by

liquid scintillation counting of 500 µl of the upper phase in a Packard Tri Carb 4000 Series spectrophotometer.

Data analysis. Data are expressed as the mean ± standard deviation (SD) from three measurements unless indicated otherwise. Where indicated, statistical significance was assessed using a two sample Student's t tests. Samples were assumed to have an unequal variance; significant differences were assigned at $p < 0.05$.

$EC_{50\ PLC\beta3}$ Estimation. To accurately quantify the potency of U73122 for activating PLCβ3 in DDM mixed micelles, concentration-effect profiles were generated for each experiment (n=4). Catalytic activities were converted to the percentage of the maximum effect (E(%)) according to Equation 1, where E_{obs} is the observed activity, E_0 is control activity in the absence of U73122, and E_{max} is the maximum activity attained with 100 µM U73122[16]. The relationship between E(%) for PLCβ3 activity and U73122 concentration (C) was fit to a standard Hill equation by nonlinear least-squares regression (WinNonlin, version 4.1) to recover estimates for $EC_{50\ PLC\beta3}$ for each concentration-effect profile (Equation 2). Estimates were also generated for the sigmoidicity factor (γ); however, 100% was substituted for E_{max}.

$$E\ (\%) = \left(\frac{E_{obs} - E_0}{E_{\max} - E_0} \right) \times 100 \tag{1}$$

$$E\ (\%) = \left(\frac{100 \times C^{\gamma}}{EC_{50}{}^{\gamma} + C^{\gamma}} \right) \tag{2}$$

Mass Spectrometry. Mass spectrometry of intact PLCβ3 was performed using reversed-phase chromatography[17] coupled to an Agilent Technologies (Santa Clara) 6210 LC-MSD-TOF mass spectrometer. Ionization was achieved using electrospray ionization with a spray voltage of -4.0 kV with heated nitrogen (350°C) serving as both a nebulizing (25 psi) and drying (12 L/min) gas. The fragmentor was set at 280 V and the instrument was set to detect

ions from mass/charge 500 to mass/charge 2500. The mass-to-charge data were transformed to the mass domain using BioConfirm software from Agilent. For peptide sequencing, solutions of unmodified and modified hPLCβ3 were reduced using DTT, alkylated with iodoacetamide, and incubated in an ammonium bicarbonate buffer with trypsin, thermolysin, or endoproteinase GluC (enzyme:substrate = 1:20) overnight at 37°C[18]. The tryptic peptides were subsequently analyzed by nano-LC/MS/MS on a Dionex UltiMate nano LC (Sunnyvale, CA) coupled to ABI-Sciex (Toronto, ON) Q-Star Pulsar i mass spectrometer using both a trapping cartridge column (0.3 x 5 mm) and a pepmap (0.075 x 150 mm, 3 μM particle size) C18 columns (Dionex). Mobile phase A contained 2% acetonitrile and mobile phase B contained 95% acetonitrile, both with 0.2% formic acid. Following a six minute load of sample onto the trapping cartridge at a flow rate of 25 μl/min with a mobile phase ratio of 95:5, flow was then switched to backflush off the trapping cartridge and onto the nano-LC column at 0.2 μl/min. The percentage of mobile phase B was changed over one hour from 5% to 40% with a linear gradient to adequately separate peptides, followed by a second linear gradient from 40% to 99% over the next seven minutes. The results were processed using MASCOT (Matrix Sciences, London, UK) protein database that had the mass of U73122 modification created as a variable modification.

RESULTS

Activity of human PLCβ3 in phospholipid-detergent mixed micelles

Rates of PIP_2 hydrolysis catalyzed by several PLCβ isozymes in DDM/PIP_2 mixed micelles have been shown to be linear when substrate mole fraction is <0.3 and ≤10% of total substrate is utilized[14, 15]. hPLCβ3 activity with respect to time at a substrate mole fraction of 0.1 is presented in Figure 4.2. Consistent with previous studies, two distinct phases of activity were observed, an initial burst of activity linear when ≤15% of PIP_2 is utilized, followed by a second, slower phase of hydrolysis. Additional studies were performed at fixed calcium and PIP_2 concentrations, and were designed such that ≤15% of total substrate was utilized in all cases when substrate consumption was linear with respect to time.

U73122, but not U73343, increases human PLCβ3 activity in a concentration dependent manner.

In DDM mixed micelles, U73122 unexpectedly increased the activity of hPLCβ3 in a concentration-dependent manner with a maximum increase of approximately eight fold with respect to control. Application of a modified Hill equation, as described in Materials and Methods, estimated an EC_{50} value of 13.6 ± 5 μM (n=4) for the observed activation (Figure 4.3). The estimated EC_{50} from these data should be interpreted within the context of the current set of results, as solubility limitations of U73122 in DMSO stock solutions prevented assessment of activation at concentrations greater than 100 μM. U73343, a close structural analog of U73122 containing N-alkylsuccinimide instead of N-alkylmaleimide moiety, (Figure 4.1) had a small but significant effect on activity at 40 μM (Figure 4.4), implicating the maleimide moiety in the observed activation of hPLCβ3 by U73122.

Thiol-containing nucleophiles, glutathione and cysteine, attenuate the U73122-mediated activation of human PLCβ3 in DDM mixed micelles.

Thiol containing compounds, glutathione and cysteine, targets for reaction with electrophilic functional groups such as maleimide, attenuated the U73122-mediated activation of hPLCβ3 in a concentration-dependent manner (Figure 4.5). The ability of thiols to prevent the increase in PLC activity caused by U73122 suggests that U73122 alkylates hPLCβ3 by reacting with nucleophiles, likely protein sulfhydryl groups (*i.e.* cysteine residues) leading to an irreversible modification of the enzyme.

U73122-mediated activation of human PLCβ3 is rapid and irreversible.

To further investigate the hypothesis that activation of hPLCβ3 by U73122 involves covalent alkylation of the enzyme, the effect of pre-incubation time on the observed activation was evaluated. Thus, hPLCβ3 was pre-incubated with U73122 in the absence of substrate and detergent, excess U73122 was removed with the addition of 1 mM glutathione. PIP_2, re-suspended in DDM, was then added to initiate the assay. Pre-incubation of hPLCβ3 with U73122 increased activity compared to control, suggesting that activation of the enzyme was caused by direct interaction of U73122 with the enzyme, likely irreversible covalent modification of one or more sulfhydryl groups. Increased activity was observed with minimal pre-incubation time, and reached a maximum increase of five fold within thirty seconds (Figure 4.6). Separate studies established that addition of 1 mM glutathione to 40 μM U73122 results in the immediate disappearance of U73122 as measured via LC/MS (data not shown). It was further confirmed that the preformed U73122-glutathione conjugate had no direct effect on the activity of hPLCβ3 in this system (data not shown).

U73122, but not U73343, alkylates human PLCβ3.

In order to unequivocally establish that alkylation of hPLCβ3 by U73122 is a requirement for its activation, mass spectra were obtained of intact hPLCβ3 alone and after incubation with either 40 μM U73122 or 40 μM U73343 for various time intervals. Mass spectra of hPLCβ3 revealed a complex protein of approximately 138kD (Figure 4.7.A); however, when incubated with 40 μM U73122, spectra revealed the presence of protein species with discrete increases in mass consistent with covalent addition of up to eight molecules of U73122 (Figure 4.7.B and 4.7.C). Extended incubation times did not lead to more than eight U73122 additions, although shorter incubation times revealed the presence of proteins with less than eight molecules of U73122 covalently attached, *i.e.* two to eight additions of U73122 were observed, and even at the shortest incubation times, protein species with a single addition of U73122 were not detected. One minute incubation revealed up to four covalently attached U73122 molecules (Figure 4.7.B) in conjunction with the near maximal activation of the protein by U73122 in DDM mixed micelles (Figure 4.6). This observation implies that not all eight U73122 molecules are required to achieve maximal activation of the enzyme. Interestingly, increasing incubation times also led to a shift in chromatographic retention time of intact hPLCβ3 (data not shown). With increasing incubation times, the observed peak began to split, and as time progressed the later half of the observed split peak became the dominant peak. This downstream shift in chromatographic retention is likely due to increased lipophilicity of intact hPLCβ3 as a result of the increased addition of the highly lipophilic U73122. Mass spectra of hPLCβ3 incubated with U73343 for extended time periods was unchanged (Figure 4.7.D), suggesting that the maleimide moiety in U73122 is responsible for the alkylation.

U73122 alkylates specific cysteine residues of human PLCβ3.

hPLCβ3 contains fourteen cysteine residues (Figure 4.8). LC/MS/MS peptide mapping of protease-digested hPLCβ3 identified peptides containing twelve of the fourteen cysteines. Following incubation of hPLCβ3 with U73122 for thirty minutes, eight of the twelve detectable cysteines were found alkylated by U73122, consistent with the observed mass changes to intact hPLCβ3 when incubated with U73122 for the same time period. The following cysteines were identified as alkylated: Cysteine (Cys)193, Cys221, Cys360, Cys516, Cys614, Cys892, Cys1176, and Cys1207.

Effect of N-ethylmaleimide (NEM) on human PLCβ3 activity, and on U73122-mediated activation and alkylation of human PLCβ3.

NEM is a small water soluble maleimide-containing compound lacking the long alkyl chain and steroid backbone present in U73122 (Figure 4.9.A). At concentrations up to 400 μM, NEM had no effect on the activity of hPLCβ3 in DDM mixed micelles (Figure 4.9.B). Despite this lack of activation by NEM in DDM mixed micelles, mass spectra of intact hPLCβ3 incubated with 400 μM NEM for extended time periods revealed protein species of increasing mass consistent with the covalent addition of up to eight NEM molecules (Figure 4.9.C). Due to the low molecular weight of NEM compared to U73122, it was not possible to distinguish discrete additions of NEM to hPLCβ3. Together, these data demonstrate that although NEM alkylates hPLCβ3, it is unable to increase its activity. On the other hand, NEM was able to attenuate the ability of U73122 to activate hPLCβ3 in a concentration dependent manner (Figure 4.9.D). Therefore, NEM blocks the U73122-mediated activation of hPLCβ3, but does not activate the protein itself. Interestingly, NEM, at concentrations as high as 1 mM, was unable to completely inhibit the U73122-mediated activation, unless hPLCβ3 was pre-incubated with NEM prior to the addition of U73122 (Figure 4.9.D). To test

the hypothesis that NEM alkylates the same cysteine residues as U73122, NEM-modified hPLCβ3 was subsequently incubated with U73122. Mass spectra of this sample revealed no further additions of U73122 to hPLCβ3 (Figure 4.9.E). Together, these data suggest that NEM attenuates the U73122-mediated activation of hPLCβ3 via alkylation at the same cysteine residues, and prevents alkylation by U73122.

PLC inhibitors 3-nitrocoumarin (3-NC) and hexadecylphosphocholine (HPC) have no effect on human PLCβ3 activity in DDM mixed micelles.

Previously reported PLC inhibitors 3-NC[13, 19] and HPC[5, 20, 21] were tested for their ability to inhibit hPLCβ3 in DDM mixed micelles. Contrary to numerous reports demonstrating inhibition of PLC by both molecules, neither had any effect on hPLCβ3 activity in DDM mixed micelles at concentrations as high as 500 μM (Figure 4.11).

DISCUSSION

U73122 has long been regarded as a prototypical inhibitor of PLC, while its inactive analog, U73343, has served as a negative control. Structurally, the difference between these two compounds is simply the substitution of a maleimide moiety in U73122 for a succinimide moiety in U73343, suggesting that the highly electrophilic maleimide is responsible for the observed effects in experimental systems, including inhibition of PLC activity. Recent studies have questioned the use of U73122 as a specific inhibitor of PLC by uncovering its "off-target" interactions, likely due to covalent modification of nucleophiles by the maleimide moiety[10-12]. These findings point to the likely complication of using U73122 as a tool to study PLC function in complex cellular systems, and suggest the need for a clearer understanding of how U73122 interacts with PLC at the molecular level.

PLCs are water soluble enzymes that hydrolyze poorly soluble phospholipids present in cell membranes. Enzymatic activity is tightly regulated in whole cells via both cell membrane and intracellular mechanisms. For example, the activity of PLCβ isozymes is tightly regulated via specific G-protein coupled receptors at the cell membrane; further, novel intracellular feedback mechanisms have recently been identified that control the amplitude and duration of PLCβ stimulation[22]. Due to the complexity of how PLC functions inside the cell, it is difficult to assess whether an observed inhibition of PLC activity is by directly interacting with the enzyme, by indirectly interfering with PLCs interaction with its substrates in the cell membrane, or by indirectly interfering with receptors and other cofactors involved in regulating its activity. In the present study, the interaction of U73122 with hPLCβ3 was investigated in a cell-free system to elucidate the mechanism by which it inhibits the enzyme in cellular systems.

In order to study PLC in cell-free systems, the presence of a suitable interface between the hydrophilic enzyme and the hydrophobic substrate is required in order to achieve measurable enzymatic activity. The enzyme must first partition between the aqueous phase and the substrate interface prior to acting on its substrate. A number of useful experimental systems have been described for assessing lipase activity, although most are unable to measure both of these discrete steps simultaneously, complicating data interpretation and kinetic analysis. The use of liposomes, vesicle based co-expression systems, membrane bilayers and monolayers, as well as mixed micellar systems have been reported, each with a unique set of advantages and limitations[14, 15, 23-25]. Perhaps the simplest of systems in which to present the substrate to PLC is a mixed micellar system, which eliminates the complexities of receptor activation that must be considered in more complex systems; on the other hand, lamellar structure is not necessarily maintained in a mixed micellar system, a characteristic that makes it less representative of physiologic cell membranes. As the purpose of the present study was to assess the direct interaction between U73122 and hPLCβ3 in the absence of confounding components that may act as nucleophilic targets for U73122, the simplest system available, *i.e.* mixed micelles, was employed. DDM is a non-ionic surfactant previously reported to be a useful inert diluent to establish an interface for presentation of insoluble substrate, PIP_2, to PLC in the absence of a phospholipid bilayer[14, 15], and was therefore utilized as detergent to form micelles in these studies.

The expectation was that U73122 would irreversibly inhibit lipase activity via a mechanism involving covalent modification of the nucleophilic residues on PLC (*i.e.* cysteines). Surprisingly, U73122 actually increased hPLCβ3 activity in a concentration

dependent manner in DDM mixed micelles (Figure 4.3), while U73343 had a minimal effect (Figure 4.4). As previously indicated, U73122 has almost exclusively been reported as a potent inhibitor of PLC action, although a recent study in whole cells observed weak and transient activation effects in addition to the expected potent inhibition[11], providing both precedence and potential relevance to cellular systems for the current observations.

The activation observed in mixed micelles reached maximal effect rapidly, and was irreversible (Figure 4.6). Furthermore, activation could be attenuated with competing nucleophiles, glutathione and cysteine (Figure 4.5). These results, along with the inactivity of succinimide containing analog U73343, strongly implicated the maleimide in the observed activation. Maleimides are inherently reactive and readily interact with thiols, such as cysteine residues, on cellular proteins. To determine if protein alkylation at cysteine residues on hPLCβ3 was a likely mechanism for the U73122-mediated activation, mass spectra of intact hPLCβ3 in the presence and absence of U73122 were collected. With increasing incubation times, the addition of up to eight U73122 molecules could easily be detected based on increases in mass/charge of intact hPLCβ3 by multiples of the molecular weight of U73122 (approximately 0.5 kDa) (Figure 4.7). These data demonstrate that U73122 reacts covalently with as many as eight distinct sites on hPLCβ3, and provides an underlying molecular mechanism for modulation of PLC activity in experimental systems.

