Response of Monovalent Cation Transporters to Pro-apoptotic Protein Kinase C Modulators in Human Lens Epithelial Cells

Michael Anthony Lepera
Wright State University

Follow this and additional works at: https://corescholar.libraries.wright.edu/etd_all

Part of the Pharmacology, Toxicology and Environmental Health Commons

Repository Citation
Lepera, Michael Anthony, "Response of Monovalent Cation Transporters to Pro-apoptotic Protein Kinase C Modulators in Human Lens Epithelial Cells" (2011). Browse all Theses and Dissertations. 480.
https://corescholar.libraries.wright.edu/etd_all/480

This Thesis is brought to you for free and open access by the Theses and Dissertations at CORE Scholar. It has been accepted for inclusion in Browse all Theses and Dissertations by an authorized administrator of CORE Scholar. For more information, please contact library-corescholar@wright.edu.
Response of Monovalent Cation Transporters to Pro-apoptotic Protein Kinase C Modulators in Human Lens Epithelial Cells

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

MICHAEL ANTHONY LEPERA
Bachelors of Science, University of Wisconsin-Green Bay, 2007

2011
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Michael Anthony Lepera ENTITLED “Response of Monovalent Cation Transporters to Pro-apoptotic Protein Kinase C Modulators in Human Lens Epithelial Cells” BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

______________________________
Peter K. Lauf, M.D.
Thesis Director

______________________________
Mariana Morris, Ph.D.
Chair, Department of Pharmacology and Toxicology

Committee on Final Examination:

_______________________________________
Peter K. Lauf, M.D.

_______________________________________
Norma C. Adragna, Ph.D.

_______________________________________
Thomas L. Brown, Ph.D.

_______________________________________
David Cool, Ph.D.

_______________________________________
Andrew T. Hsu, Ph.D.
Dean, School of Graduate Studies
ABSTRACT

Lepera, Michael Anthony, M.S., Pharmacology/Toxicology Masters Program, Wright State University, Department of Pharmacology and Toxicology 2011. Response of Monovalent Cation Transporters to Pro-apoptotic Protein Kinase C Modulators in Human Lens Epithelial Cells.

Protein kinase inhibition by staurosporine causes apoptotic volume decrease involving potassium (K) channels in immortalized human B3 lens epithelial cells (LECs). Here, the effect of two pro-apoptotic protein kinase C (PKC) inhibitors [12-O-tetradecanoyl-phorbol-13-acetate (TPA) and chelerythrine (CET)] were studied on membrane K transport in a fetal human LEC line (FHL124) by western blotting, immunofluorescence, ion flux, ATP, apoptosis, and biotinylation assays. Long term TPA exposure (0-6 h) inhibited 75% of Na+K-2Cl cotransport (NKCC). In contrast, short term (0-20 min) exposure to 50 µM CET reduced Na+K pump and NKCC by >90% and >70%, respectively, without retrieval from the membrane into the cytosol, loss of ATP and early signs of apoptosis. CET (10-30 µM) decreased cell K by 33%. Results suggest PKC modulation through the use of pro-apoptotic agents caused major early membrane changes independently affecting K channels, the Na+K pump and NKCC function, prior to rise of any significant apoptosis.
# Table of Contents

1. **Background and Literature Review**
   1.1 Growth and Development of Human Lens Epithelial Cells (HLEC) .................................. 1
   1.2. Normal Homeostasis in the Lens ................................................................. 3
   1.3. RVD and RVI .................................................................................................. 6
   1.4. Cataract Formation in the Lens ......................................................................... 11
   1.5. Structure and Function of Protein Kinase C (PKC) ............................................. 12
   1.6. Consequence of PKC inhibition: Apoptosis ....................................................... 18

2. **Rationale and Hypothesis**
   2.1. Specific Aim 1 ............................................................................................. 24
   2.2. Specific Aim 2 ............................................................................................. 24
   2.3. Specific Aim 3 ............................................................................................. 24
   2.4. Specific Aim 4 ............................................................................................. 24
   2.5. Significance .................................................................................................. 24

3. **Materials and Methods**
   3.1. Reagents ....................................................................................................... 26
   3.2. Ion Flux Solutions .......................................................................................... 28
   3.3. Cell Culture .................................................................................................. 28
   3.4. Measurement of Ion Fluxes .......................................................................... 29
   3.5. Western Blot ................................................................................................. 29
   3.6. BCA Protein Assay ....................................................................................... 30
   3.7. Immunofluorescence Staining and Fluorescence Microscopy ..................... 31
   3.8. Cell Death Detection ELISA ......................................................................... 31
   3.9. ATP Determination ...................................................................................... 32
   3.10. Mitochondrial Depolarization Detection ...................................................... 32
   3.11. TPA Depletion Studies ............................................................................... 33
   3.12. Biotinylation .............................................................................................. 33
   3.13. Statistical Analysis ...................................................................................... 34
4. Results

4.1. Characterization of PKC isoforms in FHL-124 cells .......................................................... 36
4.2. Na-K-ATPase α isoforms present in FHL-124 cells ............................................................... 37
4.3. Time course of the TPA effect on PKC cytosol/membrane distribution ................................ 38
4.4. Effect of long term exposure to TPA on cation transport in FHL124 cells ............................... 45
4.5. Time dependence of TPA action on Rb influx ...................................................................... 57
4.6. Cl-dependent Rb influxes before and after TPA and CET exposure ..................................... 58
4.7. Short term CET effects on Rb influxes ................................................................................. 62
4.8. Dose dependent CET effects on Rb influx ........................................................................ 67
4.9. ATP production and apoptosis after CET treatment .............................................................. 68
4.10. Biotinylation studies ......................................................................................................... 81
4.11. CET effect on PKC localization .......................................................................................... 82

5. Discussion

5.1. PKC isoforms in FHL-124 cells .......................................................................................... 87
5.2. TPA and its effect on ion transport in FHL-124 cells ............................................................ 88
5.3. The action of CET on K loss and Rb influxes ...................................................................... 92
5.4. Summary .......................................................................................................................... 101
5.5. Future Studies .................................................................................................................. 103

6. References ............................................................................................................................ 105

7. Appendix ............................................................................................................................... 116
List of Figures

Background

Figure 1- Early Stages of Lens Development ................................................................. 2
Figure 2- Cell volume regulation ...................................................................................... 7
Figure 3- Scheme of SPAK/WNK4 regulation of NKCC1 .................................................. 10
Figure 4- PKC structure ................................................................................................. 14
Figure 5- PKC activation ................................................................................................. 16
Figure 6- Proposed Mechanism of STP induced apoptosis ............................................... 21

Results

Figure 1- Presence of four PKC isoforms in FHL 124 cells ............................................... 39
Figure 2- Evidence of PKC isoforms in FHL 124 cells ...................................................... 40
Figure 3- Expression of Na/K-ATPase α isoforms in HLEC ............................................ 42
Figure 4- Long term TPA effect on PKC distribution in the cytosol and membrane ............ 43
Figure 5- Densitometry scans of membrane and cytosolic PKCs after TPA ......................... 44
Figure 6- Effect of TPA and CET treatment on total protein content and Kᵢ .................... 48
Figure 7- Effect of TPA and CET treatment on Rb influx ................................................ 52
Figure 8- Effect of TPA and CET treatment on total protein content and Kᵢ .................... 54
Figure 9- Effect of TPA and CET treatment on Rb influx ................................................ 56
Figure 10- Time-dependent effect of TPA on Rb Influx .................................................... 59
Figure 11- Cl dependent K loss and KCC function due to TPA treatment .......................... 64
Figure 12- Cl-dependent K loss and KCC function after CET treatment ............................ 66
Figure 13- Time dependent CET effect on Kᵢ and total protein ....................................... 70
Figure 14- Time dependent CET effect on Rb uptake ...................................................... 72
Figure 15- Dose dependent effect of CET ................................................................. 74
Figure 16- ATP determination after CET treatment ...................................................... 78
Figure 17- Mitotracker staining for mitochondrial depolarization .................................... 79
Figure 18- Cell death detection by ELISA .................................................................... 80
Figure 19- Effect of CET on Na-K-ATPase α1 subunit and NKCC1 at the membrane level .................................................................................................................. 83
Figure 20- Effect of CET on PKC isof orm distribution .................................................... 84
Figure 21- Structure of ouabain and chelerythrine ......................................................... 96
Figure 22- Proposed CET action mechanisms ............................................................. 99
1. Background and Literature Review

1.1 Growth and Development of Human Lens Epithelial Cells (HLEC)

The human lens is formed by surface ectoderm invagination (Krag et al., 2003). The lens capsule is formed by the outer most cells of the lens vesicle and is surrounded by the basement membrane of the surface ectoderm which acts as a barrier for the developing lens to provide protection from bacterial and viral invasions (Danysh et al., 2009). The lens continues to grow throughout life but slows down with age. The lens is a very unique structure, as it is the largest organ in the body that lacks a direct blood supply. Blood vessels disperse and absorb light whereas a lens devoid of blood supply can support the cornea in focusing light on the retina (Mathias et al., 2007). As a result of the lack of blood supply, the lens, over time, has developed its own micro-circulatory system to transfer and circulate ions in order to obtain nutrients (Mathias et al., 2007). After invagination, the cells become inverted so that their apical surfaces face inward to the lumen and the basal surfaces the basement membrane outward to the capsule (Bhat 2001, Krag et al., 2003). The transformation from lens epithelial cells (LECs) to lens fibre cells (LFCs) is a crucial stage for the growth and development of the lens. First, posterior LECs must differentiate into primary LFCs causing elongation that eradicates the lumen of the lens vesicle. This brings the apical surface of the elongated LFC together with the apical surface of the anterior epithelial cells to form the apical interface (Bhat 2001).
**Figure 1- Early Stages of Lens Development.** Surface ectoderm thickens and invaginates and compresses to form the lens vesicle LECs differentiate into LFCs. (Figure and legend adapted from Bhat 2001).

All future growth and development of the lens rests solely on the anterior LECs since the posterior surface is now deficient of LECs. The epithelial cells under the anterior lens form a monolayer of cells, which are essential for the overall transparency and health of the lens, and remain undifferentiated with little cell division throughout life (Li et al., 1995, Duncan et al., 1997, Krag et al., 2003,
Danysh et al., 2009). Only epithelial cells near the equator divide before transforming into secondary LFCs that make up the body of the lens (Beebe et al., 1990, Duncan et al., 1997, Krag et al., 2003, Danysh et al., 2009). This process is driven by an increase in cell volume that causes morphological changes such as elongation, loss of intracellular membrane-bound organelles, degradation of the nucleus, and causes the LFCs to stop dividing (Beebe et al., 1990, Andley et al., 1994). Once the intracellular organelles are lost, light scattering is reduced but the LFCs must survive without protein synthesis for the rest of its lifetime. As elongation continues, the LFCs become internalized and attach to Type IV collagen that runs from the center of the lens to the anterior and posterior poles (Mathias et al., 2007).

1.2. Normal Homeostasis in the Lens

Volume constancy is an essential part of all living cells requiring continuous management of ions and water passage through transporters and channels (Lauf et al., 2008).

**The Na/K pump:** Foremost, and like almost all cells, LECs contain high intracellular K and low intracellular Na maintained by the Na/K pump (Cui et al., 2002, Mathias et al., 2007). The Na/K pump is a membrane bound adenosine triphosphate (ATP) hydrolase (ATPase), consisting of a ~100 kDa molecular mass α subunit (Kaplan 2002) that through ATP-hydrolysis creates above electrochemical gradient allowing $2K^+$ to enter and $3Na^+$ to leave the cell by a ping pong mechanism (Skou 1998 Nobel lecture, reviewed in Glynn 2002, Sachs 1980, reviewed in Lauf and Adragna 2011). This exchange involves canonical
formation of a transient high energy phosphate bond at Asp 369 within the catalytic center of the enzyme (reviewed in Lauf and Adragna 2011). The Na/K ATPase membrane protein is a heterotrimer consisting of the α catalytic subunit, a β subunit for membrane insertion (Feraille et al., 2000) and a regulatory γ subunit. An alteration of the high K/Na ratio may affect the conversion of LECs to LFCs. Indeed, correlations have been established between man's age, lens K/Na ratio and cation permeability, and cataract formation (Duncan et al., 1997). In addition to the catalytic phosphorylation site, there is evidence for additional phosphorylation by serine (S), threonine (T) and tyrosine (Y) kinases and dephosphorylation by S, T and Y phosphatases. There are 2 PKC phosphorylation sites located on the α1 subunit, Ser11 and Ser18 (Feschenko et al., 1997) and their phosphorylation is proposed to be required to transport the Na/K pump from the cytosol to the membrane (Feschenko et al., 2000). Tyrosine phosphorylation at Y416 by a Y-kinase is a mechanism by which the Na/K ATPase is proposed to be inactivated during the transition from LECs to LFCs (Tamiya et al., 2007).

**Cell volume regulation** mechanisms ensure normal homeostasis in the lens, i.e., ion channels (K, Na, Cl), ion exchangers (Na/H, HCO₃/Cl), and cotransporters (Na-K-2Cl, K-Cl) (Lauf et al., 2006, 2008, Mathias et al., 2007, Chimote 2009). Regulatory volume decrease (RVD) responds to stimuli causing cellular water entrance and swelling. Regulatory volume increase (RVI) responds to stimuli causing cellular water loss and shrinkage (Lauf et al., 2008). Stretch studies done by Candia et al. (2008) have shown that a change in overall
lens volume involving the RVD and RVI mechanisms is required for lens accommodation, which is the ability to continuously focus on objects as the distances vary (Candia et al., 2008).

Together with a multitude of Na/H (NHE) exchanger isoforms, the Na-K-2Cl cotransporter (NKCC) and the K-Cl cotransporter (KCC) are responsible for the control of the volume set point (VSP) of normal cell volume in response to different kinase phosphorylation and phosphatase dephosphorylation mechanisms (Lauf et al., 2006). The VSP is defined as “a state between RVD and RVI where conservatory and dissipating ion and solute transport activities balance each other and are at their lowest activity to maintain cellular steady state” (Lauf et al., 2008). The KCC normalizes cell volume after swelling and is activated by dephosphorylation whereas the NKCC counteracts cell shrinkage and is activated by phosphorylation (Lauf et al., 2006). Both sets of cotransporters are secondary active transporters in which the cations are coupled in a 1:1 ratio with Cl and do not require ATP hydrolysis but instead rely on electrochemical gradients established by primary active transport through the Na/K pump (Lauf and Adragna 2000, Gamba 2005). Changes in cell volume are due to the movement of Cl in or out of the cell (Gamba 2005). For example, in neurons, NKCC is the primary transporter responsible for high intracellular Cl during early development but is down regulated after birth. In contrast, the KCC is absent during early development but active after birth and into adulthood (Delpire 2000, Gamba 2005).
1.3. RVD and RVI

In animal cells, osmotic swelling triggers K and Cl efflux through channels and transporters to return them to their normal size. K efflux alone causes membrane hyperpolarization that keeps K in the cells so Cl efflux is needed in parallel to regulate cell volume (Mongin et al., 2001). Aiding in RVD are the four isoforms of electroneutral K-Cl cotransporters (KCC) and their spliced variants activated by PP1 causing dephosphorylation and inactivated through phosphorylation by a kinase (Jennings et al., 1990). Strange et al. (2000) showed that mutations of a C-terminus KCC2 tyrosine kinase residue were detrimental to the cotransporter function (Strange et al., 2000). Recent evidence indicates that the C-terminus of the KCC3 has several S and T phosphorylation sites (Rinehart et al., 2009). Truncation of the C-terminus portion of KCC1 reduces significantly the NEM-induced activation of K-Cl cotransport in HEK293 transfected cells (Lauf et al., 2001). Given the chemical driving forces for K and Cl, the KCCs generally perform the outward transport of K and Cl that results in water loss and ultimately causes the cell to shrink (Flatman et al., 1996, Lauf et al., 2000, Gamba 2005).
Figure 2- Cell volume regulation. Water influx due to hyposmotic stress causing cell swelling. RVD restores normal cell volume by an efflux of K, Cl, and H₂O. Efflux of water due to hyperosmotic stress causing cell shrinkage. RVI restores normal cell volume by an influx of Na, K, Cl, and H₂O. (Figure and legend adapted from Chimote 2009).

The KCC1 isoform, located on chromosome 16.q22.1, regulates volume homeostasis and is ubiquitously expressed in all cells. KCC2, located on
chromosome 20, occurs in two spliced variants KCC2a and KCC2b. The latter was originally found only in the brain needed to remove intracellular Cl in order to assist GABA inhibiting neurons (Payne 1997) and the former also in a human LEC line (Lauf et al., *FASEB J March 17, 2011 25:657.*8). The KCC3 isoform functions as a K buffer and is located on chromosome 15.q13. It is found in the heart, brain, liver, placenta, lung, and pancreas (Lauf et al., 2000, Gamba 2005). The KCC4, located on chromosome 5p15, is present in the heart and kidney. KCC4 is pH regulated and could possibly play a role in acid-base metabolism (Lauf 2000, Gamba 2005). The KCC1, 3, and 4 but not KCC2 isoforms were detected in human lens extracts (Misri et al., 2006).

Cell swelling also causes activation of Ca-activated K channels, Cl channels, K/H and Cl/HCO₃ exchangers (Chimote 2009, Mongin et al., 2001, Okada 2004). Ca-activated K channels are divided into 3 groups; the voltage independent small conductance (SK), the inwardly rectifying intermediate conductance (IK) channel which performs RVD in human LECs (Lauf et al., 2008), and the voltage dependent big K (BK) conductance (BK) channel (Wang et al., 2003). IK channels work together with the Cl- extruding volume-sensitive outwardly rectifying anion channels (VSOR) or volume-regulated anion current (VRAC) to normalize cell volume (Okada 2004, Hoffmann et al., 2007, Chimote 2009).

When cells osmotically shrink, RVI triggers K and Cl influx into the cell and closes K channels in order for the cell to return to its normal size (Candia et al., 2008). A major player in RVI is the electroneutral NKCC. The NKCC has been
proposed to be responsible for the inward movement of Na, K, and 2Cl but its main role is Cl transport across the cell (Delpire 2000, Tang et al., 2010). There are 2 known isoforms of NKCC, NKCC1 and NKCC2, where NKCC1 is the only one found in the lens. NKCC1 is ubiquitously expressed and is located on chromosome 5q23 where it is encoded by the SLC12A gene (Gamba 2005, Tang et al., 2010). In epithelial cells, NKCC1 is localized in the basolateral membrane, except in the choroid plexus in which NKCC1 is bound to the apical membrane (Gamba 2005). The NKCC2 isoform, located on chromosome 15, occurs primarily in the luminal membrane of the thick ascending limb epithelial cells where it is responsible for NaCl reabsorption (Hebert et al., 1984). In contrast to the gliding symmetry model explaining the NaCl reabsorption through NKCC by a mechanism of orderly Na-Cl-K-Cl loading and unloading, most recently this model has been challenged by proposing that NKCC1 utilizes 1 Na and 1 Cl to bind its external aspect while transporting de facto KCl across the plasma membrane (Delpire and Gagnon FASEB J March 17, 2011 25:657.12). As opposed to the KCC, phosphorylation stimulates NKCC and dephosphorylation inhibits it. When intracellular chloride concentration falls, the NKCC1 becomes phosphorylated and its activation at the basolateral side causes Cl to be secreted across the apical membrane through Cl channels. When the chloride concentration increases, NKCC1 becomes dephosphorylated and inhibited (Gamba 2005). Ste20 related proline/alanine rich kinase (SPAK) phosphorylation along with oxidative stress response 1 (OSR1) phosphorylation increases the
activity of NKCC1 in the presence of WNK4 (with no lysine). This activation occurs when SPAK binds to WNK4 (Piechotta et al. 2003, Gagnon 2007).

Figure 3- Scheme of SPAK/WNK4 regulation of NKCC1. WNK4 phosphorylation of SPAK which in turn phosphorylates and activates NKCC1 (Figure taken from and legend modified from Gagnon et al., 2007).

Both SPAK and OSR1 interact with the amino terminus of NKCC1. NKCC1 contains two SPAK binding motifs, one of which partially overlaps with
the binding site of PP1 on NKCC1 (Piechotta et al., 2002). However, only one site is needed for the activation and regulation of NKCC1 (Gagnon et al., 2007).

NKCC2 is primarily present in the kidney, specifically in the apical membrane of the ascending loop of Henle (Delpire et al., 1999, Hoffmann et al., 2007). Also mediating RVI is the activation of Na channels and of the Na/H exchange which triggers acidification and activation of Cl/HCO₃ exchange. This allows the cells to load with Na and Cl and release H and HCO₃ (Mongin et al., 2001, Okada 2004).