The importance of cysteine residues in protein function is well established. Specific protein cysteine residues are required for activation of the ion channel, TRPA1[26, 27], are important for enzymatic activity of caspases [28, 29] as well as other lipases[30], are crucial for regulating the activity of cellular phosphatases such as PTEN[31], and are used as palmitoylation sites for integral membrane proteins such as claudin[32] and signaling proteins

like H-ras[33, 34]. There are fourteen cysteine residues in hPLCβ3 (Figure 4.8). In order to confirm cysteines as the site of alkylation by U73122, as well as to identify particular residues as specific targets, LC/MS/MS was used for peptide mapping of protease digested hPLCβ3. Intact protein was digested overnight with three different proteases (trypsin, thermolysin, and endoproteinase GluC) in order to provide greater sequence coverage of the high molecular weight hPLCβ3. Peptides containing twelve of the fourteen cysteines were detected in unmodified hPLCβ3, excluding Cys669 and Cys834. When hPLCβ3 was incubated with U73122, eight specific cysteine residues were alkylated, including Cys360 and Cys614 located in the so called 'X' and 'Y' boxes that make up the highly conserved catalytic domain of all PLCs, Cys516 in the less conserved linker region between the 'X' and 'Y' boxes, as well as Cys193, Cys221, Cys892, Cys1176, and Cys1207 outside of the catalytic domain. Interestingly, Cys193 and Cys221 are located within the PH domain, a region thought to be critical for the membrane recruitment of PLCβ isozymes, and recently implicated in the interaction of PLCβ2 with Rac1 during substrate catalysis[35]. Time-dependent activation studies (Figure 4.6) demonstrating maximum activation with very short pre-incubation of U73122 with hPLCβ3 (*i.e.* less than one minute), together with time-dependent alkylation studies (Figure 4.7.B and Figure 4.7.C) imply that incomplete alkylation (*i.e.* less than eight modifications) is sufficient to achieve the observed activation. In other words, maximal activation was observed within one minute of pre-incubation of U73122 with hPLCβ3; however, after one minute of incubation, mass spectra of intact hPLCβ3 revealed only four additions of U73122. Despite the apparent lack of specificity of U73122 for alkylating hPLCβ3, it is conceivable that alkylation at one specific residue is primarily responsible for the activation. Site-directed mutagenesis of each alkylated cysteine

residue would be useful to explore the role of individual cysteine residues in the observed activation. At this point, the question remaining to be addressed was whether the covalent modification was responsible for the activation, and if so, how?

NEM (Figure 4.9) is a small water soluble maleimide capable of modifying sulfydryl groups on proteins, and served as a useful tool to further probe the interaction between PLC and U73122. A number of interesting observations were noted in studies performed with NEM: (1) NEM alone had no effect on the activity of hPLCβ3 in DDM mixed micelles at concentrations as high as 400 μM, despite the fact that mass spectra of intact hPLCβ3 incubated with NEM identified protein species with mass/charge consistent with the addition of up to eight NEM molecules. This result indicates that although the maleimide moiety is required for the activation of PLC, it is not sufficient by itself, suggesting an important role for the hydrophobic backbone of U73122 in the mechanism of activation. This result further suggests that the eight cysteine residues alkylated by NEM are not crucial for maintaining the catalytic activity of hPLCβ3 in this system. (2) The combined treatment of NEM and U73343 had no effect on activity (data not shown), implying that the maleimide moiety and the hydrophobic backbone must be chemically bound to observe PLC activation. It would be interesting to investigate structure-activity relationships between the observed activation of PLC activity and the length of the N-alkyl chain that links the maleimide moiety to the steroid moiety of U73122. (3) Although NEM was unable to activate PLC alone, it was able to attenuate the U73122-mediated activation in a concentration dependent manner. Interestingly though, the inhibition was not complete up to concentrations as high as 1 mM, unless NEM was incubated with the enzyme prior to the addition of U73122. This result suggests a common mechanism of interaction for both molecules with hPLCβ3, and implies

that NEM alkylates the same cysteine residues. (4) To confirm that NEM alkylates the same cysteine residues, mass spectra of NEM-treated hPLCβ3 were collected before and after incubation with U73122. This study indicated that once hPLCβ3 had been exposed to NEM, U73122 was not able to react with the protein. Together, studies with NEM demonstrate that NEM alkylates hPLCβ3 at cysteine residues but does not increase its lipase activity; however, the alkylation by NEM can completely block covalent reaction of hPLCβ3 with U73122 and its subsequent activation.

The results presented herein are consistent with the following hypothesis for activation of hPLCβ3: U73122 irreversibly binds to multiple cysteine residues on hPLCβ3 and serves as either a lipid anchor or interfacial recognition site for the enzyme, facilitating adsorption to the substrate interface (*i.e.* the micelle surface), subsequently increasing the rate of lipase activity by keeping the enzyme in close proximity to multiple substrates (Figure 4.10).

The molecular mechanism of cellular PLC activation and subsequent substrate hydrolysis is not completely understood. It is interesting to speculate about the potential role of a cysteine residue in conformational changes that may occur during lipase activation and subsequent substrate hydrolysis. Conformational changes exposing the active site and increasing activity have been reported at substrate interfaces for a number of lipases (*i.e.* the opening of the lid providing access to the active site)[23, 36-42], and have recently been hypothesized for PLCβ isozymes[35]; although the molecular mechanisms associated with these changes have not yet been elucidated. Post-translational modifications, such as phosphorylation, have clearly been shown to trigger rapid conformational changes in many proteins leading to significant alterations in protein function[43-46]. In recent years, studies have

rapidly emerged implicating reversible cysteine modifications (*e.g.* S-nitrosation) in changes to protein function[47-49]. For example, nitrosation of caspase 3 by nitric oxide at a specific cysteine residue was reported to be critical for promotion of apoptosis[28, 29]. S-nitrosothiols have also been shown to oxidatively modify phosphatases such as PTEN, a PIP_3 phosphatase, leading to a reversible inhibition of its phosphatase activity[31] and supporting a hypothesis for cysteine-dependent changes in protein function. Further, S-nitrosation has been postulated as a regulatory mechanism for Ras superfamily GTPases (*i.e.* Ras and RhoA)[50, 51], two enzymes known to be directly involved in the regulation of specific PLC isozymes[52]. Perhaps a reversible modification of a cysteine residue in hPLCβ3 occurs during receptor mediated activation that leads to a protein conformational change promoting interaction with cell membranes, akin to the reported activation and conformation change of hypoxia inducible factor 1 by S-nitrosation[53]. Interestingly, a recent report suggests that low concentrations of the reactive oxygen species, H_2O_2, generates intracellular calcium oscillations in rat astrocytes by activating PLCγ1 via a sulfydryl-dependent oxidation mechanism[54].

Even more interesting are reports implicating PLC activation, not inhibition, in tight junction barrier function, as a number of agonists of receptors known to couple to PLCs have been reported to affect the functional integrity of tight junctions[55-58]. Further, increase in intracellular calcium levels[59], activation of specific PKC isozymes[60], as well as non-PLC-mediated depletion of membrane PIP_2 content[61, 62], all downstream actions resulting from PLC activation, have been demonstrated to increase paracellular permeability or alter the barrier function of junctional complexes. These studies imply that PLC activation, not inhibition, may lead to an increase in paracellular permeability due to modulation of cell-cell junctions. This hypothesis, in conjunction with current evidence suggesting U73122 may

activate PLC, provides an alternative mechanism for the observed effects of U73122 on paracellular permeability in both MDCK and Caco-2 cells (Chapter 2)[5], and explains the discrepancy between results from studies using chemical inhibitors of PLC (Chapter 2) versus results from studies employing RNAi suppression of PLC (Chapter 3). Further studies are necessary to evaluate this hypothesis and to understand why PLC activity measured in whole cell systems appears to be inhibited by U73122.

The present results also support recent evidence suggesting that U73122 is highly reactive and capable of rapidly alkylating nucleophiles[12], including cysteine residues on hPLCβ3 and BSA (data not shown). The effects of U73122, particularly in cell-based assays, will be complex and dependent on the presence of free nucleophiles in the media, in the cell membrane, and in the cytosol. Care should be taken when making conclusions based on the effects of U73122 in cellular, and non-cellular, assays. Studies intended to implicate PLC enzymes in cellular phenotypes should avoid using this compound as a probe molecule, as its reactivity with other cellular nucleophiles may lead to off-target effects that could be mistakenly attributed to PLC involvement.

As the studies reported here were carried out in a well defined cell free system, the cellular relevance of the observed activation requires further study. Interestingly, other reported inhibitors of PLC (*i.e.* 3-NC and HPC) also failed to inhibit lipase activity in this system (Figure 4.11), implying that the effects of these compounds on PLC activity in whole cells may not be mediated via direct interaction with the enzyme. In order to acquire a more complete understanding of the interaction between PLC and its reported inhibitors, and to confirm the unexpected enzymatic activation by U73122, studies should be performed in multiple assay systems to develop a more clear understanding of how purported PLC

inhibitors actually modulate the catalytic activity of this important class of regulatory signal transduction enzymes. The data reported herein provide an underlying molecular mechanism for modulation of PLC activity by U73122, whether the end-result in cellular systems is enzymatic activation or inhibition.

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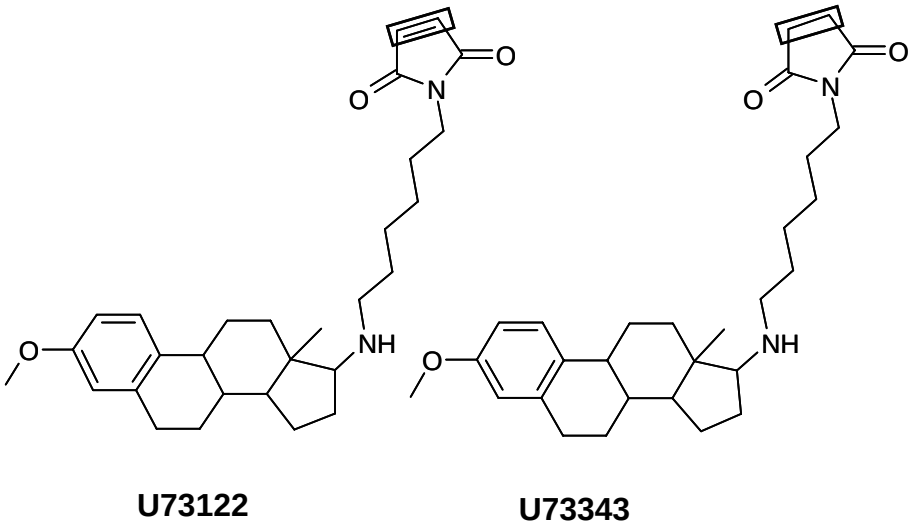


Figure 4.1. Structures of U73122 and U73343. The box highlights the only structural difference between the two compounds (*i.e.* maleimide in U73122 versus succinimide in U73343).

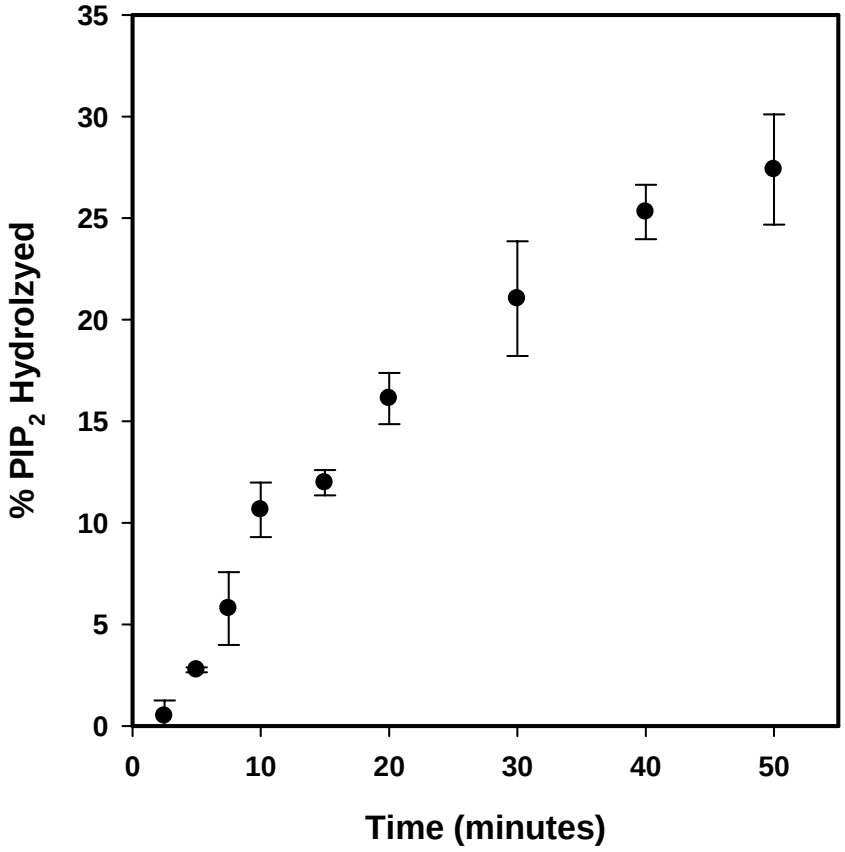


Figure 4.2. Time course of PIP_2 hydrolysis by hPLCβ3 in DDM mixed micelles. PIP_2, reconstituted in DDM, was combined with assay buffer and hPLCβ3 in a final volume of 100 µl. Assays were initiated by moving samples to a 37°C water bath and incubating for indicating times. Data represent mean ± SD from triplicate determinations from one representative experiment.

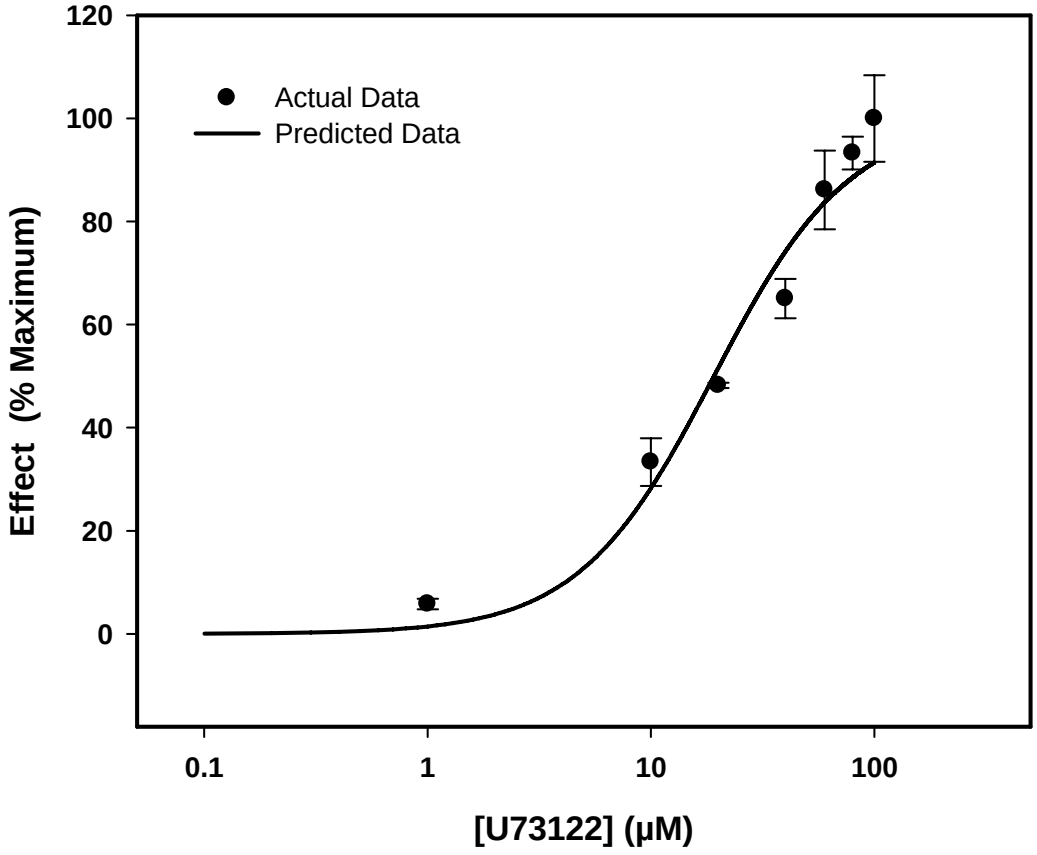


Figure 4.3. Concentration dependent activation of hPLCβ3 by U73122 in DDM mixed micelles. PIP_2, reconstituted in DDM, was combined with assay buffer and hPLCβ3 in a final volume of 100 μl. U73122, at indicated concentrations, was added to the assay mixture prior to the addition of enzyme. Assays were initiated by moving samples to a 37°C water bath. Incubation times were adjusted to ensure that less than 15% PIP_2 was hydrolyzed in all cases. U73343 at 40 μM had not effect (data not shown). Data represent mean ± SD from triplicate determinations from one representative experiment. $EC_{50\ PLC\beta3}$ was determined as described in Materials and Methods and was found to be 19.2 μM in this experiment. The estimated EC_{50} from these data should be interpreted within the context of the current set of results, as solubility limitations of U73122 in DMSO stock solutions prevented assessment of activation at concentrations greater than 100 μM.

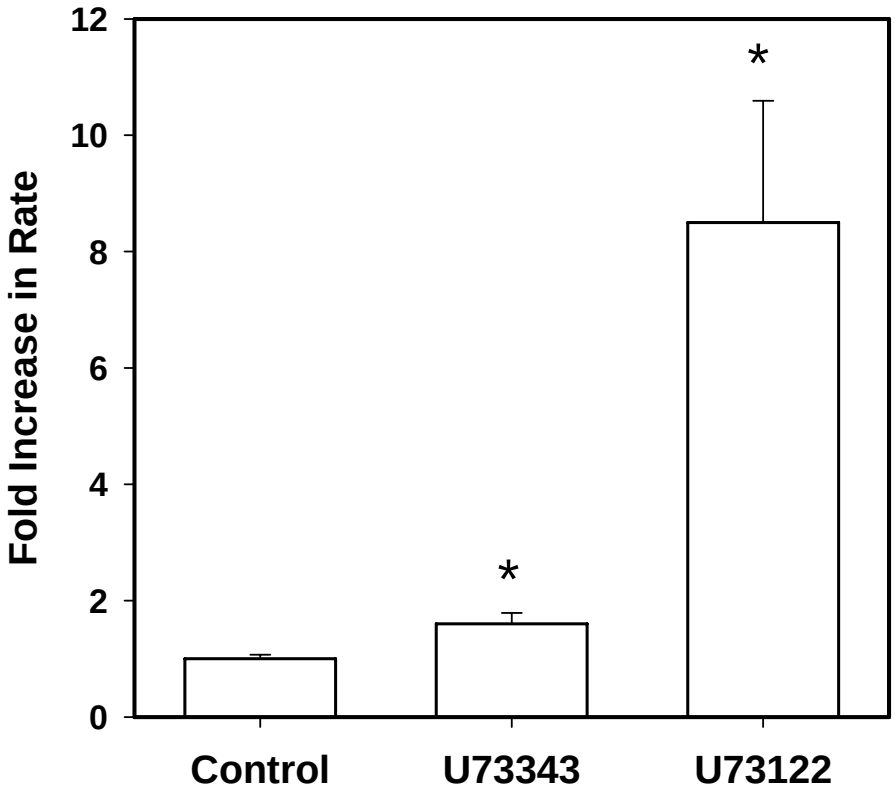


Figure 4.4. Effect of U73122 (40 μM) and U73343 (40 μM) on hPLCβ3 activity in DDM mixed micelles. PIP_2, reconstituted in DDM, was combined with assay buffer and hPLCβ3 in a final volume of 100 μl. U73122 or U73343, at indicated concentrations, was added to the assay mixture prior to the addition of enzyme. Assays were initiated by moving samples to a 37°C water bath. Incubation times were adjusted to ensure that less than 15% PIP_2 was hydrolyzed in all cases. Data represent mean ± SD from triplicate determinations from one representative experiment. Asterisks indicate significant difference ($p < 0.05$).

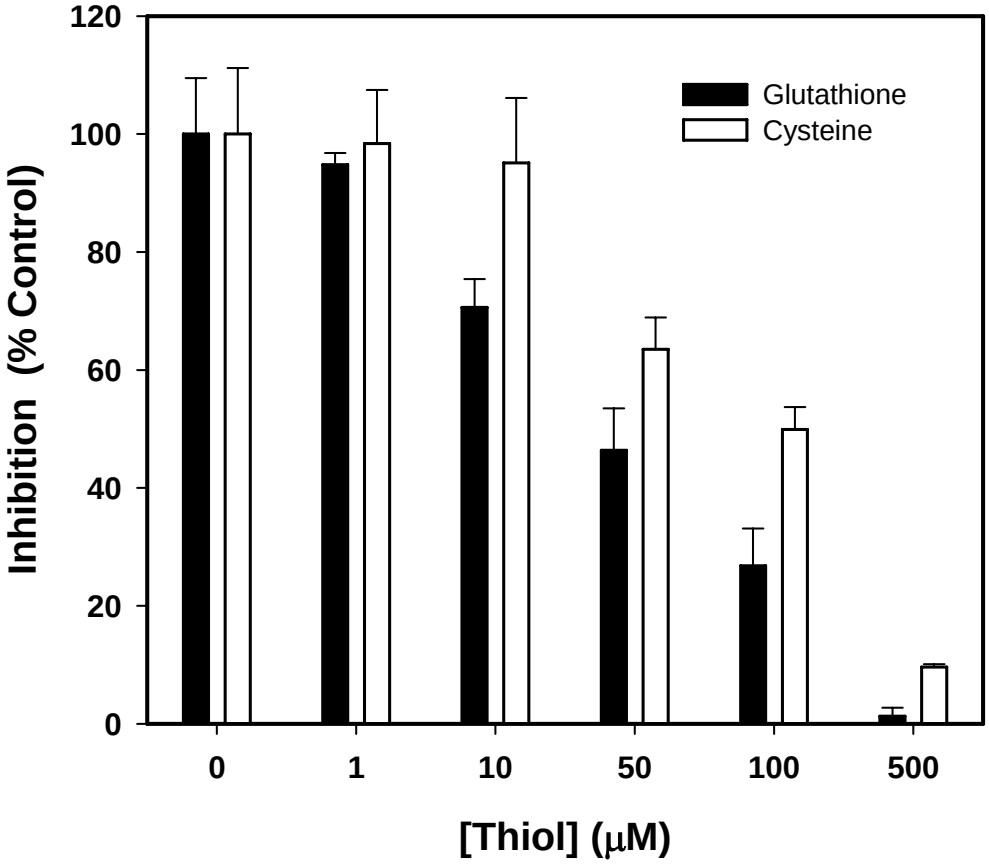


Figure 4.5. The effect of glutathione and cysteine on the U73122-mediated activation of hPLCβ3 in DDM mixed micelles. PIP_2, reconstituted in DDM, was combined with assay buffer, thiol at indicated concentration, and hPLCβ3 in a final volume of 100 µl. U73122 (40 µM) was added to the assay mixture prior to the addition of enzyme. Assays were initiated by moving samples to a 37°C water bath. Incubation times were adjusted to ensure that less than 15% PIP_2 was hydrolyzed in all cases. Data represent mean ± SD from triplicate determinations from one representative experiment.

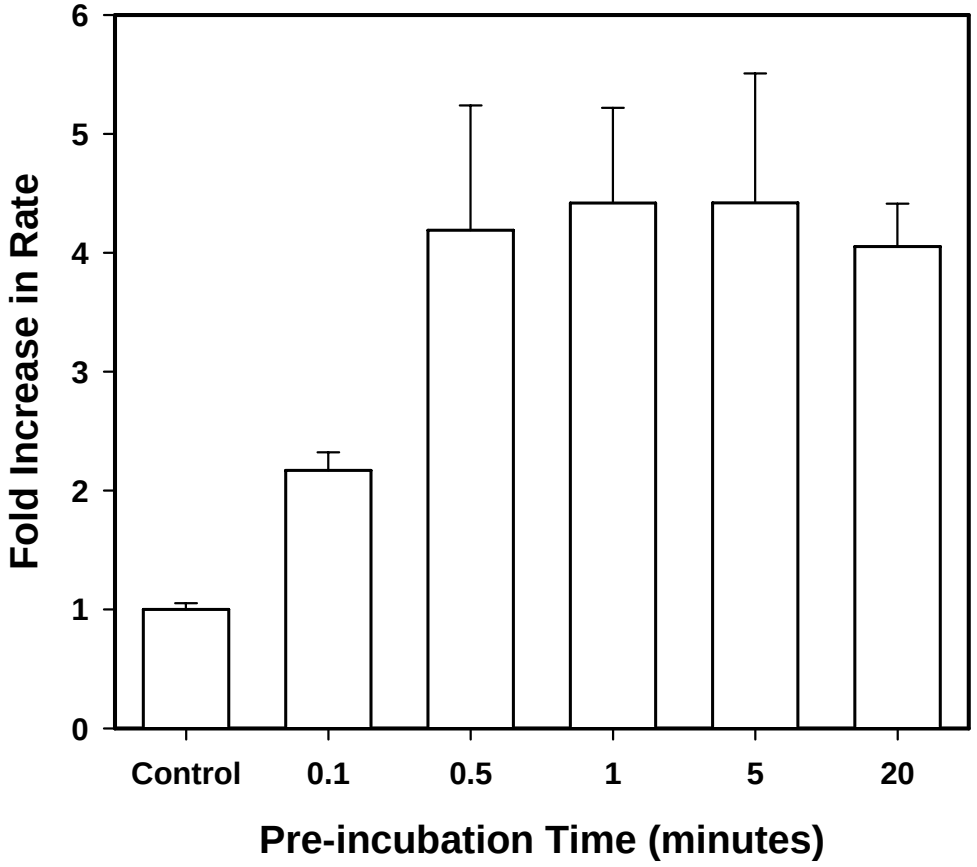
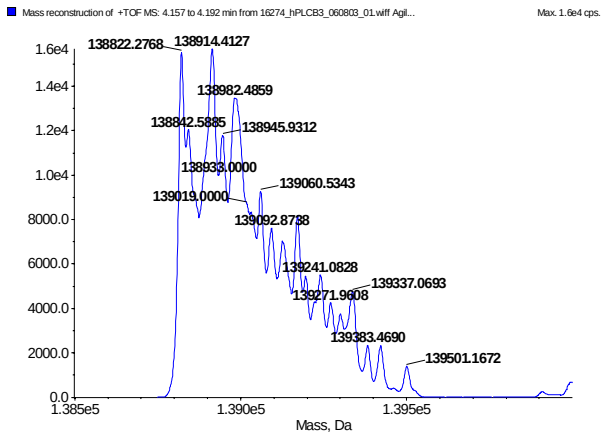


Figure 4.6. The effect of pre-incubation time (hPLCβ3 with U73122) on the U73122-mediated activation of hPLCβ3 in DDM mixed micelles. U73122 was pre-incubated with hPLCβ3 at 40 μM in assay buffer (50 μl total volume) for indicated times in the absence of PIP_2 and DDM. Pre-incubation was quenched with the addition of 1 mM glutathione. Assays were initiated with the addition of pre-incubated enzyme to mixed micelles in a final volume of 100 μl, and incubation times were adjusted to ensure that less than 15% PIP_2 was hydrolyzed in all cases. Data represent mean ± SD from triplicate determinations from one representative experiment.

A.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1.4e4
1.2e4
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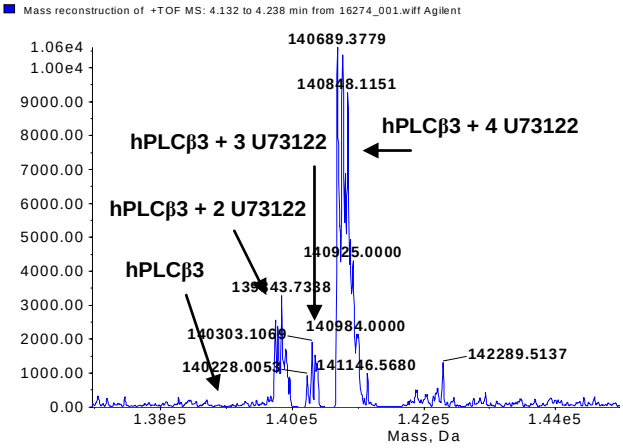
Mass, Da

B.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hPLCβ3 + 4 U73122
hPLCβ3 + 3 U73122
hPLCβ3 + 2 U73122
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hPLCβ3
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140984.0000
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142289.5137
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1.00e4
9000.00
8000.00
7000.00
6000.00
5000.00
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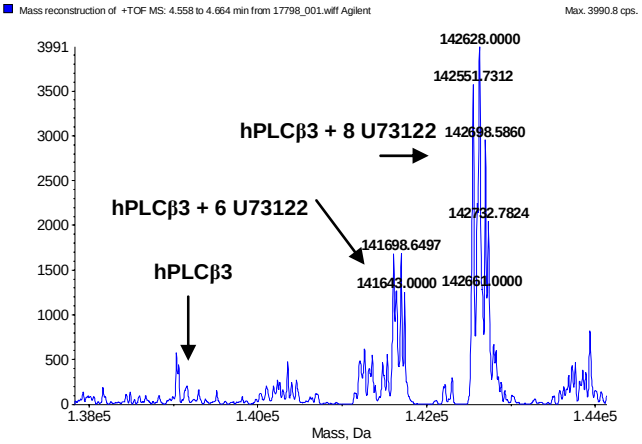
Mass, Da

C.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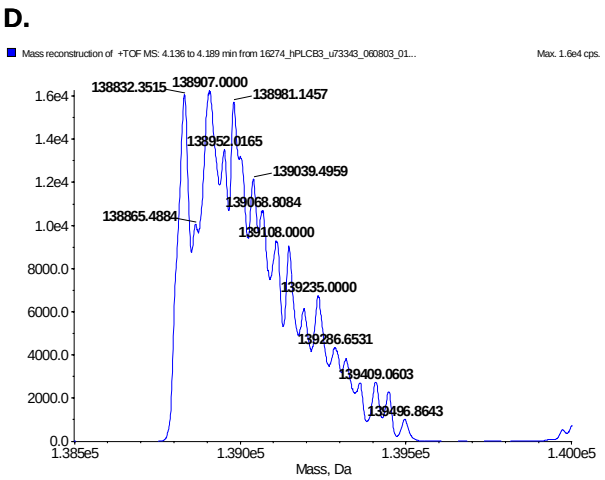


Figure 4.7. Mass spectra of intact hPLCβ3 following incubation with either U73122 or U73343. Both compounds were incubated with hPLCβ3 at 40 μM for various time intervals in assay buffer at 37°C in the absence of substrate and detergent. Incubations were terminated with the addition of 1 mM glutathione, and an aliquot was analyzed via LC/MS. (A) hPLCβ3 alone, (B) hPLCβ3 following incubation with U73122 for one minute, (C) hPLCβ3 following incubation with U73122 for fifteen minutes, or (D) hPLCβ3 following incubation U73343 for fifteen minutes. Note the differences in the x-axis between figures representing recapitulated protein mass. See material and methods for details.

```
1     MAGAQPGVHALQLEPPTVVETLRRGSKFIKWDEETSSRNLVTLRVDPNGFFLYWTGPNME
61    VDTLDISSIRDTRTGRYARLPKDPKIREVLGFGGPDARLEEKLMTVVSGPDPVNTVFLNF
121   MAVQDDTAKVWSEELFKLAMNILAQNASRNTFLRKAYTKLKLQVNQDGRIPVKNILKMFS
181   ADKKRVETALESCGLKFNRSESIRPDEFSLEIFERFLNKLCLRPDIDKILLEIGAKGKPY
241   LTLEQLMDFINQKQRDPRLNEVLYPPLRPSQARLLIEKYEPNQQFLERDQMSMEGFSRYL
301   GGEENGILPLEALDLSTDMTQPLSAYFINSSHNTYLTAGQLAGTSSVEMYRQALLWGCRC
361   VELDVWKGRPPEEEPFITHGFTMTTEVPLRDVLEAIAETAFKTSPYPVILSFENHVDSAK
421   QQAKMAEYCRSIFGDALLIEPLDKYPLAPGVPLPSPQDLMGRILVKNKKRHRPSAGGPDS
481   AGRKRPLEQSNSALSESSAATEPSSPQLGSPSSDSCPGLSNGEEVGLEKPSLEPQKSLGD
541   EGLNRGPYVLGPADREDEEEDEEEEEQTDPKKPTTDEGTASSEVNATEEMSTLVNYIEPV
601   KFKSFEAARKRNKCFEMSSFVETKAMEQLTKSPMEFVEYNKQQLSRIYPKGTRVDSSNYM
661   PQLFWNVGCQLVALNFQTLDVAMQLNAGVFEYNGRSGYLLKPEFMRRPDKSFDPFTEVIV
721   DGIVANALRVKVISGQFLSDRKVGIYVEVDMFGLPVDTRRKYRTRTSQGNSFNPVWDEEP
781   FDFPKVVLPTLASLRIAAFEEGGKFVGHRILPVSAIRSGYHYVCLRNEANQPLCLPALLI
841   YTEASDYIPDDHQDYAEALINPIKHVSLMDQRARQLAALIGESEAQAGQETCQDTQSQQL
901   GSQPSSNPTPSPLDASPRRPPGPTTSPASTSLSSPGQRDDLIASILSEVAPTPLDELRGH
961   KALVKLRSRQERDLRELRKKHQRKAVTLTRRLLDGLAQAQAEGRCRLRPGALGGAADVED
1021  TKEGEDEAKRYQEFQNRQVQSLLELREAQVDAEAQRRLEHLRQALQRLREVVLDANTTQF
1081  KRLKEMNEREKKELQKILDRKRHNSISEAKMRDKHKKEAELTEINRRHITESVNSIRRLE
1141  EAQKQRHDRLVAGQQQVLQQLAEEEPKLLAQLAQECQEQRARLPQEIRRSLLGEMPEGLG
1201  DGPLVACASNGHAPGSSGHLSGADSESQEENTQL
```

Figure 4.8. Primary sequence of hPLCβ3 (NP000923). Cysteine residues are highlighted in gray. The following cysteines were found alkylated by U73122: Cys193, Cys221, Cys360, Cys516, Cys614, Cys892, Cys1176, and Cys1207.