1.4. Cataract Formation in the Lens

Cataract is defined as a loss of transparency (opacification) in the lens and is responsible for 42% of all blindness in the world (Kyselova et al., 2004). This is a result of an accumulation of stress and damage over the years leading to the improper arrangement or stacking of new LFC over the old ones (Bhat 2001). Apoptosis has been thought by many to be an important step in the development of cataracts. Li et al. (1995), demonstrated that apoptosis is indeed associated with and precedes cataract formation (Li et al., 1995). LEC apoptosis can be triggered by UV light, oxidative stress, and increased calcium levels (Li et al., 1995). Over time, UV-B radiation exposure can cause serious damage to the eye by depleting the lens of antioxidants causing lens opacification and the development of cataracts (Chandler et al., 2010, Kyselova et al., 2004). Apoptosis was shown to be related to the development of anterior polar cataract, which is the differentiation of irregularly growing LECs into myofibroblast-like cells (Lee et al., 2002). LFCs rely on transporters to meet their nutritional
requirements. LFC nutrients are driven and maintained by the establishment of a Na gradient by the Na-K pump concentrated in the equatorial LECs. The slightest disruption in this gradient leads to a decrease in nutrient availability for the LFCs and an increase of oxidative damage leading to the formation of nuclear cataracts (Mathias et al., 2007). Another kind of cataract is the diabetic cataract. With little protein turnover in the lens, diabetes mellitus produces cataracts from the overload and metabolic effects of glucose diffusing into the lens (Kyselova et al., 2004).

Cataract surgery is always an option to improve clouded vision, but there are some complications that come with this. In 1997, it was reported that 30-50% of cataract patients developed posterior capsule opacification (PCO) following surgery (Duncan et al., 1997). PCO is a secondary cataract that arises when LECs left behind after surgery begin to proliferate, divide and migrate from the equator and anterior epithelium to the posterior capsule under the intraocular lens impeding vision (Duncan et al., 1997, Wormstone et al., 1997, Liu et al., 1996, Yadav et al., 2009).

1.5. Structure and Function of Protein Kinase C (PKC)

Many cellular and biological processes such as cell proliferation, differentiation, apoptosis, and gene activation are dependent on the function of the many isoforms of PKC, which are a group of serine/threonine kinases (Gutcher et al., 2003, Thodeti et al., 2001, Steinberg 2008). At least ten PKC isoforms have been identified to date and grouped into 3 categories according to their structure and second messenger requirements: conventional, novel, and
atypical. All isoforms require phosphatidylserine (PS) for their activation. Conventional PKCs are activated by binding diacylglycerol (DAG) in a calcium-dependent manner, while the novel PKCs bind DAG in a calcium-independent manner. The atypical PKCs only require PS for activation (Mellor et al., 1998, Gutcher et al., 2003, Thodeti et al., 2001, Steinberg 2008).

Table 1 - PKC isoform classification and functional requirements

<table>
<thead>
<tr>
<th>PKC Isoform Classification</th>
<th>Functional Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS</td>
</tr>
<tr>
<td>Conventional</td>
<td>α, βΙ, βΙΙ, γ</td>
</tr>
<tr>
<td>Novel</td>
<td>δ, ε, θ, η</td>
</tr>
<tr>
<td>Atypical</td>
<td>ζ, ι</td>
</tr>
</tbody>
</table>

All PKC isoforms consist of four conserved domains (C1-C4) and five variable domains (V1-V5). C1 and C2 are membrane binding and make up the regulatory domain to keep the enzyme in an inactive state. C3 and C4 make up the catalytic domain, which is needed for ATP and substrate binding (Dempsey et al., 2000, Gutcher et al., 2003, Steinberg 2008). The C1 domain contains two cysteine-rich zinc fingers that bind DAG. C2 binds calcium. The C3 domain serves as the ATP binding site and the C4 domain is the substrate binding site (Gutcher et al., 2003).
Figure 4- PKC structure. DAG binds to the cysteine-rich zinc fingers of C1, calcium binds to C2, ATP at the C3 region, and C4 is the substrate binding site (Figure adapted from Gutcher et al., 2003).

As shown in Figure 5, PKC activation by DAG involves a series of signalling cascades that begin with the ligation of cell surface receptors causing activation of phospholipase C (PLC) which generates DAG and inositol triphosphate (IP$_3$) through the hydrolysis of phosphatidylinositol 4, 5-biphosphate.
(PIP$_2$). IP$_3$ departs to the endoplasmic reticulum to mobilize intracellular calcium while DAG remains in the plasma membrane to recruit inactive PKC. In order for PKC to be recruited to the membrane there must be phosphorylation of three conserved sites in the catalytic domain of PKC to ensure maturity and preparation for activation: 1) Phosphorylation in the activation loop of the C-terminus by 3-phosphoinositide-dependent kinase (PDK-1). 2) Autophosphorylation in the hydrophobic region for activation of conventional and novel PKC isoforms. 3) PKC is released into the cytosol after the final phosphorylation step where it is recognized by the pseudo-substrate region. DAG brings PKC into the membrane along with the pseudo substrate region. The pseudo-substrate region is displaced, PKC is activated in the membrane, and binds to receptors for activated C kinases (RACKs), which are anchoring proteins that determine its localisation (Dempsey et al., 2000, Parekh et al., 2000, Gao et al., 2001, Gutcher et al., 2003, Steinberg 2008).
PKC isoforms can be considered pro-apoptotic or anti-apoptotic and play crucial roles in the cell but it all depends on what cell type the isoform is expressed in (Gutcher et al., 2003). In conjunctival goblet cells, PKCα and PKCε are needed for cell proliferation (Shatos et al., 2009). PKCε and PKCδ are
involved in the response of the heart to ischemia and reperfusion, where PKC\(\varepsilon\) is cardio-protective and PKC\(\delta\) causes damage to the myocardium (Murriel et al., 2003). PKC\(\varepsilon\) has been shown to be involved in muscle contraction, gene expression, and metabolism (Akita 2002).

In human leukemia cells, PKC\(\delta\) phosphorylates p53 which leads to the onset of apoptosis (Yoshida et al., 2006). PKC\(\theta\) regulates cytoskeleton stability and barrier permeability in intestinal epithelial cells (Banan et al., 2004). PKC\(\theta\) has also been implicated in T-cell activation and proliferation (Carrasco et al., 2004). In airway epithelial cells PKC\(\delta\) binds to F-actin and is needed for the regulation of NKCC1 (Liedtke et al., 1997, 2003). According to this group SPAK binds PKC\(\delta\) and down regulation of SPAK results in a loss of NKCC1 function. It was concluded PKC\(\delta\) phosphorylates SPAK acting as a mediator for NKCC1 activation (Smith et al., 2008).

Since PKC is involved in so many biological processes, it is important to study what happens when a cell is depleted of this very important kinase. Pharmacological tools have been developed for this purpose. Phorbol esters are tumor promoters that mimic DAG binding to the C1 domain of PKCs (Brose et al., 2002). Belonging to this group of phorbol esters is 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and phorbol 12-myristate 13-acetate (PMA), which can cause cell proliferation or cell arrest depending on the cell type (Isonishi et al., 2000). It has been previously shown, using Madin-Darby canine kidney (MDCK) cells, that activation of PKC by PMA retrieves NKCC1 from the membrane to the cytosol within 2.5 min through a clathrin-mediated endocytotic pathway: after 15 min
most of the NKCC1 vesicles had moved inside the cell (Mykoniatis et al., 2010). These authors also observed that F-actin and Na-K-ATPase didn’t move from the membrane of the cell when treated with PMA (Mykoniatis et al., 2010).

Other pharmacological tools like staurosporine (STP) and naphthalene sulfonamides have been shown to induce apoptosis but with little selectivity towards PKC (Chmura et al., 2000, Andersson et al., 2000). The most potent and most selective inhibitor for PKC is chelerythrine (CET), a quaternary benzophenanthridine alkaloid, which binds to the C3 domain ATP binding site of PKCs (Herbert et al., 1990, Lou et al., 2003). Like TPA, STP causes redistribution of PKC isoforms from the cytosol to the membrane (Courage et al., 1995). Inhibition of PKC with these drugs can be detrimental to the cell. In HLECs, treatment with STP leads to competition between STP and ATP resulting in dephosphorylation of kinases and possibly of the 4-amino pyridine sensitive K channel with subsequent K loss, water loss, and apoptosis (Chimote et al., 2010). CET is considered a pro-apoptotic agent that triggers the release of cytochrome c from the mitochondria by inhibiting BclXL at the BH3 domain (Chan et al., 2003). In cardiac myocytes, CET has been shown to produce reactive oxygen species leading to the development of apoptosis (Yamamoto et al., 2001).

1.6. Consequence of PKC inhibition: Apoptosis

There are many forms of cell death; necrosis, anoikis, and apoptosis to name a few, each with its own unique attributes. Necrotic cell death is energy-independent and can be attributed to the swelling of cells while anoikis is cell
death induced by the detachment of cells from their extracellular matrix (Kroemer et al., 2009, Murriel et al., 2003).

Apoptosis or programmed cell death occurs normally during development or as a response to stress or toxically induced events to remove excess or unwanted cells. Removal of cells during eye development is needed for the timely separation of the lens from the future corneal epithelium and for the development the retina (Harocopos et al., 1998). The problem arises when uncontrollable cell death occurs. Apoptosis has been linked to the development of cataracts, AIDS, and cancer (Li et al., 1995, Harocopos et al., 1998, Gutcher et al., 2003, Lionetto et al., 2010). There are many indications that a cell has turned apoptotic: cell membrane blebbing, cell shrinkage, caspase activation, DNA fragmentation, disturbance of membrane asymmetry by the translocation of phosphatidylserine (PS) by scramblases from the inner to the outer leaflet of the plasma membrane, mitochondrial depolarization, and formation of apoptotic bodies (Gutcher et al., 2003, Chimote 2009, Lang et al., 2010, Lionetto et al., 2010). Potassium (K) has been shown to be an important ion in the regulation of apoptosis. As stated earlier, a normal cell contains high levels of intracellular K. When cells start to lose K, this causes apoptotic volume decrease (AVD) with subsequent apoptosis (Bortner et al., 2007).

Emerging over the years have been the two popular pathways of apoptosis, intrinsic and extrinsic. The intrinsic pathway, also known as the mitochondrial pathway, is initiated through disruption within the cell of certain cell functions by DNA damage and different kinds of stress signals. In this pathway,
damage causes cytochrome c release from the mitochondria and activation of caspase-3 through caspase-9 leading to cellular apoptosis (Fulda et al., 2006). The extrinsic pathway, also known as the death receptor pathway, is initiated by the signalling of receptors outside the cell. The death receptors include CD95 and tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL). Activation of the death receptors leads to recruitment of the Fas ligand-associated death domain (FADD) and inactive procaspase-8. Once bound, caspase-8 becomes activated, cleaves effector caspases like caspase-3 and stimulates the caspase cascade leading to apoptosis (Fulda et al., 2006).