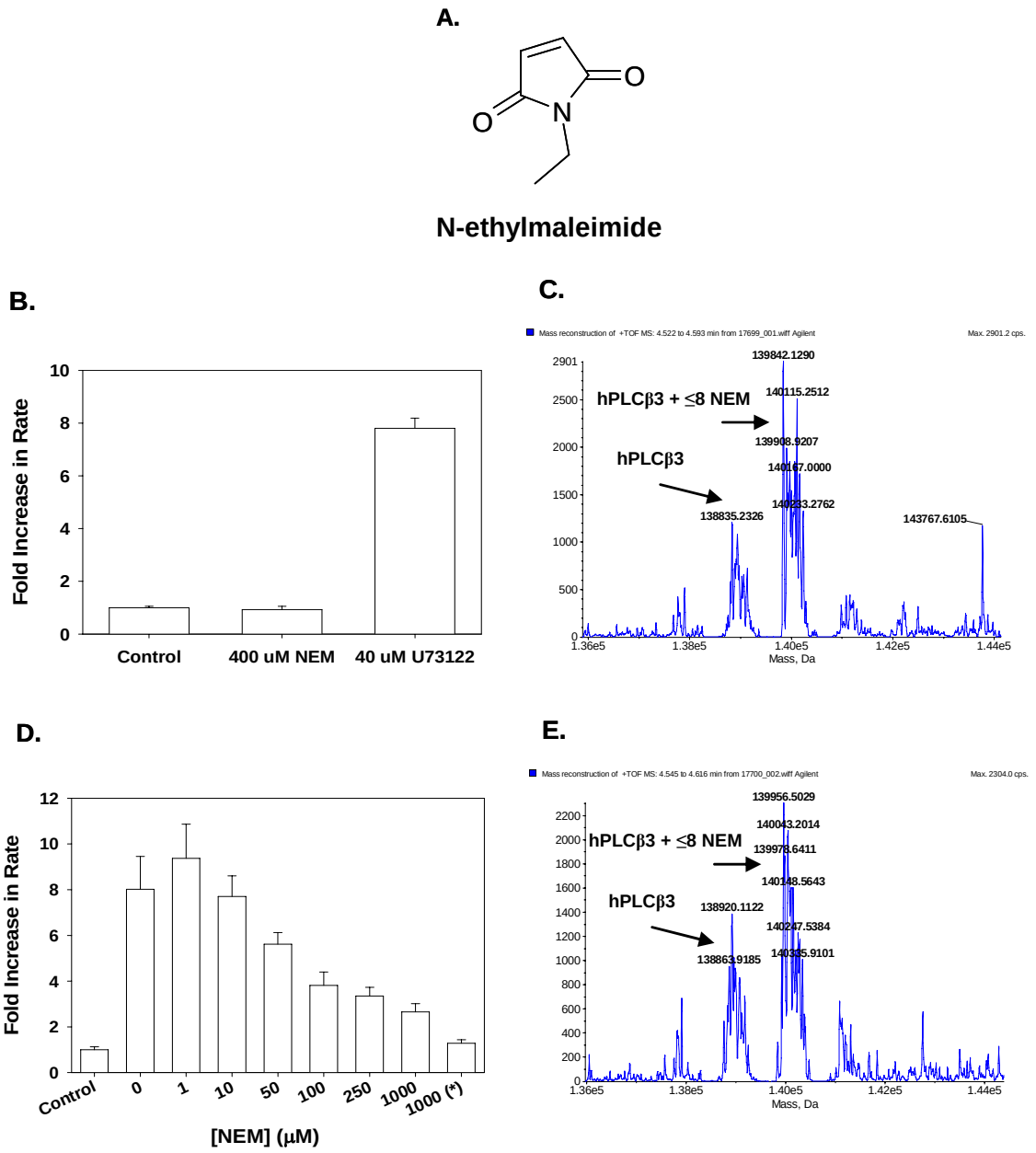


Figure 4.9. The effects of N-ethylmaleimide (NEM) on hPLCβ3 in DDM mixed micelles. (A) Structure of NEM. (B) The effect of NEM on hPLCβ3 activity in DDM mixed micelles. PIP_2, reconstituted in DDM, was combined with assay buffer and hPLCβ3 in a final volume of 100 µl. NEM or U73122, at indicated concentrations, were added to the assay mixture prior to the addition of enzyme. Assays were initiated by moving samples to a 37°C water bath. Incubation times were adjusted to ensure that less than 15% PIP_2 was hydrolyzed in all cases. Data represent mean ± S.D. from triplicate determinations. (C) Mass spectra of intact

hPLCβ3 following incubation with NEM. NEM was incubated with hPLCβ3 at 400 μM for fifteen minutes in assay buffer at 37°C in the absence of substrate and detergent. Incubations were terminated with the addition of 1 mM glutathione, and an aliquot was analyzed via LC/MS. (D) The effect of NEM on the U73122-mediated activation of hPLCβ3 in DDM mixed micelles. PIP_2, reconstituted in DDM, was combined with assay buffer and hPLCβ3 in a final volume of 100 μl. NEM, at indicated concentrations, and U73122 (40 μM) were added to the assay mixture prior to the addition of enzyme. * indicates that NEM was pre-incubated with enzyme prior to adding the enzyme to the assay mixture. Assays were initiated by moving samples to a 37°C water bath. Incubation times were adjusted to ensure that less than 15% PIP_2 was hydrolyzed in all cases. Data represent mean ± SD from triplicate determinations from one representative experiment. (E) Mass spectra of intact hPLCβ3 following sequential incubation with NEM and U73122. NEM was incubated with hPLCβ3 at 400 μM for fifteen minutes in assay buffer at 37°C in the absence of substrate and detergent, followed by the addition of U73122 at 40 μM for an additional fifteen minutes. Incubations were terminated with the addition of 1 mM glutathione, and an aliquot was analyzed via LC/MS. Treatment of hPLCβ3 with NEM (400 μM) followed by treatment with U73122 (40 μM) does not further modify hPLCβ3, consistent with almost complete inhibition of U73122-mediated activation by NEM at concentrations greater than 250 μM.

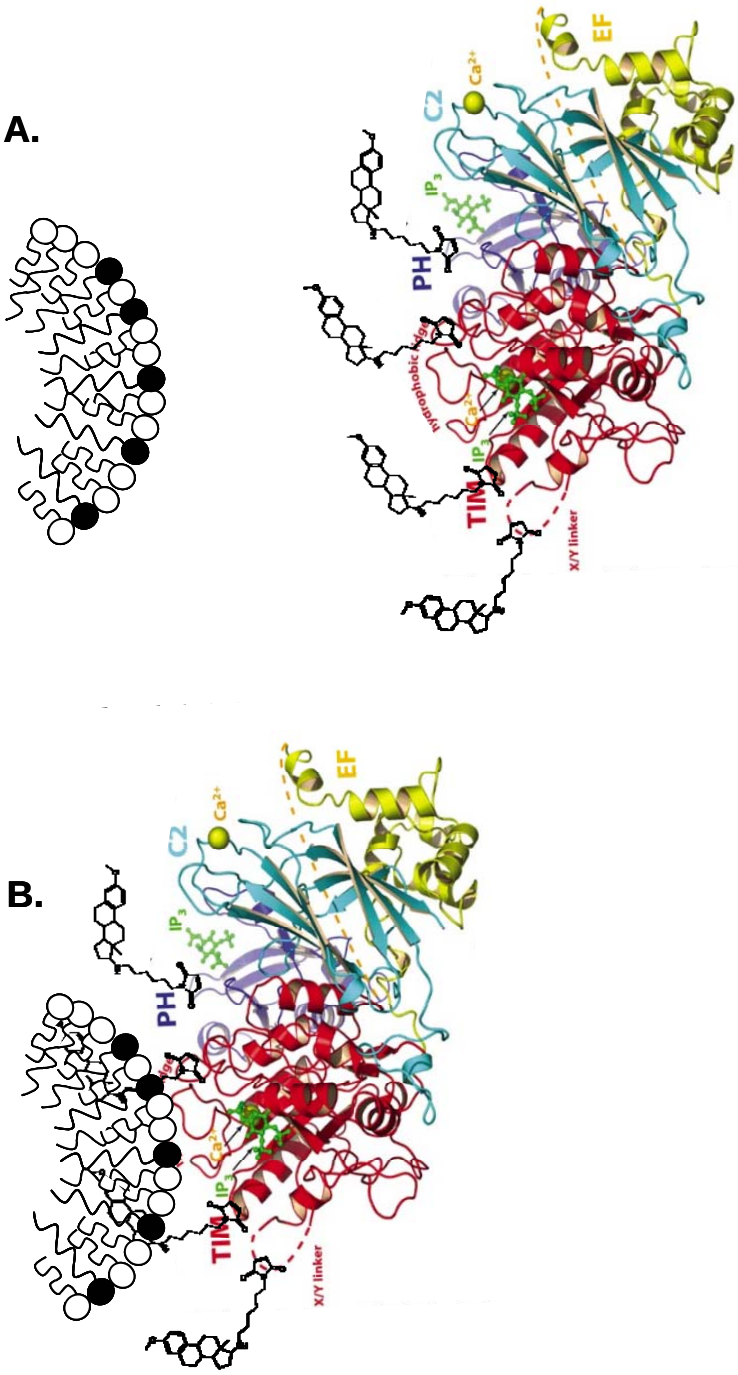
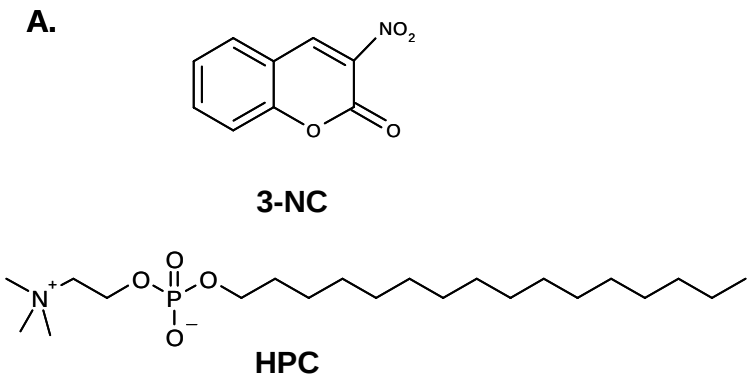


Fig. 4.10. Proposed model for the observed activation of hPLCβ3 in DDM mixed micelles. Open circles in micelle represent detergent (DDM) while closed circles represent substrate (PIP_2). Ribbon structure of PLCδ1 reproduced with permission from Harden and Sondek (2006). (A) Representative alkylation of PLC at multiple sites by U73122 prior to micelle

interaction. (B) A U73122-modified PLC anchored to the micelle via the steroid backbone of U73122 that facilitates enzyme interaction with substrate. Points of attachment of four representative U73122 molecules are not necessarily representative of identified alkylation sites in hPLCβ3.



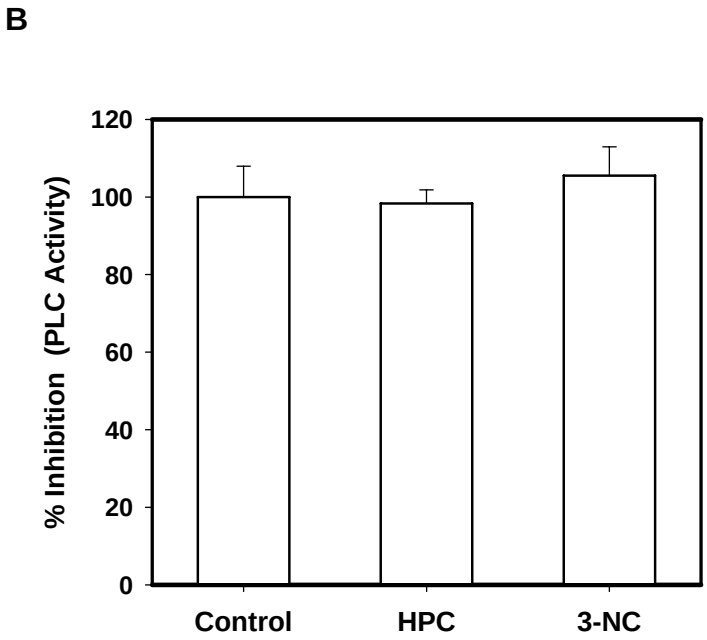


Figure 4.11. The effect of hexadecylphosphocholine (HPC) and 3-nitrocoumarin (3-NC) on hPLCβ3 activity in DDM mixed micelles. (A) Structures, (B) PIP_2, reconstituted in DDM, was combined with assay buffer and hPLCβ3 in a final volume of 100 µl. Both compounds (500 µM) were added to the assay mixture prior to the addition of enzyme. Assays were initiated by moving samples to a 37°C water bath. Incubation times were adjusted to ensure that less than 15% PIP_2 was hydrolyzed in all cases. Data represent mean ± SD from triplicate determinations.

CHAPTER 5

ALKYLPHOSPHOCHOLINES INCREASE PARACELLULAR PERMEABILITY VIA A MECHANISM INVOLVING MEMBRANE PERTURBATION

ABSTRACT

Phospholipase C (PLC) isozymes comprise a large family of key cell signaling proteins that hydrolyze inositol containing phospholipids to form two cellular second messengers, diacylglycerol (DAG) and inositol triphosphate (IP_3). Recently, PLCβ isozymes have been implicated in the regulation of epithelial tight junction permeability in both MDCK and Caco-2 cells based on the observed relationship between the potency of a series of alkylphosphocholines (APCs) as PLC inhibitors, and as PPEs. Evidence exists in the literature suggesting that APCs may be potent modulators of membrane fluidity and stability. The purpose of the present study was to determine whether APCs cause increased tight junction permeability via direct inhibition of PLCβ, or by perturbing cell membrane integrity. Hence, potency of amphiphilic APCs in a homologous series to partition into Caco-2 cell membranes and cause release of intracellular LDH was determined. Further, the ability of APCs to directly inhibit PLC activity was assessed in a phospholipid-detergent mixed micellar system. The ability of APCs to perturb cell membranes was dependent on the length of the alkyl chain, and was correlated to their potency as both enhancers of paracellular permeability and PLC inhibitors in Caco-2 cells. Further, although they all inhibited hPLCβ1 activity in a concentration dependent manner, their potency as inhibitors was inconsistent with their potency as PPEs. Finally, the ability of hexadecylphosphocholine to inhibit hPLCβ3 in mixed micelles was detergent dependent, with no observable inhibition when non-ionic dodecylmaltoside was used as detergent. Collectively, these data suggest that the ability of APCs to increase paracellular permeability may not be the direct result of PLC inhibition, but instead may be related to their ability to partition into cell membranes.

INTRODUCTION

Alkylphosphocholines (APCs) containing a polar zwitterionic head group attached to a variable length lipophilic alkyl chain have been reported to increase the permeability of the paracellular marker mannitol across epithelial monolayers of both MDCK and Caco2 cells (Chapter 2)[1-3], as well as across rat ileum *in situ*[4]. It has been suggested that these compounds, with structural similarities to membrane phospholipids, may be potential intestinal absorption enhancing agents for hydrophilic drugs and macromolecules in humans[1, 3, 5, 6].

Hexadecylphosphocholine (HPC), a prototypical APC with a sixteen carbon alkyl chain, was initially hypothesized to be an inhibitor of phospholipase C (PLC) more than fifteen years ago[7], with subsequent studies confirming this hypothesis[8, 9]. Recent studies have extended this hypothesis and demonstrated that APCs of variable chain length inhibited PLCβ in MDCK and Caco-2 cell monolayers with different potencies, and that a strong relationship existed between their potency as PLCβ inhibitors and as enhancers of paracellular permeability across these cell monolayers (Chapter 2)[3]. These observations implicated PLC in the regulation of epithelial tight junction barrier function. Specific downstream events have been attributed to this PLC inhibition-mediated increase in paracellular permeability by APCs, including reorganization of the cortical actin ring[3] and redistribution of tight junction proteins ZO-1[10] and claudin-1[1]. On the other hand, a more recent study contradicts the hypothesis that PLC isozymes regulate epithelial paracellular permeability, as RNAi mediated knockdown of both PLCβ3 and PLCγ1 in MDCK cells had no effect on the assembly of tight junctions, or on the permeability function of established tight junctions (Chapter 3). Therefore, the observed increase in paracellular permeability

following treatment of epithelial cells with APCs may occur via a mechanism other than PLC inhibition.

Changes to the structure of phospholipid membranes can lead to indirect effects on the catalytic activity of membrane associated enzymes[11-14]. Furthermore, tight junctions form between adjacent cells in specialized apical membrane microdomains called lipid rafts[15], and serve to separate the distinctly different lipid and protein composition of apical and basolateral membranes. Therefore, it is reasonable to hypothesize that alterations to the stability and integrity of the apical membrane may adversely affect both the activity of membrane associated PLC isozymes, as well as the barrier function of apically directed tight junctions. For example, depletion of cholesterol from the membrane of Caco-2 cells leads to a loss of barrier function, presumably by altering the distribution of specific tight junction proteins as a result of changes to the composition of the membrane[16]. Additional evidence suggests that APCs may disrupt the integrity of the apical membrane in Caco-2 cells[1, 5, 10, 17] at concentrations similar to those that have been shown to increase paracellular permeability (Chapter 2).

The purpose of this study was to evaluate the ability of APCs to inhibit PLCβ activity in a cell free system to determine if the inhibition was caused by direct interaction with the protein, or indirectly by perturbation of the substrate/enzyme interface (*e.g.* the cell membrane). Further, the ability of APCs to perturb integrity of the apical membrane in Caco-2 cells was quantified. Together, these studies would yield an improved understanding about the mechanism underlying the paracellular permeability enhancement by APCs.

MATERIALS AND METHODS

Materials. Purified cholate, as well as purified phospholipase Cβ1 and β3, were generously provided by the laboratory of Dr. T. K. Harden (Department of Pharmacology, School of Medicine, UNC Chapel Hill). Dodecylmaltoside was purchased from Fluka. APCs with various alkyl chain lengths were synthesized as previously described[3]. N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES), Hank's Balanced Salt Solution (HBSS), and other cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Twelve well Transwells™ were obtained from Costar (Cambridge, MA). CytoTox-ONE™ Homogeneous Membrane Integrity Assay was purchased from Promega Corporation (Madison, WI). [^{3}H]-PIP_2 (phosphatidylinositol 4,5-bisphosphate, [myo-inositol-2-^{3}H(N)]) (20 Ci/mmol) was purchased from American Radiolabeled Chemicals. PIP_2 was purchased from Avanti Polar Lipids. U73122, fatty acid free bovine serum albumin (BSA), and all other reagents were purchased from Sigma unless otherwise indicated.

Cell Culture. The Caco-2 cell line, Caco-2 cell clone HTB-37 was obtained from American Type Tissue Culture at passage 20. Caco-2 cells, derived from human colorectal carcinoma cells[18], were cultured at 37°C in minimum essential medium, supplemented with 10% FBS, 1% NEAA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B in an atmosphere of 5% CO_2 and 90% relative humidity. Caco-2 cells (passage number 25 to 35) were seeded at a density of 60,000 cells/cm^2 in Transwells™. Medium was changed the day after seeding, and every other day thereafter (apical volume 0.5 ml, basolateral volume 1.5 ml). Cells were grown to confluence for at least 21 days during which time they differentiated into epithelial cell monolayers as evidenced by the establishment of a stable

transepithelial electrical resistance (TEER) between 200-500 Ω*cm^2. Cells were used between 21-27 days after seeding in Transwells™.

Determination of lactate dehydrogenase leakage. Leakage of lactate dehydrogenase (LDH) into extracellular compartments is a measure often used as an indicator of cell membrane integrity. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay was used to assess the leakage of intracellular LDH from Caco-2 cells into the apical compartment. Cell media was aspirated from both compartments of Transwell™ inserts, replaced with transport buffer, and cells were pre-incubated at 37°C for 30 minutes. Integrity of the tight junctions was confirmed with the measurement of TEER prior to the experiment. Experiments were initiated by aspirating transport buffer from the apical compartment and replacing it with pre-warmed transport buffer (0.5 ml) containing test compound or vehicle. Cells were incubated at 37°C and TEER values were measured after 30 minutes. At the conclusion of the treatment period, an aliquot was removed from the apical compartment of each well, diluted ten fold with transport buffer, and assayed for LDH according to the manufacturer's instructions. Briefly, the diluted sample was transferred to a 96-well plate, mixed with an equal volume of reagent, and incubated for 10 minutes at room temperature. The reaction was stopped with 50 µl of stop solution and the fluorescent end product, resorufin, was detected with a Cytofluor fluorescent plate reader (Applied Biosystems, Foster City, CA) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Fluorescence readings of samples obtained from treated monolayers were normalized to the maximum amount of LDH released from monolayers, and reported as % LDH leakage ± SD of three experiments performed in triplicate. The maximal LDH leakage was determined by treating the monolayers with 0.18% (2.9 mM) Triton-X 100, as specified by the

manufacturer. The effect of test compounds on LDH leakage was evaluated at several concentrations, and $EC_{50\ LDH}$ values, defined as the concentration causing half maximal leakage of LDH from Caco-2 cell monolayers, were determined.

Mixed Micellar PLC Assays. The activity of hPLCβ1 and hPLCβ3 in a cell free system was evaluated by an adaptation of previously published methods[19, 20]. Briefly, [^{3}H]-labeled and unlabeled PIP_2 (5 nmol) was reconstituted in a 1% cholate, or 1 mM dodecylmaltoside (DDM) solution, and mixed with assay buffer containing 40 mM Hepes (pH 7.4), 480 mM KCl, 40 mM NaCl, 8 mM EGTA, 23.2 mM $MgSO_4$, and 8.4 mM $CaCl_2$. For assays in DDM, 4 mM $MgCl_4$, and 7.6 mM $CaCl_2$ were used in lieu of 23.2 mM $MgSO_4$, and 8.4 mM $CaCl_2$. Compounds at indicated concentrations, and purified enzyme in 1% fatty acid-free BSA and 10 mM Hepes (pH 7.0), were subsequently added to the mixture. To initiate assays, samples were moved to a 37°C (or 30°C for studies in DDM) water bath and incubated for 2-10 minutes such that less than ten percent total substrate was hydrolyzed. At designated times, reactions were stopped by addition of either 200 µl trichloroacetic acid and 100 µl of 10 mg/ml fatty acid free BSA. For cholate assays, 750 µl $CHCl_3$:MeOH:HCl (40:80:1), 100 µl water, 250 µl CHCl3, and 250 µl 0.1M HCl were added for precipitation instead. Samples were vortexed and centrifuged at 3000 rpm for 10 minutes at 4°C. The amount of formed [^{3}H]-inositol phosphates was measured by liquid scintillation counting of the supernatant in a Packard Tri Carb 4000 Series spectrophotometer. The $IC_{50\ (PLC\beta)}$, defined as the concentration causing a 50% decrease in the formation of inositol phosphates, was determined.

Data analysis. Data are expressed as the mean ± standard deviation (SD) from three measurements unless indicated otherwise. Where indicated, statistical significance was assessed using a two sample Student's t tests. Samples were assumed to have an unequal

variance; significant differences were assigned at $p < 0.05$. For determination of $EC_{50\ LDH}$ values, the relationship between LDH leakage (% Cytotoxicity) and enhancer concentration was fit to a four parameter Hill equation by nonlinear least-squares regression (WinNonlin, version 4.1). Estimates were also generated for the sigmoidicity factor (γ), E_0, and E_{max}. For determination of $IC_{50\ PLC\beta1}$ values, the relationship between hPLCβ1 activity (% Inhibition) and enhancer concentration was fit to a three parameter Hill equation by nonlinear least-squares regression. Estimates were also generated for the sigmoidicity factor (γ) and E_{max}. Relationships between parameters estimating paracellular permeability enhancement and LDH release were assessed via linear regression analysis, and the correlation was expressed by the Pearson correlation coefficient (r).

RESULTS

Activity of human PLCβ1 in phospholipid-detergent mixed micelles.

hPLCβ1 activity with respect to time (using PIP_2 as substrate) was measured using cholate as a surfactant to establish an interface for presentation of the insoluble substrate to the enzyme in the absence of a cell membrane phospholipid bilayer (Figure 5.1). Consistent with previous studies with dodecylmaltoside as detergent (Chapter 4), two distinct phases of activity were observed, an initial burst of activity linear when ≤ 10% PIP_2 is utilized, followed by a second, slower phase of hydrolysis. Additional studies were performed at fixed PIP_2 concentrations and were designed such that ≤ 10% of total substrate was utilized in all cases.

Alkylphosphocholines inhibit hPLCβ1 activity in cholate mixed micelles.

In cholate mixed micelles, HPC (Figure 5.2) inhibited the activity of hPLCβ1 in a concentration-dependent manner (Figure 5.3). The concentration of HPC that caused 50% inhibition of activity was estimated from a plot of % inhibition versus concentration and is reported in Table 5.1. The $IC_{50\ hPLC\beta1}$ determined in cholate mixed micelles was similar to the $IC_{50\ PLC\beta}$ determined in Caco-2 cells (Table 5.1), and therefore, the potency of HPC for inhibiting PLC in both systems is greater than its potency as a PPE in Caco-2 cells (quantified using $EC_{50\ TEER}$ and EC_{10X}).

Because HPC inhibited hPLCβ1 in cholate mixed micelles, the ability of all APCs (Figure 5.2) to inhibit hPLCβ1 activity in this cell free system was evaluated. All APCs inhibited hPLCβ1 activity in a concentration dependent manner (Figure 5.4). $IC_{50\ hPLC\beta1}$ values are reported in Table 5.1. The potency of APCs for inhibiting the activity of hPLCβ1 activity was dependent on chain length and increased with the length of the alkyl chain, with

maximum potency occurring at a chain length of twenty carbons. This chain length dependent potency of APCs as inhibitors of hPLCβ1 is different than the relationship between chain length of APCs and potency as both PLCβ inhibitors and PPEs in Caco-2 cells, where a maximum potency was achieved with approximately fourteen to sixteen carbons in the alkyl chain (Table 5.2).

Hexadecylphosphocholine inhibition of hPLCβ3 in phospholipid-detergent mixed micelles is detergent dependent.

To further assess the interaction between APCs and PLC isozyme activity, the ability of HPC, as a representative APC, to inhibit hPLCβ3 activity was assessed in both cholate and DDM mixed micelles, systems composed of detergents with (cholate) or without (DDM) a net charge. The ability of HPC to inhibit hPLCβ3 activity was detergent dependent (Figure 5.5), as it was unable to inhibit hPLCβ3 activity in DDM mixed micelles at concentrations as high as 500 μM, yet it inhibited hPLCβ3 activity in cholate mixed micelles in a concentration dependent manner.

Alkylphosphocholines cause leakage of lactate dehydrogenase from Caco-2 cells.

To evaluate the effect of APCs on Caco-2 cell membranes, the ability of HPC, as a representative APC, to disrupt the barrier function of cell membranes and cause release of intracellular LDH was evaluated (Figure 5.6). Apical HPC treatment led to a concentration dependent release of LDH from Caco-2 cell monolayers into the apical compartment. The concentration that caused 50% of total LDH leakage ($EC_{50\ LDH}$) was estimated from the plot of % LDH release versus HPC concentration, and is reported in Table 5.2. The potency of HPC for causing LDH leakage ($EC_{50\ LDH}$) was approximately three-fold higher than its potency for increasing paracellular permeability ($EC_{50\ TEER}$ and EC_{10X}) in Caco-2 cells.

Similar to HPC, all APCs caused release of LDH from Caco-2 cells in a concentration dependent manner (Figure 5.7). $EC_{50\ LDH}$ values are reported in Table 5.2. Interestingly, increasing concentrations of C10 never released more than approximately 30% of the total cytosolic LDH. Control studies were performed to ensure that this result was not due to interference with the LDH assay at the high concentrations of C10 required to observe LDH release (data not shown).

The relationship between the potency of APCs as cytolytic agents and their potency as both PLC inhibitors and PPEs was evaluated (Figure 5.8). The $EC_{50\ LDH}$ is linearly related to both $EC_{50\ TEER}$ a ($r > 0.99$, $p < 0.0001$) and EC_{10x} ($r > 0.99$, $p < 0.0001$). Interestingly, their potency as PPEs was approximately 2.5 fold greater than their potency as cytolytic agents in Caco-2 cells.

Non-amphiphilic PPE and PLC inhibitor, U73122 (Chapter 2) (Figure 5.9), did not cause any increase in LDH leakage into the apical compartment after up to ninety minutes of exposure to Caco-2 cells at concentrations that also increase paracellular permeability (*i.e.* up to 30 μM), suggesting that the release of LDH caused by APCs is not the direct results of PLC inhibition.

DISCUSSION

The PLC-dependent pathway has long been postulated to be involved in the regulation of epithelial tight junctions[6, 21-23]. More recent studies have directly implicated this important class of signaling molecules in the regulation of epithelial paracellular permeability (Chapter 2)[3, 24]. However, results from Chapter 3 show that suppression of the expression of both PLCβ3 and PLCγ1 in MDCK cells by RNAi had no effect on either the assembly of tight junctions, or on the permeability of established tight junctions. These results cast some doubt on the involvement of PLC enzymes in the regulation of tight junctions, although they cannot rule out the possibility that PLC isozymes other than PLCβ3 and PLCγ1 may be involved. Clearly, these results raised questions about whether inhibition of PLCβ by APCs in both Caco-2 cells and MDCK cells was responsible for their activity as enhancers of paracellular permeability in these cell systems, and suggested that the observed increase in paracellular permeability following APC treatment may occur via a mechanism other than PLC inhibition.

HPC (miltefosine) is the first orally effective treatment for visceral leishmaniasis and has proven to be highly effective in phase III clinical trials[25]. The major side effects associated with oral administration of miltefosine include frequent but mild nausea, vomiting, and diarrhea[26]. These local gastrointestinal side effects may be due to the reported ability of APCs to effectively partition into phospholipid membranes[27-29], a characteristic exploited in cancer therapy, where their anti-neoplastic properties are believed to be related to their interaction with cell membranes rather than DNA[30]. This is supported by the finding that the rate of HPC oral absorption is extremely slow, and an unusually high percentage of HPC is recovered from intestinal tissue as much as twenty four hours after oral

administration to mice[31-34]. Additional evidence has established the ability of APCs to alter the fluidity and barrier function of apical membranes in Caco-2 cells[1, 5, 10, 17]. Thus, an alternative hypothesis is considered in this study that the observed increase in paracellular permeability following APC treatment is due to disruption of tight junctions caused by alteration in cell membrane fluidity or integrity. Therefore, the inhibition of PLC enzymes caused by the APC treatment may not necessarily have a cause-effect relationship with the enhancement of paracellular permeability.