Previous work has shown that apoptotic cell shrinkage induced by the pro-apoptotic general kinase inhibitor staurosporine (STP) is initiated by apoptotic volume decrease (AVD) and mediated by 4-AP-sensitive voltage-gated K channels in human lens epithelial cells (Chimote et al., 2010). Although lipid changes with PS externalization occurs within 15 min after adding STP to HLECs, caspase inhibitors appear without effect on this process suggesting early membrane changes including Kv channel activation (Chimote et al., 2010). The actual target kinases are unknown nor whether the STP effect includes the participation of the so called mitochondrial (intrinsic) pathway of apoptosis. There are many protein kinases found throughout the human lens such as serine/threonine, tyrosine kinases, MAPK and others, all involved in a wide variety of physiological processes. A tyrosine kinase-mediated phosphorylation of the Na/K pump has been proposed to be responsible for the Na/K pump inactivation during the LEC to the LFC transition (Tamiya et al., 2007). All could
be a target for STP, a fungal derivative displacing ATP, but in order to link early membrane changes to a particular kinase group, the serine/threonine protein kinases C, are of great interest as they are important in the regulation of various membrane transport processes.

Figure 6- Proposed Mechanism of STP induced apoptosis
Proposed scheme of events during AVD. STP inhibits kinases, especially serine threonine kinases (1), causing wide-spread dephosphorylation with activation of 4-AP-sensitive $K_v$ channels (2, 3A) and scramblases (2), with externalization of PS from the inner cell surface (3B) and phospholipid randomization (5). The
resulting membrane lipid symmetry is accompanied or followed by depolarization (4,5) furthering K efflux. The loss of K_i triggers activation of effector caspases (6), DNA fragmentation (7) and cell death. (Figure and legend taken from Chimote et al., 2010).
2. Rationale and Hypothesis

Human lens epithelial cells contain at least 4 different protein kinase (phosphorylase) PKC isoforms, of which at least two (α and ε) are anti-apoptotic and two (δ and θ) pro-apoptotic. A hallmark of cell volume control is that major K transporters such as the Na/K pump, the Na-K-2Cl (NKCC) and K-Cl (KCC) cotransporters are under reciprocal phosphorylation / dephosphorylation control during their activation and inactivation. There is evidence from other cell systems that at least one PKC isoform contributes via another kinase to the phosphorylation and hence activation of NKCC (Liedtke et al., 2002, 2003), and another PKC might phosphorylate the Na/K pump (Feraille et al., 1999) suggesting a crucial role of PKCs in the regulation of membrane transport.

The following hypothesis is proposed:

In lens epithelial cells various pro-apoptotic stressors target PKC kinases and thus modify important membrane transport functions causing loss of cellular K through channels and KCC or inhibition of inward K transport by the Na/K pump and the NKCC, resulting in activation of the caspase cascade followed by apoptosis associated with membrane phospholipid changes, mitochondrial depolarization, and DNA fragmentation. These changes affect the normal cellular homeostasis of lens epithelial cells and their ultimate trans-differentiation into lens fiber cells and thus might contribute to cataract formation.
2.1. **Specific Aim 1**

Demonstrate the presence of PKC isoforms in LECs and follow their temporal distribution between the cytosol and the plasma membrane after activation and depletion by phorbol esters or exposure to selective inhibitors.

2.2. **Specific Aim 2**

Compare by Rb uptake and K loss measurements how pharmacological interventions at the PKC level will affect membrane transport.

2.3. **Specific Aim 3**

Assess changes in mitochondrial depolarization, ATP levels and DNA fragmentation and correlate these changes with those in the ion transport mechanisms after exposing the cells to stress simulating substances such as phorbol esters and selective PKC inhibitors.

2.4. **Specific Aim 4**

Establish the functionality of at least one membrane transporter localization, that of the Na/K pump and its isoform, at the membrane level and verify its continued supply with ATP.

2.5. **Significance**

By altering PKC function through different pharmacological approaches, it is anticipated to gain insight into the transport mechanisms of the Na/K pump and
cation co-transporters (NKCC, KCC) upon PKC depletion. Results could offer valuable insight into the transformation of LECs to LFCs and lead to additional treatments for cataracts and possibly even a cure. Since PKCs play crucial roles in the regulation of ion transporters of all tissues and cells, findings made here may be extrapolated to other research areas under study in biomedicine such as cancer and vascular atherosclerosis.
3. Materials and Methods

3.1. Reagents

3.1.1. Chemicals

Sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride (MgCl₂), dimethyl sulfoxide (DMSO), tris (hydroxymethyl) aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 70% perchloric acid (PCA) were purchased from Fisher Scientific (Fair Lawn, NJ). Calcium chloride (CaCl₂) was purchased from J.T. Baker Chemical CO (Phillipsburg, NJ). Glucose and 3-[N-morpholino] propane sulfonic acid (MOPS) were acquired from Sigma Chemicals (St.Louis, MO). Rubidium chloride (RbCl) was obtained from Alfa Aesar (Ward Hill, MA) and rubidium nitrate from Johnson Matthey Materials Technology U.K. (England). Horse serum, 0.05% trypsin, and medium 199/EB SS were purchased from Hyclone laboratories Inc (Logan, Utah) while KGM-2 and Fetal Clone III were procured from Lonza (Walkersville, MD). Gentamicin reagent solution and cesium chloride (CsCl) were purchased from Invitrogen (Carlsbad, CA).

3.1.2. Inhibitors

Chelerythrine chloride was purchased from LC Laboratories (Woburn, MA), ouabain from Santa Cruz Biotechnology (Santa Cruz, CA), bumetanide and
12-O-tetradecanoylphorbol-13-acetate (TPA) was from Sigma- Aldrich (St.Louis, MO).

3.1.3. Molecular and Immunological Tools

M-PER mammalian protein extraction kit, HALT protease inhibitor cocktail kit, and Bicinchoninic acid (BCA) protein assay reagents A & B were purchased from Pierce Biotechnology (Rockford, IL). Primary antibodies and blocking peptides against PKCα, ε, δ, θ isoforms were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies included horseradish peroxidise-coupled donkey anti-rabbit IgG and Cy3-conjugated donkey anti-rabbit IgG both purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Na/K ATPase α and phosphoserine (pSer) primary antibodies were purchased from Santa Cruz biotechnology while the NKCC1 primary antibody was obtained from cell signalling technology (Danvers, MA). Cell death ELISA kit and Lumi-light western blotting substrate were obtained from Roche Applied Science (Indianapolis, IN). EZ-Link Sulfo- NHS Biotinylation Kit was purchased from Pierce Biotechnology (Rockford, IL). Mitotracker CMX-ROS was purchased from Invitrogen (Carlsbad, CA). Vectashield mounting medium with DAPI for fluorescence was bought from Vector Laboratories (Burlingame, CA). Tissue culture plates were procured from Costar (Corning, NY) and from Sarstedt (Newton, NC) and 75cm² culture flasks from Fisher Scientific (Fairlawn, NJ).
3.2. Ion Flux Solutions

A flux solution is used to introduce outside Rb (K) to the cell. It’s used to measure the amount of K entering the cell and allows for the comparison of the control cells vs treated cells. Balanced salt solution (BSS) consisted of 20 mM Hepes-Tris, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and NaCl as a filler salt. BSS has an osmolarity of 300 mosM, measured using an Advanced Micro-osmometer model 330 from Advanced Instruments (Norwood, MA) and a pH of 7.4 at 37°C. 10 mM RbCl flux solution consisted of 20 mM Hepes-Tris, 10 mM RbCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 0.1% bovine serum albumin (BSA), and NaCl as a filler salt with an osmolarity of 300 mosM and a pH of 7.4 at 37°C. The isosmotic washing solution consisted of 10 mM MOPS/Tris and MgCl₂ as a filler salt yielding an osmolarity of 300 mosM and a pH of 7.4 at room temperature. The cation extraction solution consisted of 5% PCA and 4 mM CsCl. 1 M NaOH was used for protein dissolution.

3.3. Cell Culture

FHL-124 cells were grown in medium 199/EB SS containing 72% KGM-2, 5% fetal clone III, 5% horse serum, and gentamicin. Cells were grown to confluence in a 75 cm² flask and in an incubator containing 5% CO₂ and 95% air at 37°C. Once confluent, the cells were trypsinized using 3 ml of 0.05% trypsin and incubated for 2 min at 37°C. The cell/trypsin mixture was added to more growth medium and centrifuged at 700 rpm for 5 min. The pellet was re-suspended in fresh medium and its cells counted using a haemocytometer. The
cells were then plated into 100 mm petri dishes, 6 well plates, or 12 well plates and grown until they reached 100% confluence.

3.4. Measurement of Ion Fluxes

Intracellular rubidium uptake (Rb$_i$) and K content (K$_i$) in nmoles/mg protein were measured according to Lauf et al. (2008). FHL-124 cells were grown to confluence in 12 well plates and washed with the warm (37°C) BSS-NaCl solution stated above at room temperature. Cells were exposed to the appropriate treatments for anywhere between 0-6 h according to specific protocols. After treatment, the Rb-Cl flux media mentioned above was added to the cells. The influx was terminated by removal of flux media and was followed by five washes in the MgCl$_2$ solution at room temperature. Ions were extracted using a mixture of 5% PCA and 4 mM CsCl for 15 min at 4°C and then 1 N NaOH was added to the plates for 30 min at room temperature for protein dissolution. Protein was measured using the BCA protein assay while Rb$_i$ and K$_i$ were measured using a Perkin-Elmer 5000 Atomic Absorption spectrophotometer (Perkin-Elmer, Boston MA). After absorbance was measured, K$_i$ and Rb$_i$ were calculated as described by Adragna 1991.

3.5. Western Blot

FHL-124 cells were grown to 100% confluence in 100 mm tissue culture plates according to Chimote et al. (2010). Cell lysis was performed using M-PER® mammalian protein extraction kit in the presence of Halt™ protease inhibitor cocktail according to manufacturer’s instructions. The cell lysates were
combined in a 1:1 ratio with 2x Laemmli SDS electrophoresis sample buffer (Sigma Aldich, St.Louis, MO). 5µg of protein was loaded into each lane of a 7.66% polyacrylamide gel for 1.25 h run in a Mini-V 8.0 Vertical Gel System (GIBCO®BRL, Invitrogen, Carlsbad CA) at room temperature and with a current of 100 mA. The protein bands were transferred to polyvinylidene difluoride (PVDF) (Millipore Ireland Ltd) membranes at 4°C overnight with the current set to 90 mA. The membrane was placed in a blocking solution consisting of 10% milk and TBST (10 mM Tris-Cl, 150 mM NaCl, and 0.05% Tween-20) for 1 h at room temperature and moderate shaking on a plate rocker. The membrane was incubated overnight at 4°C with primary antibodies against the various PKC isoforms in dilutions ranging from 1:200 to 1:1000. Primary antibody dilutions for Na/K ATPase α, pSer, and NKCC1 were 1:500, 1:200, and 1:200 respectively. Next, the membrane was incubated with HRP-coupled donkey anti-rabbit IgG or anti-mouse secondary antibody in a 1:3000 dilution for 1.5 h at room temperature and was washed several times with TBST. The membrane was coated with Lumi-Light Western Blotting Substrate for 45 seconds and the protein bands were analysed using a Fuji Las-4000 imaging system.