As discussed in Chapter 4, PLCs are water soluble enzymes that hydrolyze poorly soluble membrane phospholipids. In the absence of whole cells, these proteins require the presence of a suitable interface between hydrophilic enzyme and hydrophobic substrate to achieve measurable enzymatic activity. A number of useful experimental systems have been described for assessing lipase activity, perhaps the simplest of which is a mixed micellar system. The potency of APCs to inhibit hPLCβ1 activity was evaluated in cholate mixed micelles to assess their direct interaction with PLC. A concentration dependent effect of HPC on hPLCβ1 activity was observed. The $IC_{50\ PLC\beta}$ was estimated to be 95 ± 9 μM, similar to its potency as a PLCβ inhibitor in Caco-2 cells, 40 ± 19 μM (Table 5.1). The ability of the entire APC series to inhibit hPLCβ1 activity was determined in triplicate and $IC_{50\ hPLC\beta1}$ values were determined. Within the series of APC, the ability to inhibit hPLCβ1 activity was clearly dependent on alkyl chain length with a maximum potency exhibited by APC containing twenty carbons in the alkyl chain (Table 5.1), inconsistent with the reported relationship between chain length of APCs and their potencies as PLCβ inhibitors in both MDCK and Caco-2 cells, where a chain length of fourteen to sixteen carbons achieved maximum inhibitory potency. Interestingly, their potency as hPLCβ1 inhibitors in mixed micelles was

more closely related to their reported critical micelle concentrations (Table 5.1), implying that their ability to inhibit PLC activity in this system may be dependent on the micellar form of APCs causing the disruption of the detergent/substrate mixed micelles. To further probe this hypothesis, the non-ionic detergent, dodecylmaltoside (DDM), was used instead of cholate, a negatively charged detergent, in the mixed micellar system. Previous reports have established the use of DDM as a suitable inert diluent to assess PLC activity in lipid-detergent mixed micelles (Chapter 4)[19, 20]. In cholate mixed micelles, HPC inhibited the activity of hPLCβ3 in a concentration dependent manner; however, in DDM mixed micelles, HPC was unable to inhibit hPLCβ3 activity at concentrations up to 500 μM (Figure 5.5). This result supports the hypothesis that APC inhibition of PLC activity is indirect, and dependent on the ability of APCs to perturb the interface between water soluble enzyme and lipid soluble substrate. However, this hypothesis contradicts the observation that PLC can metabolize HPC in mice, as well as *in vitro* with purified enzyme, hydrolyzing HPC as if it were a glycerophospholipid composed of phosphocholine and hexadecanol[31], and implying that inhibition of PLC activity by HPC may be via competitive binding to the enzyme active site. Additional studies are required to further address this apparent contradiction.

Due to the inconsistency between the potency of APCs to inhibit PLC in a cell free system as compared to the whole cells systems (*i.e.* MDCK and Caco-2 cells), the potency of APCs to cause release of LDH into the apical compartment of cultured Caco-2 cell monolayers was evaluated. Within the series of APCs, their ability to cause release of LDH was dependent on chain length; this dependence followed the order C14, C16, C18, C20, C12, and C10, identical to the chain length dependence for both increasing paracellular permeability and inhibiting PLCβ activity (Figure 5.7 and Table 5.2). The potency of APCs

to cause membrane disruption was approximately 2.5 fold less than the potency for increasing paracellular permeability, consistent with previous studies reporting a narrow window (*i.e.* two to three fold) between the efficacy and cytotoxicity of APCs in cultured cells[1, 4, 10]. Notably, small but significant amounts of LDH release from Caco-2 cell monolayers were apparent at concentrations that had minimal effect on TEER (*i.e.* 100 μM) (Figure 5.6), demonstrating that the increase in permeability and release of LDH occur at similar concentrations, and may be related events. Further, there is a strong correlation between the potency of APCs for causing LDH release and for increasing paracellular permeability (Figure 5.8), similar to the correlation between their potency as paracellular permeability enhancers and PLCβ inhibitors. These established relationships between the three measurable effects of APCs on Caco-2 cells cloud any cause-effect interpretation between any two of the effects, namely PLC inhibition and paracellular permeability enhancement. The results from Chapter 3, demonstrating that specific suppression of PLC expression by RNAi has no impact on tight junction barrier function further raise questions regarding the role of PLC enzymes in regulating tight junction permeability. The initial pharmacological effect of APCs on epithelial cell monolayers may be disruption of the integrity of the apical membrane, followed by two dependent downstream events: (1) loss of tight junction barrier function and (2) indirect inhibition of membrane associated PLC. This hypothesis is not without precedent, as the absorption enhancing effect of other amphiphilic PPEs, such as acylcarnitines, has been shown to be dependent on the length of the lipophilic carbon chain and the ability of this series of amphiphiles to interact with cell membranes[35]. In this regard, specific membrane composition of different cell types may dictate the potency of APCs as PLC inhibitors, or as PPEs, in different models of epithelia and in different tissues.

It is worth noting that non-amphiphilic PPE and PLC inhibitor, U73122 (Figure 2.10), did not release LDH from Caco-2 cells at concentrations that increased paracellular permeability (Figure 5.9), implying potentially different mechanisms of action for these structurally distinct PPEs (*i.e.* U73122 and HPC).

Given the damage to Caco-2 cell membranes at increasing concentrations of APCs allowing the release of relatively large intracellular proteins such as LDH, the observed increase in transport of the paracellular marker mannitol could be due an increase in transcellular transport rather than paracellular transport, particularly at higher concentrations of APCs. Consistent with this hypothesis, HPC has previously been shown to dramatically alter the cellular distribution of a fluorescent paracellular probe from a strictly paracellular localization to one with an extensive transcellular as well as paracellular localization[17]. However, a more extensive evaluation of the effects of apically dosed APCs on the integrity of the basolateral membrane would need to be assessed, as the basolateral membrane serves as an additional barrier to the transcellular transport of hydrophilic molecules such as mannitol, and could prevent the movement of mannitol into the basolateral compartment via the transcellular route. Therefore, the increased mannitol permeability induced by APCs could be strictly paracellular, as the apical membrane perturbation could lead to de-stabilization of the tight junction barrier allowing increased paracellular transport across the monolayer as well as increased access to the cytosol from the apical compartment, but not increased transcellular permeability.

Collectively, these data suggest that APCs may not directly interact with the active site of PLC, but instead may indirectly inhibit lipase activity by interfering with the quality of

the enzyme/substrate interface via a process referred to as “competitive interfacial inhibition”[36].

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Table 5.1. Potency of alkylphosphocholines (APCs) as inhibitors of hPLCβ1 in cholate mixed micelles, reported critical micelle concentrations[37], and potency as inhibitors of ATP-stimulated PLCβ activity in Caco-2 cells.

APC	$IC_{50\ PLC\beta1}$ (μM) (cell free)	CMC (μM)	$IC_{50\ PLC\beta}$ (μM) (Caco-2) *
C10	507 ± 137	10930 ± 2890	1304 ± 837
C12	288 ± 51	1278 ± 171	123 ± 28
C14	176 ± 32	110 ± 11	29 ± 13
C16	95 ± 9	13 ± 2.8	40 ± 19
C18	112 ± 21	1.4 ± 0.099	70 ± 38
C20	58 ± 24	0.14	104 ± 8

* Reproduced from Table 2.3. Specific details surrounding the determination of these parameters are also provided in Material and Methods, Chapter 2.

Table 5.2. Potency of alkylphosphocholines (APCs) as paracellular permeability enhancers ($EC_{50\ TEER}$ and EC_{10X}), as inhibitors of PLCβ activity ($IC_{50\ PLC\beta}$), and as compounds that cause LDH leakage and damage to the apical cell membrane ($EC_{50\ LDH}$) in Caco-2 cells.

APC	$EC_{50\ LDH}$ (μM)	$EC_{50\ TEER}$ (μM) *	EC_{10X} (μM) *	$IC_{50\ PLC\beta}$ (μM) *
C10	ND	9800 ± 1375	8333 ± 1320	1304 ± 837
C12	1494 ± 126	735 ± 62	576 ± 160	123 ± 28
C14	685 ± 86	213 ± 23	261 ± 90	29 ± 13
C16	745 ± 44	248 ± 52	317 ± 26	40 ± 19
C18	1093 ± 51	299 ± 19	384 ± 94	70 ± 38
C20	1254 ± 28	429 ± 77	481 ± 45	104 ± 8

* Reproduced from Table 2.3. Specific details surrounding the determination of these parameters are also provided in Material and Methods, Chapter 2.

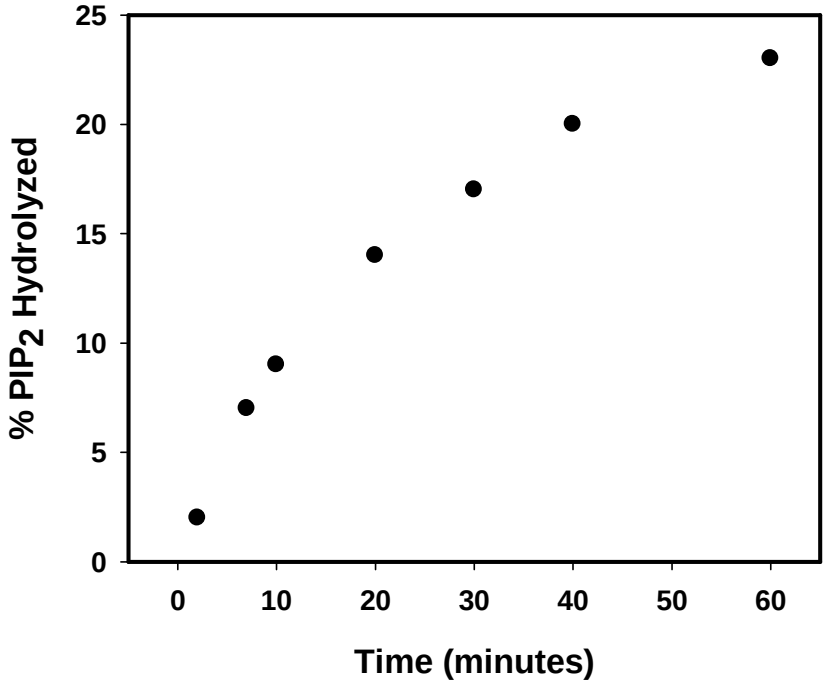

Figure 5.1. Time course of PIP_2 hydrolysis by hPLCβ1 in cholate mixed micelles. PIP_2, reconstituted in cholate, was combined with assay buffer and hPLCβ3 in a final volume of 100 µl. Assays were initiated by moving samples to a 30°C water bath and incubating for indicating times. Data represent mean from duplicate determinations from one representative experiment.

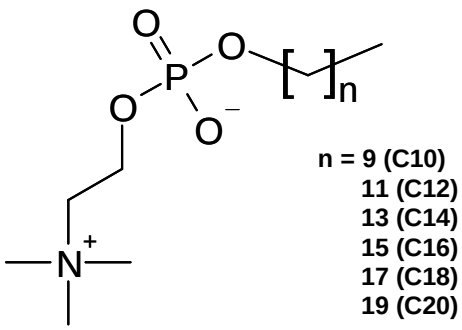

Figure 5.2. Structures of alkylphosphocholines.

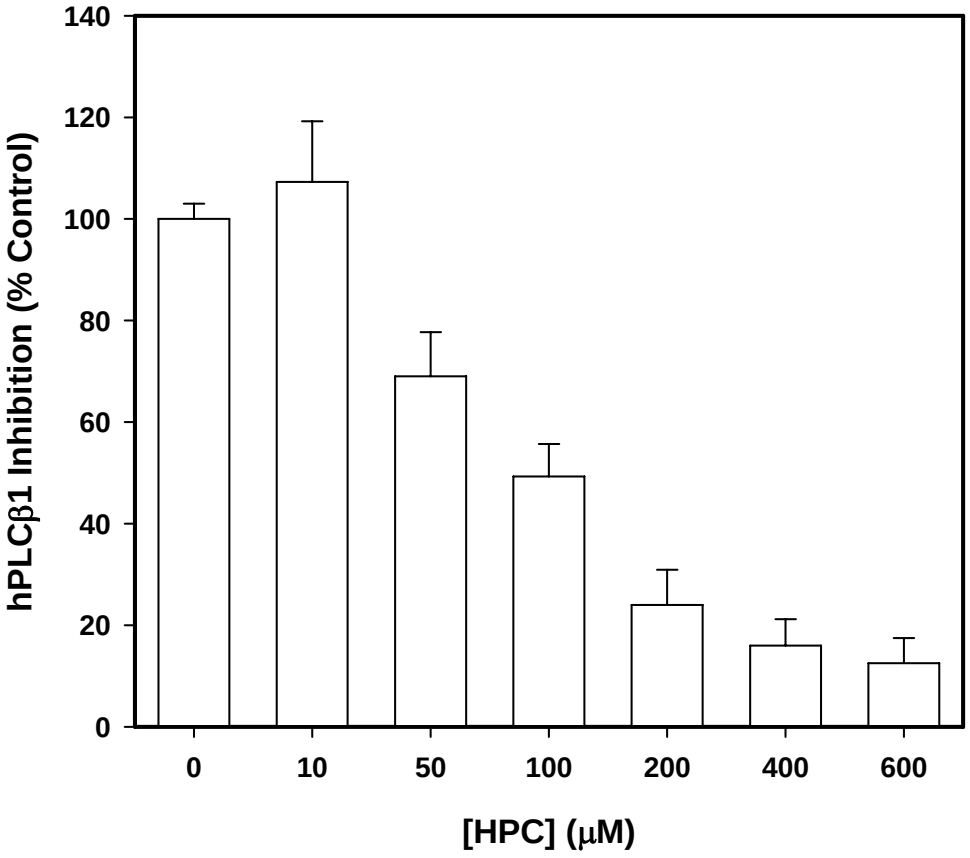

Figure 5.3. Concentration dependent inhibition of hexadecylphosphocholine (HPC) on hPLCβ1 in cholate mixed micelles. PIP_2, reconstituted in DDM, was combined with assay buffer and hPLCβ1 in a final volume of 100 μl. HPC, at indicated concentrations, was added to the assay mixture prior to the addition of enzyme. Assays were initiated by moving samples to a 30°C water bath. Incubation times were adjusted to ensure that less than 10% PIP_2 was hydrolyzed in all cases. Data represent mean ± SD from triplicate determinations from one representative experiment.

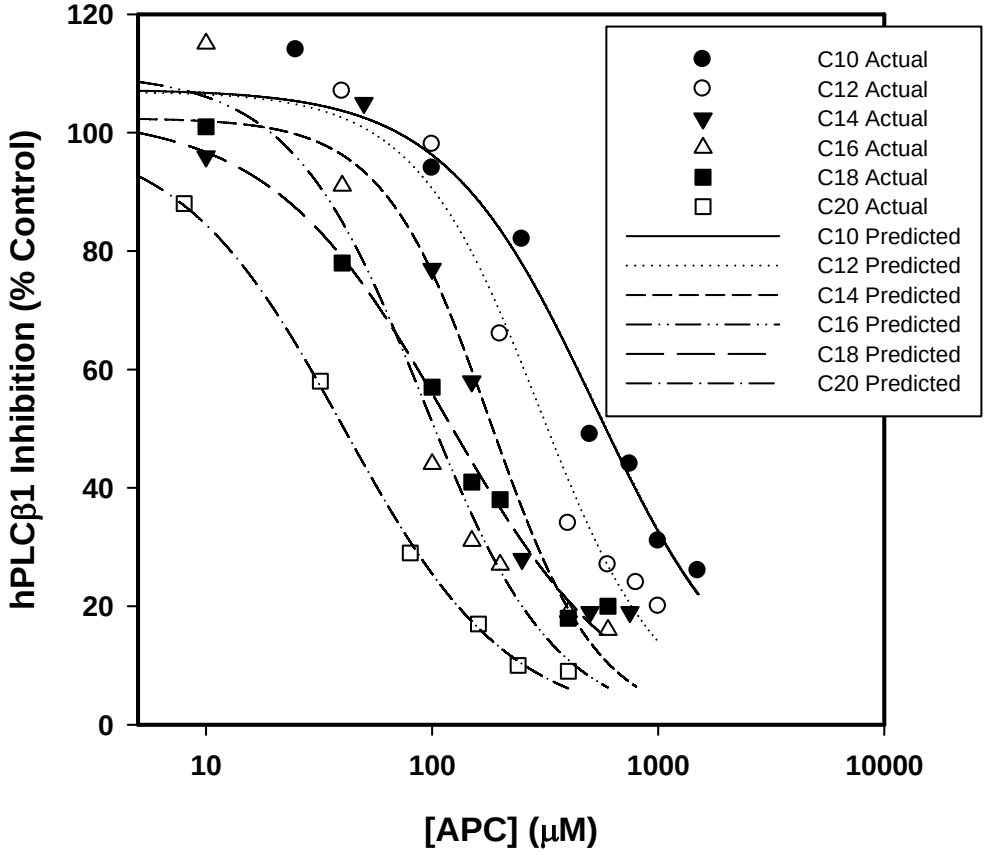

Figure 5.4. Concentration dependent inhibition of alkylphosphocholines (APCs) on hPLCβ1 in cholate mixed micelles. PIP_2, reconstituted in DDM, was combined with assay buffer and hPLCβ1 in a final volume of 100 μl. APCs, at indicated concentrations, was added to the assay mixture prior to the addition of enzyme. Assays were initiated by moving samples to a 30°C water bath. Incubation times were adjusted to ensure that less than 10% PIP_2 was hydrolyzed in all cases. $IC_{50\ PLC\beta1}$ values are reported in Table 5.1. Data represent mean from duplicate determinations from one representative experiment for each APC.

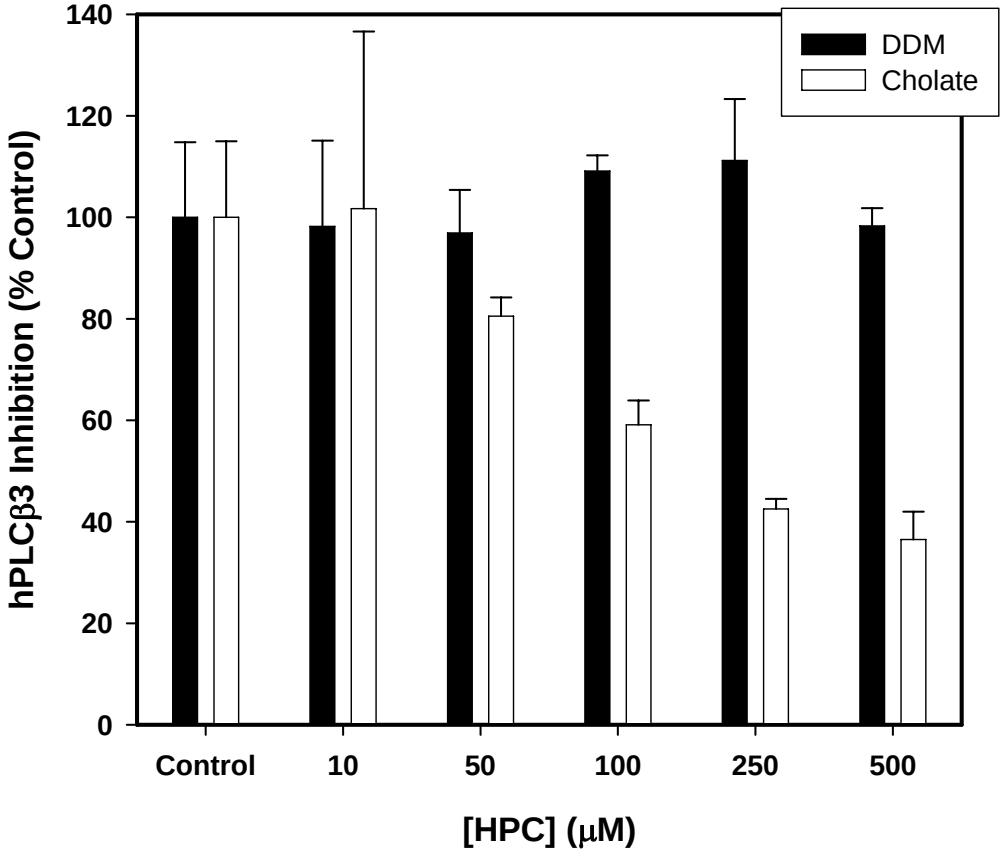

Figure 5.5. Concentration dependent inhibition of hexadecylphosphocholine (HPC) on hPLCβ3 in cholate mixed micelles and in DDM mixed micelles. PIP_2, reconstituted in detergent, was combined with assay buffer in a final volume of 100 µl. HPC, at indicated concentrations, was added to the assay mixture prior to the addition of enzyme. Assays were initiated by moving samples to a 30°C or 37°C water bath for cholate and DDM assays respectively. Incubation times were adjusted to ensure that less than 10% PIP_2 was hydrolyzed in all cases. Data represent mean ± SD from triplicate determinations from one representative experiment.

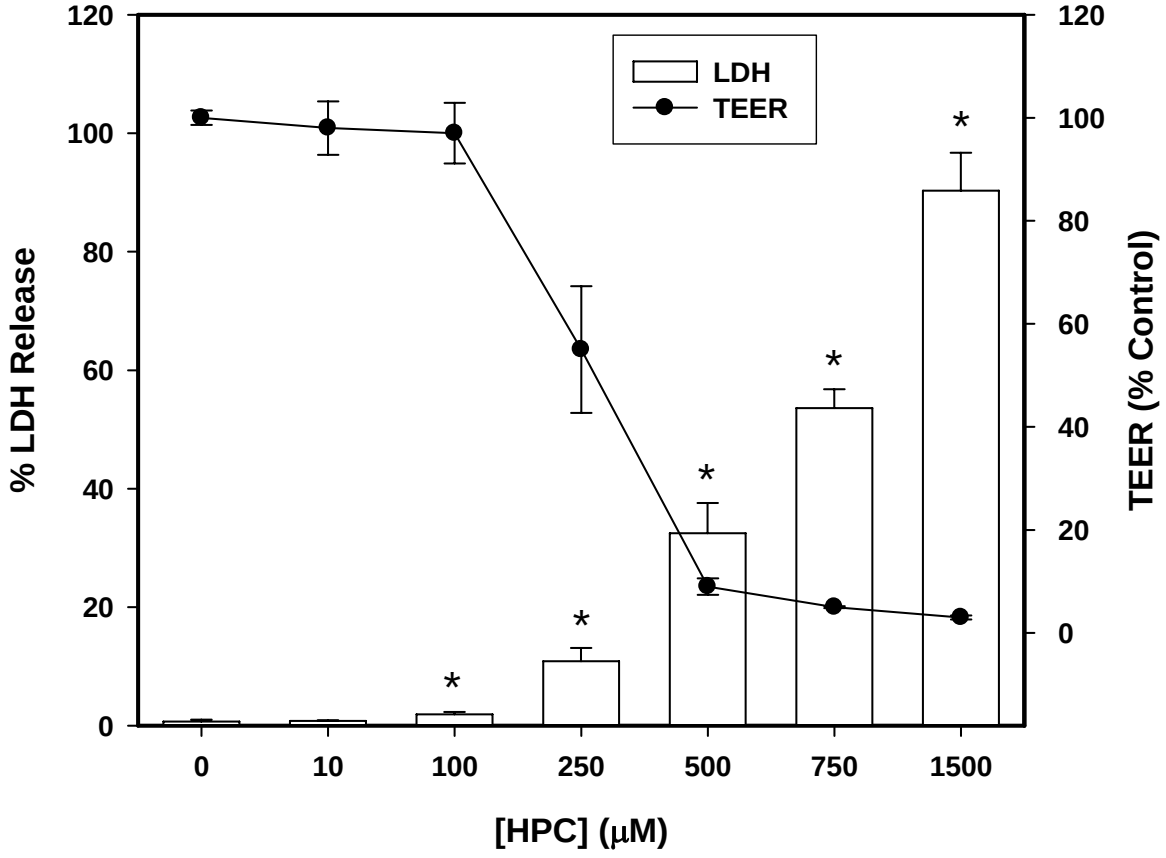

Figure 5.6. Concentration dependent effect of hexadecylphosphocholine (HPC) on LDH release from Caco-2 cells. Cells were treated apically with HPC at indicated concentrations for thirty minutes and the amount of LDH released into the apical compartment was determined. Data represent mean ± SD from triplicate determinations from one representative experiment. Asterisks indicate significant difference ($p < 0.05$) for LDH release.

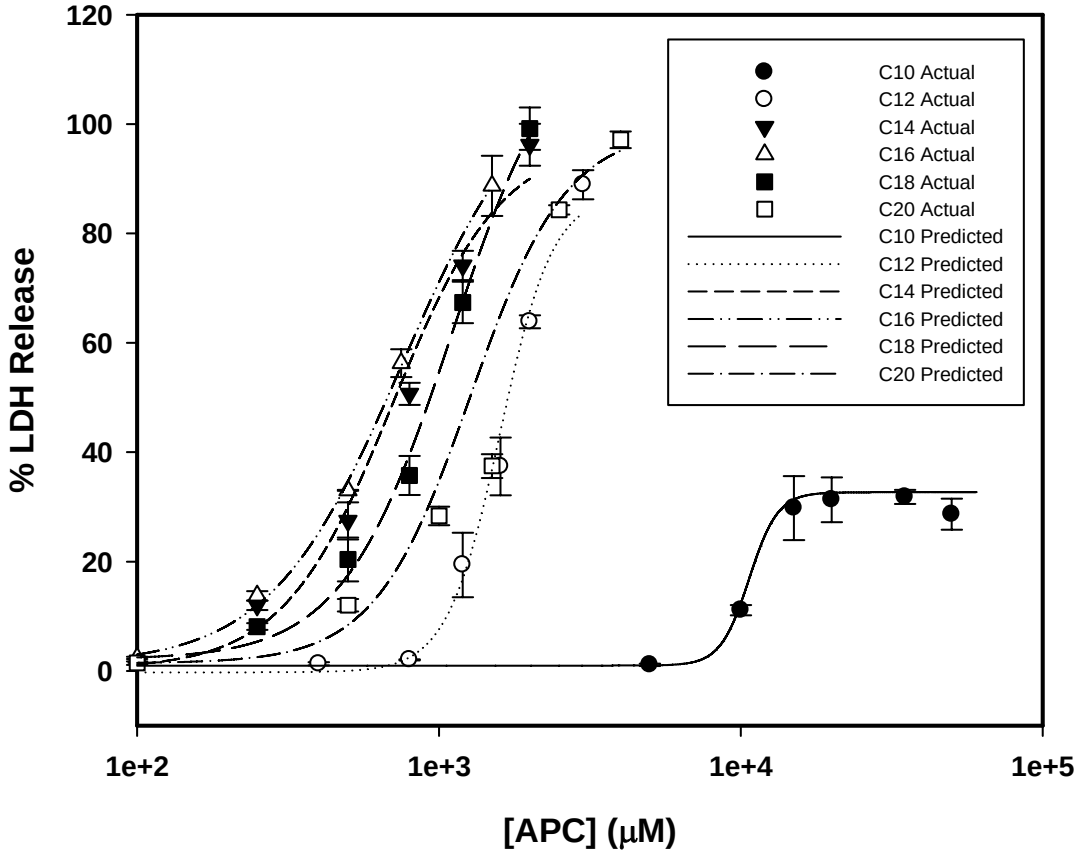

Figure 5.7. Concentration dependent effect of alkylphosphocholines (APCs) on LDH release from Caco-2 cells. Cells were treated apically with APCs at indicated concentrations for thirty minutes and the amount of LDH released into the apical compartment was determined. Data represent mean ± SD from triplicate determinations from one representative experiment for each APC.

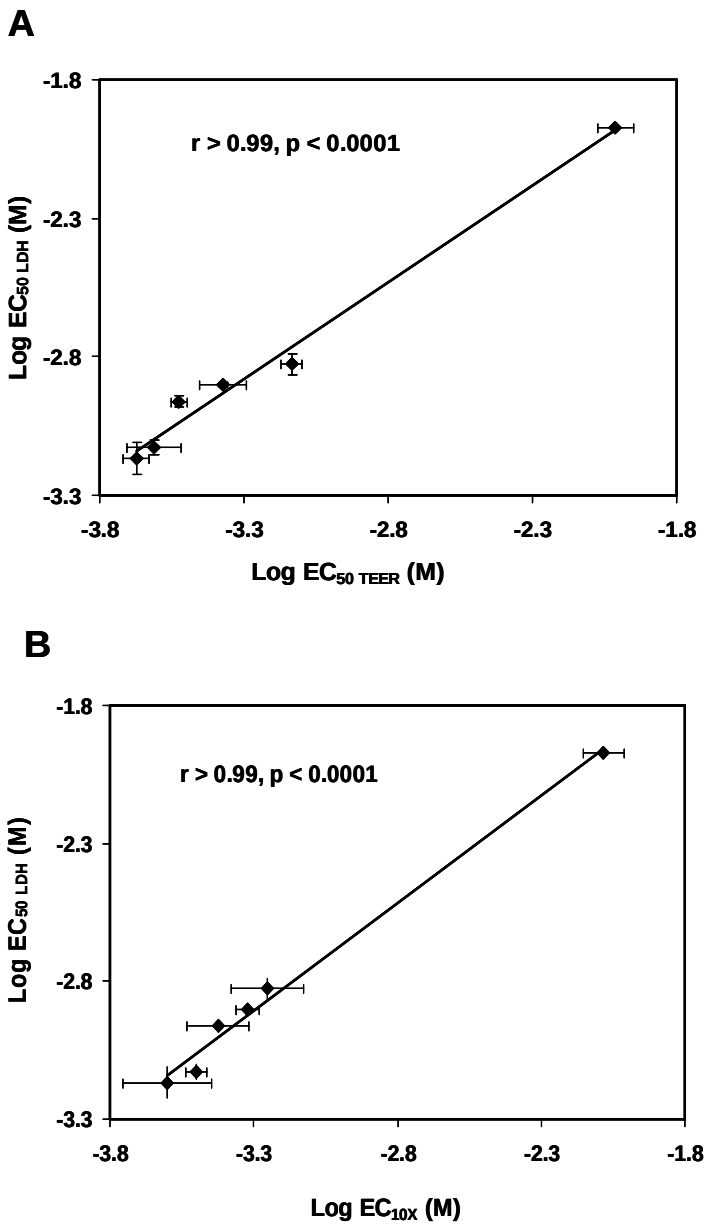

Figure 5.8. The relationship between LDH release and increase in paracellular permeability by alkylphosphocholines in Caco-2 cell monolayers. (A) The relationship between the $EC_{50\ LDH}$ and $EC_{50\ TEER}$, and (B) the relationship between the $EC_{50\ LDH}$ and EC_{10X}. See Table 5.2 for data. The correlation is expressed by the Pearson correlation coefficient (r).

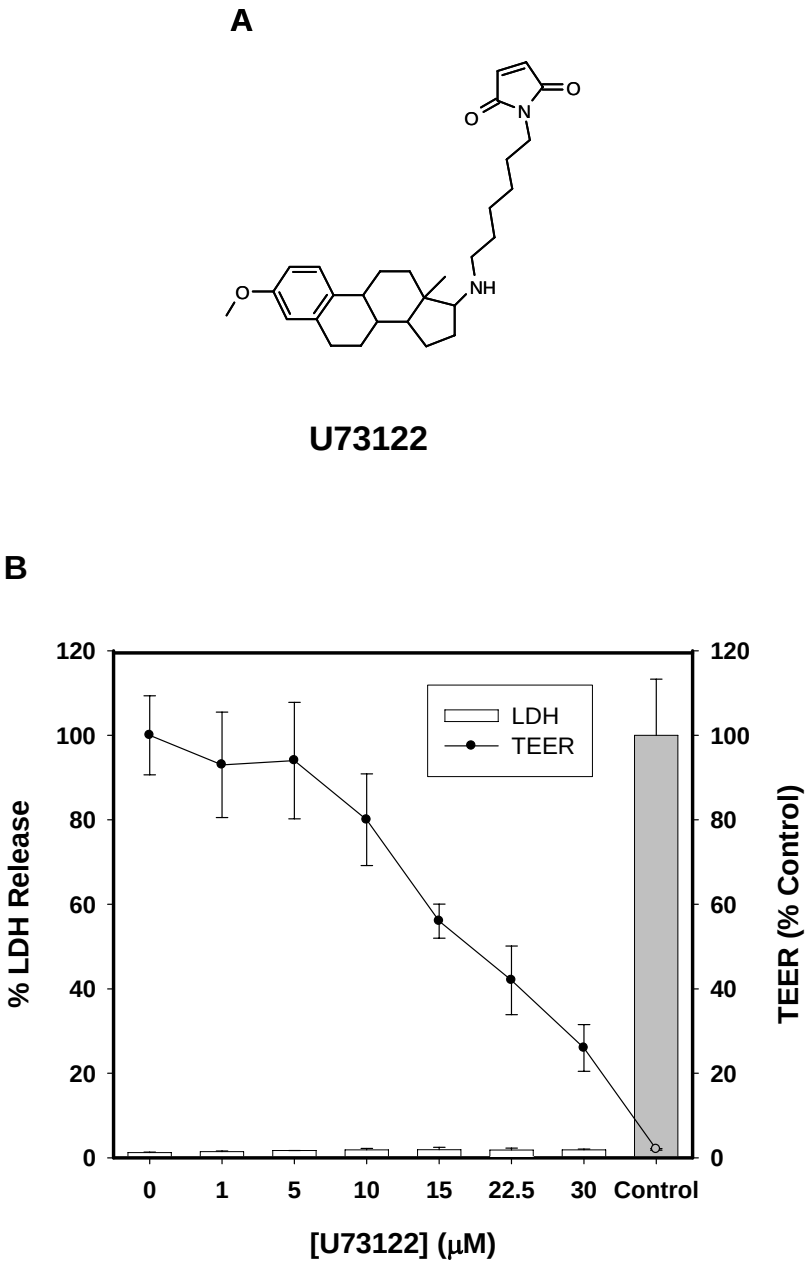

Figure 5.9. Concentration dependent effect of U73122 on LDH release from Caco-2 cells. (A) Structure of U73122, and (B) Cells were treated apically with U73122 at indicated concentrations for ninety minutes and the amount of LDH released into the apical compartment was determined. Data represent mean ± SD from triplicate determinations from one representative experiment.