3.6. BCA Protein Assay

Samples of 12.5 µL were loaded into a 96 well plate and combined with BCA™ Protein Assay Reagent A and B according to manufacturer's instructions. The plate was incubated at 37°C for 30 min and the developed color quantified using Labsystems multiskan MCC/340 plate reader.
3.7. Immunofluorescence Staining and Fluorescence Microscopy

FHL-124 cells were grown on an 8 well Lab-Tek Chamber Slide™ (NUNC) as described by Chimote et al. (2010). Once confluence was reached the cells were fixed with a 4% paraformaldehyde solution containing 0.1% saponin for permeabilization for 30 min at 4°C. Chambers were washed with 1X PBS and blocked with 3% normal goat serum for 1 h at 4°C and then incubated overnight at 4°C with primary antibody in a 1:200 dilution. The cells were then incubated in a 1:250 dilution of a CY3-conjugated secondary antibody for 90 min in a light-protected box at 4°C. Cells were washed with 1X PBS and mounted with Vectashield™ containing DAPI and analysed using the Nikon Eclipse E400 epifluorescence microscope. Objectives of 20X, 40X, 60X, and oil immersion 100X were used. Images were taken with a Canon rebel camera and overlaid using GIMP 2.6 imaging software.

3.8. Cell Death Detection ELISA

Cell Death Detection ELISA Kit (Roche Applied Science Indianapolis, IN) was used for this experiment. Protocol was adapted from (Chimote et al., 2010 and Kulkarni et al., 2006) and changed to prevent the use of trypsin. FHL-124 cells were grown to confluence in 6 well tissue culture plates and underwent a treatment of 50 µM CET for 0-3 h at 37°C. Following CET incubation, the cells were placed in incubation buffer for 30 min, scraped into new tubes, and centrifuged at 16,000 x g for 10 min. ELISA was performed on the supernatants according to manufacturer’s instructions using an anti-histone antibody and anti-
DNA peroxidise-conjugated antibody. Cell death was evaluated in the Labsystems multiskan MCC/340 plate reader (ThermoFisher Scientific) at 405 nm and 490 nm for reference.

3.9. ATP Determination

Protocol was adapted from Brown 1982. FHL-124 cells were grown to confluence in 12 well plates as described by and treated with 50µM CET for 0-30 min at 37°C. The rest of the experiment was done on ice to keep solutions and samples cooled at 0-4°C to minimize ATP hydrolysis. Cells were extracted with 5% PCA and combined with d-H₂O to give a 1.49 x dilution. A KOH mixture (200 mM Hepes, 100 mM KCl, and 700 mM KOH) was added to give a pH of 7.0-7.4. Samples were analysed in a Beckman LS 6000TA Scintillation Counter (Brea, CA) after vials were set up with combination buffer (20 mM Hepes, 25 mM MgCl₂, and 5 mM Na₂HPO₄) and luciferin-luciferase from ground-up firefly lanterns (Sigma Aldrich).

3.10. Mitochondrial Depolarization Detection

FHL-124 cells were plated at 16 x 10⁴ cells/well in an 8 well Lab-Tek Chamber Slide™ (NUNC). Cells were treated with either 50µM CET or BSS as the control and at concentrations (nM) of 50, 100, 200, and 300 of Mitotracker® Red CMXROS for 30 min at 37°C according to manufacturer’s instructions (Invitrogen, Carlsbad CA). After treatment, cells were fixed with a 4% paraformaldehyde solution for 15 min at 4°C and then mounted with Vectashield.
containing DAPI. Cells were observed using the Nikon Eclipse E400 epifluorescence microscope. Objectives of 20X, 40X, 60X, and oil immersion 100X were used. A decrease in the red staining is an indication of membrane depolarization. Images were taken and processed as described in 3.7.

3.11. TPA Depletion Studies

FHL-124 cells were grown to 100% confluence in 60 mm culture dishes. Cells were incubated with BSS and 100 nM TPA for 0-6 h at 37°C (Thodeti et al., 2001). Cells were washed and lysed using M-PER® mammalian protein extraction kit in the presence of Halt™ protease inhibitor cocktail according to manufacturer's instructions. Cells were centrifuged at 16,000 x g for 10 min and the supernatant and pellet which was washed 3 x in BSS were analysed by Western blotting.

3.12. Biotinylation

FHL-124 cells were grown to 90-95% confluence in 49 cm² culture dishes according to manufacturer's instructions (Pierce Biotechnology). Cells were washed quickly with ice cold PBS to prevent rounding and detachment. Cells were then incubated with BSS ± 50 µM CET for 30 min at 37°C. Thereafter 2 µM okadaic acid was added and incubated for 10 min at 37°C. Sulfo-NHS Biotin was dissolved in PBS and added to each culture dish and incubated at 4°C for 30 min. An amine buffer was then added to quench any free biotin. Cells were lysed and centrifuged for 1 min at 10,000 x g. Resulting supernatant was removed and
pellet solubilized in a solution containing lysis buffer and 1% CHAPS. Pellet was sonicated and centrifuged for 2 min at 10,000 x g. Resulting supernatant was added to 1 ml of StreptAvidin, incubated at 4°C for 1 h, and centrifuged for 2 min at 5,000 x g. 50 mM DTT was added to 2x SDS and incubated for 60 min at RT to help elute the proteins. After incubation, eluted proteins were centrifuged for 2 min at 5,000 x g and resulting supernatant was analyzed by Western Blotting.

3.13. Statistical Analysis

T-tests and one-way ANOVA were calculated using STATISTIX 7 software (Analytical software, Tallahassee FL) and Graphpad Prism 5 (Graphpad Software, La Jolla CA). Charts and graphs were plotted using Origin 7 software and Origin Pro 8.5 Student Version (Origin Labs, Northampton MA). Data with p-values < 0.05 were considered significant.
4. Results

In a previous study it was shown how the general protein kinase inhibitor staurosporine, by opening a voltage-dependent K (Kv1.4) channel, presumably through dephosphorylation, causes a fall of the intracellular K concentration associated with membrane phosphatidylserine externalization followed by apoptosis (Chimote et al., 2010). To narrow down this effect, the present study was aimed at the effect of pro-apoptotic PKC modulators, such as TPA and the selective inhibitor CET, on cation transport mechanisms in LECs hoping to identify a specific PKC isoform in control of the apoptotic volume regulatory Kv1.4 channel. From other cell systems there is ample evidence that PKCs are regulating primary and secondary active cation transport mechanisms (Pierre et al., 2002), especially the Na/K pump and the cation-chloride cotransporters (Liedtke et al., 2002, 2003).

Hence, the results address first the cellular presence of four PKC isoforms in LECs discussed in section 4.1, and the effect of TPA and CET on their distribution between the cytosol and the plasma membrane, the two main compartments of their functional equilibration (section 4.3). Second, previous work already established the presence of the NKCC1 and KCC1, 3, and 4 isoforms in human LECs (Misri et al., 2006), however, it was unknown which of the 4 Na/K pump catalytic α subunits occurs in these cells. Therefore, to complete the picture, also the Na/K pump isoform responsible for primary active Na/K exchange transport was immunologically determined. Third, based on the time course of the PKC changes in response to the two pharmacological
interventions, subsequent ion flux studies were designed to capture and explain
their changes from steady state requirements that ultimately may or may not lead
to apoptosis (sections 4.9). Fourth, the significant effects of one of the two PKC
modulators on the Na/K pump and NKCC activities, required additional
approaches to clarify the role of PKCs in the maintenance of steady state
functions of these transporters, or their failure potentially preceding apoptosis.
The results of this work thence constitute new insights into the functioning of
Na/K pump and cation chloride cotransporters in human LECs which are
compromised by a challenge with pro-apoptotic PKC inhibitors.

4.1. Characterization of PKC isoforms in FHL-124 cells

Many PKC isoforms have been identified and classified according to their
structure and second messenger requirements (Mellor et al., 1998, Gutcher et
al., 2003, Thodeti et al., 2001, Steinberg 2008). The present study looks at 4
different PKC isoforms, 2 of which are pro-apoptotic (δ, θ) and 2 are anti-
apoptotic (α, ε). Figure 1 shows the Western blot analysis of each PKC isoform
tested with its blocking peptide to compete for the epitope which the primary
antibody recognizes. This ensures that the primary antibody can no longer bind
to the epitope and hence constitutes a negative control. The WB shows that
FHL124 cells possess PKC isoforms α, ε, δ and θ. Protein bands of ~78 kDa
molecular weight were obtained and abolished (α, ε, δ) or attenuated (θ) by
incubation with isoform-specific peptides against which the antibodies were
raised. Figure 2A shows the presence of the four PKC isoforms by immunofluorescence (IF) staining. Strongest IF staining occurred with anti-PKCε and was primarily cytoplasmic diffuse (α, ε) and punctuate (δ and ζ), and generally was abolished (α, ε, δ) or attenuated (ζ) by the blocking peptides (Figure 2B).

### 4.2. Na-K-ATPase α isoforms present in FHL-124 cells

The Na-K-ATPase enzyme consists of the catalytic α and the supportive and regulatory β and γ subunits (reviewed in Lauf and Adragna, 2011). The α subunit exists in 4 isoforms, α1-α4 (Pierre et al., 2002, Sottejeau et al., 2010). To test which α isoforms were present in the FHL-124 cells, whole cell extracts were prepared as outlined in Materials and Methods and sent for analysis by Yoann Sottejeau and Qiming Duan at the University of Toledo. Figure 3 shows the WB of FHL-124 cells and of B3 lens epithelial cells, another but SV-40 transformed cell line, compared to the positive control of rat brain homogenate. Figure 3A shows that both cell lines contained a single band at 100 kDa of the Na-K-ATPase α1 but not the α2 or α3 isoforms (Figure 3B & 3C).