CHAPTER 6

CONCLUSIONS

The overarching goal of this dissertation research was to develop a clearer understanding of the molecular mechanisms involved in the regulation of intestinal epithelial tight junctions via the PLC-catalyzed signal transduction cascade. Specifically, the goal was to explore whether a cause-effect relationship exists between the inhibition of PLC activity and increased paracellular permeability via specific modulation of epithelial tight junctions. The outcome of this investigation is of considerable interest, as PLC isozymes have been postulated for many years as potential regulatory enzymes of tight junction function[1-4]; however, unequivocal evidence to support such a role for any PLC isozyme has yet to be established. A cause-effect relationship between the activity of specific PLC isozymes and tight junction permeability would allow mechanism based approaches to selectively and reversibly increase the permeability of intestinal tight junctions. Such a technology would have significant implications in the pharmaceutical industry, leading to the development of novel formulations for hydrophilic drugs, including small to midsize peptides and oligonucleotides that are typically very poorly absorbed following an oral dose. The search for a safe and effective paracellular permeability enhancer (PPE) for clinical use in humans has not yet yielded success, partly due to the lack of a mechanistic understanding of how mature epithelial tight junctions are regulated. Therefore, an improved understanding of the underlying molecular mechanisms governing tight junction barrier function would make the attainment of this goal an achievable reality.

Recent studies using chemical inhibitors have, for the first time, provided direct evidence supporting a role for PLCs as regulators of epithelial tight junction function[5, 6]; however, other recent studies have questioned the alleged specificity of these PLC inhibitors in cellular systems[7-11]. Therefore, an additional aim of this research was to determine whether

these putative specific PLC inhibitors interact directly with PLC at the molecular level. This was an important objective, as these inhibitors (*e.g.* U73122) have repeatedly been utilized in the literature as pharmacological probes to implicate the activity of PLC isozymes in cellular phenotypes. If these molecules are not specifically inhibiting the catalytic activity of PLCs, their interactions with other cellular proteins may lead to off-target effects that could be mistakenly attributed to PLC, confounding our understanding of the cellular roles of this important class of signaling molecules.

Validation of Caco-2 Cell Monolayers as a Model to Study PLC-mediated Modulation of Tight Junction Permeability in the Human Intestinal Epithelium

Caco-2 cells are an *in vitro* cell culture model routinely used to assess the absorption potential of new drug candidates[12-16]. This cell line has also often been used to assess the potency of candidate PPEs *in vitro*[17-20]. In fact, a recent study has described novel adaptations to this model that provide increased predictability of the *in vivo* efficacy for certain classes of PPEs, depending on their physicochemical properties[21]. Results generated in this dissertation (Chapter 2) have characterized the expression profile of PLC isozyme mRNA in Caco-2 cells, and notably, determined that these specific PLCs are also expressed in the human small intestine. Thus, this work has established Caco-2 cell monolayers as an appropriate *in vitro* model in which to assess the role of PLC as a potential pharmacological target for increasing intestinal absorption in humans. These findings provide support for the previously published hypothesis that sodium caprate increases paracellular permeability in Caco-2 cells via modulation of PLC-dependent signaling[2, 3, 22].

Mechanisms Underlying PLC Inhibition and Paracellular Permeability Enhancement by Alkylphosphocholines

As Caco-2 cells were originally derived from a human colon adenocarcinoma, they were further utilized to specifically establish PLC inhibition as a viable mechanism for increasing paracellular permeability across the human intestine. Results from Chapter 2 demonstrated the existence of a significant correlation between the potency of a series of homologous APCs as PLCβ inhibitors and as paracellular permeability enhancers in the Caco-2 cell culture model. The relationship was dependent on the length of the alkyl chain, and was similar to the previously established relationship in MDCK cells[6]. However, a five to nine fold reduction in PPE potency was observed in Caco-2 cells as compared to MDCK cells, despite the fact that their potency as PLCβ inhibitors was comparable between the two cell culture models. This observation suggested several possibilities: (1) that different mechanisms regulate paracellular permeability between the two cell lines, (2) that additional regulatory mechanisms are present in Caco-2 cells, or (3) that the observed paracellular permeability enhancement was mediated via a mechanism independent of PLC inhibition.

Given the amphiphilic nature of this series of compounds, direct interaction with the apical cell membrane leading to changes in membrane fluidity and lipid composition presented a plausible alternative mechanism to explain the observed increase in paracellular permeability[10, 23, 24]. Therefore, differences in the apical membrane phospholipid composition between cells lines could explain the observed differences in the potency of APCs as PPEs. Indeed, apical cell membranes in the intestine have been reported to contain glycosphingolipids[25, 26] that serve to increase the stability of the apical membrane facing the intestinal lumen through increased intermolecular hydrogen bonds[27-29]. Therefore, Caco-2 cell membranes, derived from the human intestine, may be more resistant to membrane

perturbing effects of APCs than are MDCK cell membranes, a cell line derived from canine kidney.

Evidence in the literature has suggested that changes to the structure of phospholipid membranes can lead to indirect effects on the catalytic activity of membrane associated enzymes[30-33]. This is certainly likely for enzymes in the PLC family since the interactions between the cytosolic enzyme and the substrate embedded in the cell membrane must occur for catalysis. Thus, one explanation for the observed inhibition of PLC activity by APCs in both cell lines is the disruption of the structural integrity of the apical membrane and interference with the quality of the enzyme/substrate interaction via a process referred to as "competitive interfacial inhibition"[34]. Relatively little difference observed between cell lines with respect to the potency of APCs as PLCβ inhibitors, in contrast to the substantial difference between cells lines with respect to the potency of APCs as PPEs, may be due to the possibility that the activity of membrane bound enzymes is more sensitive to membrane destabilization events than is paracellular permeability. Clearly, further studies will be required to test this hypothesis.

In Chapter 5, the validity of the above hypothesis was tested by evaluating the ability of APCs to directly inhibit PLC in the absence of whole cells. Although all APCs inhibited the activity of hPLCβ1 in a concentration dependent manner, the chain length dependence for inhibiting PLC in the cell free system was not entirely consistent with the chain length dependence for inhibiting PLC in whole cells. In addition, the ability of HPC to inhibit hPLCβ3 in the cell free system was dependent on the detergent used in the assay, *i.e.* HPC inhibited the activity of hPLCβ3 in cholate mixed micelles, but had no effect on activity in dodecylmaltoside mixed micelles, implying that the inhibition was not via a direct interaction

with the enzyme in these assays, but instead was dependent on the environment in which the substrate was presented to the enzyme. Additional results presented in Chapter 5 confirmed the hypothesis that APCs disrupt apical cell membranes in Caco-2 cells, as each APC caused a concentration dependent leakage of the intracellular enzyme LDH across the apical membrane. Interestingly, a strong relationship existed between the potency of APCs to cause LDH leakage and their potency as PPEs, implicating apical membrane disruption in the observed effects on paracellular permeability. Further, HPC concentrations that caused significant increases in mannitol permeability (i.e. 100 μM; Figure 2.7.B) also caused significant leakage of LDH into the apical compartment (Figure 5.6)

Collectively, results generated in both cellular and non-cellular systems with APCs raised questions with respect to the proposed cause-effect relationship between PLC inhibition and tight junction function, suggesting the need for a more direct approach to inhibit PLC activity in whole cells. Further, these results implied that the ability of APCs to increase paracellular permeability is due to their ability to disrupt the normal order of the apical cell membrane. HPC (miltefosine) is the first orally effective treatment for visceral leishmaniasis[35]; interestingly, its major side effects include nausea, vomiting, and diarrhea[36], likely related to local irritation of the gastrointestinal tract, a hypothesis substantiated in this dissertation.

Activation of PLC by U73122 in a Cell Free System

Studies in Chapter 2 confirmed the ability of another reported PPE and inhibitor of PLC activity in the MDCK cell model, *i.e.* U73122[6], which is not amphiphilic and is structurally distinct from APCs, to both increase paracellular permeability and inhibit PLC activity in the Caco-2 cell model. Importantly, the reported inactive analog of U73122,

U73343, did not affect either parameter, providing support for a role for PLC inhibition in the regulation of intestinal tight junction function. However, the potency of U73122 as both a PPE and PLC inhibitor was reduced in Caco-2 cells as compared to MDCK cells, as was the case with APCs. Interestingly, a recent report implied that the highly electrophilic maleimide moiety of U73122, which is not present in U73343, may be responsible for off target effects in cellular models[11], and suggested that differences in available nucleophiles between different cellular systems may lead to differences in the potency of U73122 towards measured phenotypes. This report provided an explanation for the observed differences in potency of U73122 between MDCK and Caco-2 cells, with the implicit assumption that Caco-2 cells have a greater number of nucleophiles accessible to an apical dose of U73122 than do MDCK cells, a hypothesis consistent with reports that Caco-2 cells express transporters that actively efflux glutathione[37].

Despite the numerous studies reporting U73122 as an inhibitor of PLC, as well as its widespread use as a probe molecule to implicate the involvement of PLC in signaling pathways and phenotypic cellular response, little work had been done to understand the interaction of U73122 with PLC at the molecular level. This observation was particularly surprising given the presence of the highly reactive maleimide, a structural moiety that readily interacts with thiols. A reaction between U73122 and nucleophilic residues on PLC represented a potential molecular mechanism that could explain the oft reported inhibition of PLC activity by U73122. Studies in Chapter 4 were designed to study the direct interaction between U73122 and PLC in a cell free system. These studies uncovered for the first time that U73122 increases the activity of a PLC isozyme, hPLCβ3, by several fold in a cell free system, as opposed to its long reported ability to inhibit the activity of PLC. In addition,

studies in Chapter 4 have established for the first time that U73122 directly alkylates hPLCβ3 at up to eight different cysteine residues; alkylation of at least some of these cysteines appeared to be responsible for the increase in activity. Interestingly, a recent study reported an apparent weak and transient activation of PLC action following treatment of cells with U73122[8], an unexpected result that these authors could not explain; the results presented in this dissertation offer an explanation to support the observations from this report.

These results demonstrate a novel mechanism of enzyme activation in this cell free system that may provide insight into mechanisms of regulation and subsequent activation of PLC isozymes in whole cells. The molecular mechanism of cellular PLC activation and substrate hydrolysis is not completely understood. Reversible modifications of proteins at cysteine residues have been implicated in conformation changes; thus, the present results may be indicative of events occurring in whole cells during receptor mediated activation, membrane binding, and subsequent substrate hydrolysis. Further, results presented herein, in combination with the recent report that U73122 also reacts with components of cell media[11], clearly indicate that this molecule should no longer be used as a pharmacological probe to implicate PLC signal transduction in cellular response, and suggest that previous conclusions based solely on inhibition of PLC activity by U73122 treatment may warrant reconsideration.

RNA Interference Studies to Determine the Role of PLC in Tight Junction Function

Because studies in both cellular and non-cellular systems with previously reported PLC inhibitors raised questions about the relationship between PLC activity and tight junction permeability, a more definitive approach was required to evaluate the hypothesized cause-effect relationship between the inhibition of PLC activity and increased paracellular permeability in epithelial cells. Studies in Chapter 3 utilized RNA interference technology to

specifically suppress the expression of individual PLC isozymes expressed in MDCK cells in order to more directly assess this possibility. MDCK cells were used in these studies due to their expeditious growth rate and tendency to rapidly establish confluent monolayers, characteristics which provided the opportunity to assess the effect of PLC depletion on both the assembly and maintenance of tight junction barrier function following transient transfection of cells with short interfering RNA. Results from these studies provided convincing evidence that a cause-effect relationship does not exist between inhibition of two major isozymes of PLC present in MDCK cells and tight junction function. Specific depletion of both PLCβ3 and PLCγ1 (the only isozymes of their respective families found in these cells), alone and in combination, had no impact on either tight junction assembly or on the barrier function of already formed tight junctions. However, as isozymes from other PLC families that may also be expressed in MDCK cells were not suppressed, these results do not rule out the possibility that inhibition of other PLC families leads to the observed changes in paracellular permeability. A report that HPC inhibits the activity of PLCδ1 provides support for such a hypothesis[38]. Further, results from Chapter 3 do not completely rule out the possible involvement of PLCβ and PLCγ isozymes in the regulation of tight junction function. A number of studies have indirectly suggested that PLC activation may lead to changes in the function of junctional complexes. For example, a number of agonists of receptors known to couple to PLCs have been reported to affect the functional integrity of tight junctions[39-42]. Further, increase in intracellular calcium levels[43, 44], activation of specific PKC isozymes[45, 46], as well as non-PLC-mediated depletion of membrane PIP_2 content[47, 48], all downstream actions of PLC activation, have been demonstrated to increase paracellular permeability or alter the barrier function of junctional complexes in a number of systems.

This hypothesis, in conjunction with evidence from Chapter 4 suggesting U73122 may activate PLC, leaves open the possibility of an alternative mechanism for the observed effects of U73122 on paracellular permeability in both MDCK and Caco-2 cells (Chapter 2)[6], and may explain the discrepancy between results from studies using chemical inhibitors of PLC (Chapter 2) versus results from studies employing RNAi suppression of PLC (Chapter 3).

In summary, studies in this dissertation have significantly enhanced the current understanding of the role PLC enzymes play in the regulation of tight junction function. They have demonstrated that previous hypotheses implicating specific inhibition of PLCβ and PLCγ isozyme families as a cause for increased paracellular permeability via specific modulation of tight junction structure and function need to be re-examined. They have further provided convincing evidence that the effects of reported PLC inhibitors, APCs and U73122, on paracellular permeability are not mediated via direct inhibition of PLC activity. Evidence was presented to suggest that APCs modulate paracellular permeability via their interaction with and disruption of apical membrane integrity in Caco-2 cells, an interaction reported to be responsible for their anti-bacterial and anti-neoplastic activity. Evidence was also presented to support an alternative hypothesis to explain the observed increase in paracellular permeability following treatment of cells with U73122, a mechanism involving a novel interaction between PLC and U73122 leading to the unexpected activation of a PLC isozyme in a cell free system.

Thus, significant contributions were made in this dissertation to various scientific disciplines: (1) to the field of pharmaceutics and drug delivery by developing significant new information regarding a proposed mechanism for enhancing the oral absorption of hydrophilic drugs and macromolecules, (2) to the field of tight junction physiology by

providing evidence that regulation of epithelial tight junction barrier function does not appear to be affected by inhibition of PLCβ and/or PLCγ isozyme families, and (3) to the area of phospholipase C biochemistry by demonstrating a novel mechanism of enzyme activation in a cell free system that may provide insight into mechanisms of regulation and activation in whole cells.

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