Previous work from this laboratory has shown by western blots, and by reverse transcriptase and polymerase chain reaction (RT-PCR), the expression of NKCC1 and KCC1, 2, 3 and 4 isoforms in FHL124 cells and hence these data are not reproduced in this section (Misri et al., 2006, Lauf et al., 2008).
4.3. Time course of the TPA effect on PKC cytosol/membrane distribution

The phorbol ester TPA is known to cause redistribution of PKC after prolonged exposure in MCF-7 human breast cancer cells (Issandou et al., 1988). Such TPA effects are unknown in human LECs. TPA mimics the behavior of DAG by binding to PKC, initially activating it, and progressively degrading the kinase, which alters the cell signalling cascade (Kolb et al., 2004). A TPA dose response curve was not done because 100 nm of TPA was the dose used by Thodeti et al. (2001) to give positive results in human intestinal cells. Figure 4 and 5 show that TPA depleted all 4 PKC isoforms (α, ε, δ, ζ) after 2 h of treatment by removing the PKCs from the cytosol and redistributing them into the membrane particulate fraction. In the membrane, PKCα was increased at 2 h by 600% and then decreased thereafter. After remaining constant from 0-4 h, PKCε increased to almost 400% from 4-6 h. PKCδ and PKCθ were not completely removed from the cytosol. In the membrane, PKCδ increased and decreased in staining-intensity throughout the 6 h treatment while PKCθ gradually decreased. These findings are consistent with that of Thodeti et al. (2001) in human intestinal cells who studied the effect of TPA on the cytosolic expression of PKC ε.
Figure 1- Presence of four PKC isoforms in FHL 124 cells

Western blot of protein extracts from FHL 124 cells for the presence of various PKC isoforms using rabbit polyclonal IgG 1º antibody (Ab) and blocking peptides. Lanes 1 and 2 are a double staining for (A) PKCα 1:2000. (B) PKCε 1:500. (C) PKCδ 1:200. (D) PKCθ 1:500. 2º Ab was horse radish peroxidase (HRP)-coupled donkey anti-rabbit IgG (1:3000). Lanes 3 and 4 are a double staining of the primary antibody with its blocking peptide for each isoform.
**Figure 2- Evidence of PKC isoforms in FHL 124 cells.** FHL 124 cells fixed in 4% paraformaldehyde and 0.01% saponin revealed the presence of (A) PKCα, ε, δ, θ(100x magnification). (B) Primary antibody + blocking peptide (60x magnification)(by immunofluorescence microscopy. DAPI for nuclear stain (blue) and CY3-conjugated donkey anti-rabbit antibody (red) as 2⁰ antibody at a 1:250 dilution.
Figure 3: Expression of Na/K-ATPase α isoforms in HLEC. WB analysis of FHL-124 cells and B3 human lens epithelial compared with rat brain homogenate for the presence of Na-K-ATPase α isoforms. FHL-124 cell extracts prepared as outlined in Material and Methods were kindly analyzed by Yoann Sottejeau and Qiming Duan at the University of Toledo. Protein extracts of 25 µg were used. Isoforms A) α1, B) α2, C) α3.
Figure 4: Long term TPA effect on PKC distribution in the cytosol and membrane. FHL cells were treated with 100 nM TPA for times 0, 2, 4, and 6 h. Thereafter, cells were lysed and their supernatants (lane 2-5) and washed membrane pellets (lane 6-9) analyzed by WB. Identical protein amounts (4µg), 1ºAb concentration (1:200), and 2ºAb concentration (1:3000) were used per gel. (A) PKCα. (B) PKCε. (C) PKCδ. (D) PKCθ. 2º Ab was (HRP)-coupled donkey anti-rabbit IgG (1:3000).
Figure 5: Densitometry scans of membrane and cytosolic PKCs after TPA.

Densitometry x Area (D x A) were calculated from Figure 4 by multiplying the net intensity and interior area (pixels) of the gel bands and dividing by $10^8$. (A) PKCα. (B) PKCε. (C) PKCδ. (D) PKCθ.
4.4. Effect of long term exposure to TPA on cation transport in FHL124 cells.

Previous work established the initial velocity conditions for Rb uptake in the presence and absence of 0.1 mM ouabain and 0.01 mM bumetanide, the specific Na/K pump and selective NKCC inhibitors, respectively, in FHL124 cells (Lauf et al., 2008). In addition, using Cl replacement by NO₃ in the presence of ouabain and bumetanide, the KCC activity was determined from the difference of the Rb influx in the presence and absence of Cl. Applying this technique, FHL124 cells were first incubated with 100 nM TPA for 6 h, the dose and maximum time period used in the WB experiments, to assess any major change in the total Rb influx, and its subcomponents, the Na/K pump, the NKCC and the KCC activity. We also measured cellular K content at the end of the incubation times to assess whether any pre-apoptotic K loss had occurred. In addition to TPA, the selective PKC inhibitor CET was present for 20 min at the beginning and the end of the long term TPA incubation. A more detailed description of the short term effects of CET is given in section 4.7.

In order to assess whether any cell loss due to apoptosis occurred, the total protein was determined in all experiments henceforth described in each well that was subsequently analyzed for its ionic composition. Figures 6A and B reveal the long term action of TPA on protein and K content in FHL124 cells within a time frame that led to a redistribution of the PKC isoforms from the cytosol to the membrane (see Figure 5).
Figure 6A shows that 6h exposure in the absence of 100 nM TPA (control) led to ~20% loss of total protein that was only significantly different from the BSA zero pre-incubation in the absence of any inhibitor (None) and presence of ouabain, but not when both (ouabain + bumetanide) were present. When the incubation was carried out for 6 h with TPA, all three conditions (None, ouabain, ouabain plus bumetanide) were significantly different from the BSA zero time control but not from the 6 h control. Thus long term incubation per se lowered the protein content, i.e. led to cell loss, which was not further augmented by the presence of TPA. The additional presence of CET had no further significant effect whether CET was applied before or after the 6 h incubation in presence or absence of TPA.

Figure 6B reveals that the cellular K content also fell in the 6 h control as compared to the zero time BSA control which is expected if cells are lost due to prolonged incubation. However, TPA did not change this outcome, indicating that 6 h incubation with TPA did not lead to cell loss. In contrast, the addition of 50 µM CET, while not affecting at all the 20 min K content of the zero time BSA (except in the presence of ouabain and bumetanide), caused a further loss of K totalling 33% when added after 6 h of TPA treatment (compare TPACET v s TPA) which was real since the protein concentration did not change (see Figure 6A). Thus 6 h TPA treatment had little effect on cellular K, but when followed by CET exposure for 20 min, cell K content fell significantly. This finding is consistent with K loss preceding protein loss, and hence may anticipate apoptosis at subsequent times as shown earlier (Chimote et al., 2010).
Figure 6: Effect of TPA and CET treatment on total protein content and $K_i$
FHL cells were incubated at 37°C in the presence and absence of 0.1 mM ouabain, and (ouabain + 10 µM bumetanide). Note that the y-axis scales are different for each panel. **A)** Protein (mg/well) was measured by BCA assay and **B)** cell K (nmol K/mg protein) by atomic absorption spectrometry, at 20 min. **BSA:** Incubation with BSA for 30 min. **BSACET:** Pre-incubation with BSA for 10 min followed by 50 µM CET for 20 min. **Control:** 6 h incubation in BSS. **ContCET:** 6 h BSS followed by 50 µM CET for 20 min. **TPA:** 6 h incubation in BSS with 100 nM TPA. **TPACET:** 6 h 100 nM TPA followed by 50 µM CET. Thereafter, cells were incubated at 37°C for 20 min in a flux solution containing 10 mM Rb (see Materials and Methods). Means ± SD for n=4.

* p<0.05, ** p<0.01, *** p<0.001
Figure 7 shows Rb influx before and after 6 h treatment with TPA with and without CET. Table 1 computes the statistical significances for the data shown in Figures 6 and 7. In Figure 7A, compared to the zero time BSA data, the total Rb influx before 6 h TPA was greater which was due to a significant increase of Rb influx in the presence of ouabain without a substantial change in the bumetanide inhibition. This means that the NKCC activity, shown in Figure 7B, was significantly augmented by 75%. After 6 h TPA exposure both total (none) and ouabain-insensitive (ouabain) Rb influxes were significantly reduced as compared to the 6h control values, meaning that primarily the NKCC activity was reduced to 35% of the 6h control value as shown in Figure 7B. Addition of CET after 6 h incubation in control medium also reduced total and ouabain-insensitive Rb influx, suggesting effective inhibition of NKCC by 45% rather than the Na/K pump, as seen in panel B. However, after 6 h TPA treatment, CET reduced primarily the total Rb influx (panel A) which led to a greater than 85% inactivation of NKCC (see Figure 7B).

Figure 7C amplifies the Rb influx data of panel A in the presence of ouabain and bumetanide, i.e. the Rb inward leak flux through K channels and KCC. Note that whereas 6 h TPA had no effect on this parameter, the addition of 50 µM CET for 30 min either to the zero time BSA samples, or to both the 6h control or 6 h TPA treatment reduced the "leak" Rb influx significantly by 36%.

Thus the effect of TPA was primarily on the NKCC and not on the Na/K pump activity in these experiments. The CET-sensitive Rb influx difference was
independent of the time of application and, as shown below, was explored further to identify whether KCC or K channels were affected by this drug.

The experiment in Figure 7 was done in the absence of CO₂ which could have caused a change in pH, cell volume and hence greater NKCC transport activity. To test this, the cells were treated with 50 µM CET and 100 nM TPA in the presence of CO₂ for 6 h, however, no difference was found when the results were compared to the cells incubated without CO₂ (data not shown). To test whether BSA was having an effect on the K content and the Rb flux, an experiment was designed to replace the BSA with BSS. The results of this experiment are displayed in Figure 8 and 9. As in Figure 7, so shows Figure 8A no difference in total protein content and Figure 8B CET reduced the K content in controls and after 6 h TPA significantly as seen before in Figure 7B while TPA had no effect. Comparing Figures 9A, B, and C, CET inhibited the Na-K-pump activity by 47% after 30 min but after 6 h there was only a slight decrease in activity. However, TPA decreased NKCC activity by 70% and when TPA was combined with CET, the NKCC was again inhibited by almost 80%. CET caused a decrease in the leak while TPA had no effect. These results suggest CET may have caused an initial and marked effect on the Na/K pump while TPA a more prolonged effect on the NKCC. Comparing the zero time BSA data with the 6h control another important conclusion can be drawn, namely that simply exposing FHL124 cells to 6 h in BSA at 37 °C reduced significantly the Na/K pump-mediated flux to levels comparable to those seen with CET at zero time.
Figure 7: Effect of TPA and CET treatment on Rb influx. FHL cells were incubated at 37°C in the presence and absence of 0.1 mM ouabain and (ouabain + 10 µM bumetanide). A) Cellular Rb\textsubscript{i} (in nmol Rb/mg protein) was measured by atomic absorption spectrometry after 20 min. B) Na/K pump, NKCC and C) Leak were calculated from the results of Rb\textsubscript{i} in A). BSA: Incubation with BSA for 30 min. BSACET: Pre-incubation with BSA for 10 min followed by 50 µM CET for 20 min. Control: 6 h incubation in BSS. ContCET: 6 h BSS followed by 50 µM CET for 20 min. TPA: 6 h incubation in BSS with 100 nM TPA. TPACET: 6 h with 100 nM TPA followed by 50 µM CET. Thereafter, cells were incubated at 37°C for 20 min in a flux solution containing 10 mM Rb (see Materials and Methods). Data are means ± SD for n=4. * p<0.05, ** p<0.01, *** p<0.001
Figure 8: Effect of TPA and CET treatment on total protein content and $K_i$.

FHL cells were pre-incubated at 37$^\circ$C in the presence and absence of 0.1 mM ouabain and (ouabain + 10 µM bumetanide). **A)** Protein (mg/well) was measured by BCA assay and **B)** cellular $K$ (in nmol K/mg protein) by atomic absorption spectrometry. **BSS**: Incubation with BSS for 30 min. **BSSCET**: Pre-incubation with BSS for 10 min followed by 50 µM CET for 20 min. **Control**: 6 h incubation in BSS and ContCET followed by 50 µM CET for 20 min. **TPA**: 6 h incubation in BSS with 100 nM TPA and TPACET followed by 50 µM CET. Cells were then incubated at 37$^\circ$C for 20 min in a flux solution containing 10 mM Rb (see Materials and Methods). Data are means ± SD for n=4. * p<0.05; ** p<0.01; *** p<0.001.
Figure 9: Effect of TPA and CET treatment on Rb influx.

FHL cells were incubated at 37°C in the presence and absence of 0.1 mM ouabain and (ouabain + 10 µM bumetanide). **A)** Cellular Rb (in nmol Rb/mg protein) was measured by atomic absorption spectrometry after 20 min. **B)** Na/K pump, NKCC and **C)** Leak were calculated from the results of Rb in A). **BSS:** Incubation with BSS for 30 min. **BSSCET:** Pre-incubation with BSS for 10 min followed by 50 µM CET for 20 min. **Control:** 6 h incubation in BSS. **ContCET:** 6 h BSS followed by 50 µM CET for 20 min. **TPA:** 6 h incubation in BSS with 100 nM TPA. **TPACET:** 6 h BSS with 100 nM TPA followed by 50 µM CET. Thereafter, cells were incubated at 37°C for 20 min in a flux solution containing 10 mM Rb (see Materials and Methods). Data are means ± SD for n=4. * p<0.05;** p<0.01;*** p<0.001
4.5 Time dependence of TPA action on Rb influx.

The preceding experiments were based on the maximum depletion effect of TPA on the PKC isoforms seen by WB analysis (see 4.1), and showed that 6h after TPA treatment NKCC was significantly inactivated and that the Na/K pump was less affected. Also shown was that CET in combination with TPA decreased further NKCC and the Na/K pump in controls and after TPA, and diminished significantly the (ouabain plus bumetanide)-insensitive "leak" Rb transport. It was therefore of interest to first focus separately on the time dependency of the TPA effects on these transport mechanisms at a much earlier time frame, and later on the short term time dependence of the CET effects (see section 4.7).

Figure 10A shows that the Na/K pump flux in the control media decreased gradually over a time window of 6 h and significantly by 33% at the last time point of 340 min, while TPA increased the Na/K pump significantly at the earliest time point which subsequently was followed by a similar decay to about 57% of its initial value with TPA. In contrast, Figure 10B reveals that the control NKCC activity significantly increased at early time points to some 60% at 150 min. Furthermore, TPA caused a 50% inhibition of the NKCC activity at the first time point of 10 min (zero time after TPA exposure) and 70% at 30 min following TPA treatment, which, however, gradually recovered to ~75% of the initial flux in control media, by 6h. This finding suggests an early TPA-sensitive inhibitory component and a later TPA-insensitive phase leading to recovery of NKCC transport function. Finally, Figure 10C displays that the remaining (ouabain plus bumetanide)-insensitive Rb leak influx decreased significantly after 40 min in the
control and stayed unchanged thereafter. This Rb influx time dependence was exacerbated further by TPA which caused a 40%, statistically significant, decrease by 340 min, as calculated in Table 2.

4.6. Cl-dependent Rb influxes before and after TPA and CET exposure.

The experiments with TPA and CET shown in section 4.2 and 4.3 indicated that both reagents reduced the (ouabain plus bumetanide)-insensitive Rb leak influx. In order to answer the question, whether these effects are due to an inactivation of K channels or of KCC which earlier was shown in sheep red cells to be inhibited by a PKC of yet undefined isoform (Ferrell et al., 2000), it was important to measure the PKC inhibitor effects on Rb influx in two flux solutions of RbCl and RbNO₃, the latter a known inhibiting anion. The Cl dependent K loss and leak (KCC) was calculated by simply taking the difference between the fluxes done in Cl and NO₃.
**Figure 10: Time-dependent effect of TPA on Rb Influx.** FHL cells were treated with or without 100 nM TPA for 0 min to 340 min and pre-incubated for 10 min at 37°C in the presence and absence of 0.1 mM ouabain and ouabain + 10 µM bumetanide. Thereafter, cells were placed in a flux solution containing 10 mM Rb (see Materials and Methods) for 20 min. Na/K Pump (A), NKCC (B), and remaining leak (C). Data are means ± SD for n=4. * p<0.05
In the experiment shown in Figure 11, advantage was taken of the K content determined in Cl and NO₃-equilibrated LECs before and after treatment with 100 nM TPA for 3 h, in addition to measuring Rb influx. Figure 11A shows a calculated (positive on the y-axis) Cl-dependent K loss measured within 20 min in control BSS media that was largest in the presence of ouabain and smallest with (ouabain plus bumetanide) present, respectively, indicating a KCC component in all three conditions. However, after 3 h exposure to TPA, the calculated Cl-dependent K loss became negative, indicating that long term incubation with TPA not only inhibited KCC but also activated a higher K permeability in NO₃ media presumably due to channels. Figure 11B shows a small but significant KCC in the control which decreased 40% upon TPA exposure. Thus, although not statistically significant, TPA appeared to inhibit rather than stimulate KCC as predicted for a PKC-inhibited transport system (Ferrell et al., 2001, Adragna et al., 2006 review).

It was shown in Figure 7C that 50 µM CET added for 20 min prior to the flux inhibited by ~30% the Rb leak influx in Cl media, but it was unclear whether this effect is on the KCC system rather than on K channels. Figure 12A demonstrates that the calculated total Cl-dependent K loss, presumably due to KCC activity, was positive being statistically significantly lower when both ouabain and bumetanide were present than either in the absence of these two drugs (None) or in the presence of ouabain alone. Addition of CET did not alter significantly this result, suggesting that CET like TPA before exerted no clearly defined effect on KCC function, even though, like in the TPA experiment, CET
inhibited the Cl-dependent Rb influx through the KCC system by about 30%, which was statistically not significant (Figure 12B). Not shown are the total protein content values for TPA and CET experiments which did not change from the control.

In summary, although both long term incubation (3h) with 100 nM TPA and short term exposure (20 min) to 50 µM CET decreased the KCC activities about 30%, the findings were not statistically significant to conclude at this time that PKC regulates KCC in LECs.

**4.7. Short term CET effects on Rb influxes.**

The previous experiments (sections 4.2-4.5) were to study long term effects of TPA to elucidate the effect of the cytosol to membrane redistribution of PKC isoforms on cation transport in FHL124 cells. CET has been shown to be a selective PKC inhibitor (Chmura et al., 2000). In the course of these experiments, CET was studied in combination with or without TPA to further augment the effect of PKC modulation on cation transport. The short term exposure to a fixed concentration of CET (50µM) led to the conclusion that there were some, perhaps significant, effects of CET on Na/K pump, NKCC and KCC fluxes, requiring a deeper analysis of this phenomenon by short term time- and concentration-dependent studies in FHL-124 cells.
Figure 11: Cl dependent K loss and KCC function due to TPA treatment.
FHL cells were incubated at 37°C ± 100 nm TPA for 3 h in the presence and absence of 0.1 mM ouabain, and (ouabain + 10 µM bumetanide). A) Cellular K (in nmol K_i/mg protein) and B) Rb (in nmol Rb_i/mg protein) were measured by atomic absorption spectrometry at 20 min. Flux solutions with RbCl and RbNO_3 were used. Data were calculated by taking the difference of the Cl and NO_3 values. Data are means ± SEM for n=8 for 2 independent experiments done in quadruplicates. * p<0.05; ** p<0.01; *** p<0.001.
Figure 12: Cl-dependent K loss and KCC function after CET treatment. FHL cells were incubated at 37°C ± 50 µM CET for 20 min in the presence and absence of 0.1 mM ouabain and (ouabain + 10 µM bumetanide). A) Cell K (in nmol K/mg protein) and B) Rb (in nmol Rb/mg protein) were measured by atomic absorption spectrometry at 20 min. Flux solutions with RbCl and RbNO₃ were used. Data were calculated by taking the difference of the Cl and NO₃ values. Data are means ± SEM for n=12 for 3 independent experiments done in quadruplicates. * p<0.05; ** p<0.01; ***p<0.001.
At variance with the experimental conditions in section 4.2-4.4, cells were first pre-equilibrated for 10 min in BSS and then pre-treated with ± 50 µM CET from 0 to 20 min and, after a 10 min flux time, total protein, K content, and Rb uptake were measured. In the control (Figure 13A and C), there was a tendency for both K and protein content to increase with time whereas in the CET treated samples both cell K and protein decreased slightly, but significantly only (see Table 3) at 20 min. Figure 14 shows the time-dependent CET effect on Rb flux. In Panel A no significant changes of Rb influx in the absence and presence of ouabain, and ouabain and bumetanide occurred. In sharp contrast, pre-incubation with 50 µM CET (Panel B) immediately reduced both the total Rb influx (after 5, 10 and 20 min 31%, 35% and 85%, respectively) and ouabain-inhibited Rb flux without affecting the Rb leak influx in the presence of ouabain and bumetanide. Figure 14C and D show that CET primarily and solely reduced Rb influx through the Na/K pump and the NKCC as calculated from the results of Figure 14A and B. After 5, 10 and 20 min the Na/K pump was inhibited 26%, 33% and 90%, respectively. Likewise, the NKCC was inhibited by 25% and 95% at 5 min and 20 min, respectively. The remaining leak did not change significantly after 20 min of CET. Statistical significances of the experiments shown in Figure 14 are given in Table 3.

4.8. Dose dependent CET effects on Rb influx.

Based on the established time to reach maximum inhibition of both Na/K pump and NKCC fluxes, FHL-124 cells were treated with increasing concentrations of CET ranging from 0-50 µM for 20 min. Figure 15A
demonstrates a small but significant change in protein content, however, a large and significant (33%) K content decrease between 10 and 30 μM CET returning to control levels at 40-50 μM. This finding suggests that between 10 and 30 μM CET, K exceeds protein loss, apparently by a K channel affected by this range of CET concentrations, reminding of the STP effect on Kᵢ1.4 (Chimote et al., 2010). Figure 15C reveals continuous decrease of the total and ouabain-insensitive, but not the leak Rb influx signifying primarily inhibition of both the Na/K pump by 90% and the NKCC by 72% at 50 μM CET (Figure 15D). Statistics for these experiments are given in Table 4. These data suggest that CET, via its action on PKC isoforms, directly inhibited the Na/K pump and the NKCC.

4.9. ATP production and apoptosis after CET treatment

ATP is important for driving most cellular processes like the hydrolysis of ATP by the Na/K pump to maintain high K and low Na inside the cell (Contreras et al., 1999, Lauf and Adragna, 2011). Without ATP, the cell would practically shut down due to the inability of the energy dependent processes to function. Furthermore, all PKC isoforms utilize ATP during the phosphorylation and regulation of many cell processes, and inhibition of PKC has been shown to trigger apoptosis (Gutcher et al., 2003). The mitochondria are involved in 50-90% of all ATP production in a cell (Piccoli et al., 2004).
Figure 13: Time dependent CET effect on $K_i$ and total protein. FHL cells were pre-equilibrated at 37°C in BSS for 10 min followed by a second pre-incubation ± 50µM CET for 0-20 min in the presence and absence of 0.1 mM ouabain, and (ouabain + 10 µM bumetanide). Cells were then placed for 10 min in a fluxing solution containing 10 mM RbCl (see Materials and Methods) after which protein was measured by BCA assay (expressed as mg total protein/well) and cell K (in nmol K/mg protein) by atomic absorption spectrometry. K content in A) BSS Control and B) after CET. Total protein in C) BSS control and D) after CET. Data are mean ± SEM for n=12 for 3 independent experiments done in quadruplicates. Significance at p<0.05 is represented as follows: * None; + Ouabain; # OuaBum
Figure 13

(A) Change in nmol K$_i$/(mg protein at 10 min) over time in the Control group with different treatments.

(B) Change in nmol K$_i$/(mg protein at 10 min) over time in the 50 μM CET group with different treatments.

(C) Change in Total Protein (mg/well) over time in the Control group.

(D) Change in Total Protein (mg/well) over time in the 50 μM CET group.
**Figure 14: Time Dependent CET effect on Rb uptake.** FHL cells were pre-equilibrated at 37°C in BSS for 10 min followed by a second pre-incubation ± 50µM CET for 0-20 min in the presence and absence of 0.1 mM ouabain, and (ouabain + 10 µM bumetanide). Cells were then placed for 10 min in a fluxing solution containing 10 mM RbCl (see Materials and Methods) after which protein (mg total protein/well) was measured by BCA assay and cell K (nmol K/mg protein) by atomic absorption spectrometry. Rb uptake for **A)** BSS control and **B)** after CET treatment. **C)** Calculated transport activities in BSS control, and **D)** after CET. Data are a mean ± SEM for n=12 for 3 independent experiments done in quadruplicates. Significance at p<0.05 is represented as follows: * None; + Ouabain; # OuaBum.
Figure 15: Dose Dependent effect of CET. FHL cells were pre-equilibrated at 37°C in BSS for 10 min followed by a second pre-incubation ± 0-50 µM CET for 20 min in the presence and absence of 0.1 mM ouabain, and (ouabain + 10 µM bumetanide). Thereafter, cells were placed in a fluxing solution containing 10 mM RbCl (see Materials and Methods) for 10 min after which protein (mg total protein/well) was measured by BCA assay and cell K (nmol Ki/mg protein) and Rb (nm/mg protein) by atomic absorption spectrometry. A) Total Protein, B) K content, C) Rb uptake, D) Transporter function. Data are a mean ± SEM for n=8 for 2 independent experiments done in quadruplicates. Significance at p<0.05 is represented as follows: * None; + Ouabain; # OuaBum.
Molecular probes have been developed to offer an additional way to study mitochondria. Mitotracker dyes accumulate in the active mitochondria but leak out, once membrane potential depolarization occurs (Piccoli et al. 2004). It has been shown in murine embryonic fibroblasts that CET initiates apoptosis through a direct effect on the mitochondria (Wan et al. 2008). Cytochrome c is released from the mitochondrial permeability transition pore leading to a loss of mitochondrial membrane potential and a decrease in ATP. Thus it was important to measure cellular ATP, mitochondrial integrity and apoptosis by cell death ELISA.

Figure 16 displays the results of ATP measurements in nmoles ATP/mg protein before and after CET treatment. The ATP levels remained constant (or increased at 20 min in control but not CET-treated cells) and decreased thereafter at 30 min. However, this decrease is only apparent because the total protein was higher in the 30 min samples. Figure 17 illustrates that CET apparently did not affect significantly the mitochondrial membrane potential since there was no recognizable change in the levels of Mitotracker dye staining. Thus CET appears not to have acted via mitochondria.

Cell death ELISA measures DNA fragmentation through a complex formed by the binding of a histone antibody to fragmented DNA. Any changes in the complex’s absorbance (measured at 405 nm and 490 nm for reference) between the control and CET-treated cells is an indication of DNA fragmentation and apoptosis (Chimote et al., 2010, Kulkarni et al., 2006). Figure 18 shows that over a time span of 3 h the 405-490 nm absorbance ratio of CET/control remained
around unity indicating there was no significant apoptosis after the cells were treated with CET. However, a small but insignificant evidence for necrosis was detected in the culture supernatants at 3 h.
Figure 16: ATP Determination after CET treatment. See Materials and Methods for procedure. ATP was measured in nmoles ATP/ mg protein from 0-30 min. Insert: total protein (mg/well). Data are a mean ± SEM for n=9 for 3 independent experiments done in triplicates. * p<0.05, **p<0.01, *** p<0.001.
Figure 17: Mitotracker staining for mitochondrial depolarization. FHL-124 cells were stained with Mitotracker® Red CMXROS and treated with either 50 µM CET or BSS (control) for 30 min at 37°C. Cells were then fixed in 4% paraformaldehyde and mounted with Vectashield® containing DAPI. Cells were observed using the Nikon Eclipse E400 epifluorescence microscope. A) BSS control 60x and B) CET treated cells 40x.
Figure 18: Cell death detection by ELISA.

See materials and methods section for procedure. Data are a mean ± SEM for n=9 for 3 independent experiments done in triplicates to test apoptosis. Mean ± SD for n=3 for 1 experiment done in triplicates to test necrosis. * p<0.05; ** <0.01; *** p<0.001.
4.10. Biotinylation studies

It has been recently shown in Madin-Darby canine kidney (MDCK) cells that exposure to phorbol 12-myristate 13-acetate (PMA), which is like TPA, causes a cytosolic retrieval of NKCC1 after just 2.5 min, whereas the Na-K-ATPase stayed in the membrane (Mykoniatis et al., 2010). The purpose of this study was to see whether after biotinylation the Na-K-ATPase α1 subunit remained in the membrane after PKC was inhibited by CET. Since the Na-K-pump function decreased by 90% after 30 min of CET exposure, it was of interest to study whether, like NKCC1 in MDCK cells, the Na-K-ATPase was internalized into the cytosol.

The results displayed in Figure 19A show that, as compared to the control, there was a reduction of the Na-K-ATPase α1 subunit but there was still a significant amount of the α1 subunit present in the membrane after treatment with CET for 30 min, yielding strong protein bands with a molecular weight of approximately 100 kDa. Figure 19B shows positive staining in the membrane before and after CET treatment at about 100 kDa for the phosphoserine antibody. Figure 19C shows positive and about equivalent membrane staining at about 175 kDa for NKCC1 before and after CET exposure, and a stronger smaller molecular weight protein band at ~100 kDa which must be a decompositional product of NKCC1. These data highly suggest that the Na-K-ATPase α1 subunit only partially left the plasma membrane upon CET treatment, and most likely, the NKCC1 molecules not at all.
4.11. CET effect on PKC localization

As seen earlier in these studies, TPA caused a redistribution of PKC from the cytosol to the membrane when the cells were exposed to TPA. To test if CET has a similar effect on the PKCs, FHL-124 cells were treated for 30 min with CET. The results in Figure 20 display the CET effect on each of the 4 PKC isoforms tested. Figure 20A shows that the PKCα signal, when treated with CET, was decreased in the cytosol and increased in the membrane. Figure 20B shows PKCε displaying the same characteristics as PKCα, the isoform was depleted from the cytosol and compartmentalized in the membrane. Figure 20C and 20D show PKCδ and PKCθ being depleted from the cytosol remained in the membrane after CET treatment. Each isoform yielded a molecular weight of approximately 78 kDa.
Figure 19: Effect of CET on Na-K-ATPase α1 subunit, pSer and NKCC1 at the membrane level.

FHL-124 cells were treated with ± CET for 30 min and subjected to biotinylation (see materials and methods). The cell lysates were analysed using Western blotting techniques and primary antibodies of A) Na-K-ATPase α1 at 1:500 dilution B) pSer at 1:200 and C) NKCC1 at 1:200 dilution were used.
**Figure 20: Effect of CET on PKC isoform distribution.** FHL-124 cells were treated ± 50 µM CET and incubated at 37°C for 30 min. Western blots of membrane and cytosol fractions for the presence of various PKC isoforms using rabbit polyclonal IgG 1° Ab with the following dilutions:  (A) PKCα 1:200.  (B) PKCε 1:200.  (C) PKCδ 1:200.  (D) PKCθ 1:200. β-actin was the loading control.

<table>
<thead>
<tr>
<th></th>
<th>Cytosol</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS</td>
<td>CET</td>
<td>BSS</td>
</tr>
<tr>
<td>95 kDa</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>B-actin</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Cytosol</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS</td>
<td>CET</td>
<td>BSS</td>
</tr>
<tr>
<td>95 kDa</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>B-actin</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>
5. Discussion

The original purpose of this study was to narrow down the kinases responsible for early membrane changes in human lens epithelial cells when treated with the pro-apoptotic kinase inhibitor staurosporine (STP) which causes early membrane changes like the activation of amino-pyridine-inhibited Kv 1.4 channels (Chimote et al., 2010). STP is a broad spectrum inhibitor of OH-amino acids and tyrosine kinases. The initial goal was to determine which type of kinase was responsible for these reported changes. The PKCs are serine/threonine kinases, known major players in many physiological processes (Gutcher et al., 2003, Thodeti et al., 2001, Steinberg 2008). PKC isoforms are present in a wide variety of cell lines: Human intestinal cells, vascular smooth muscle cells, human leukemia T-cells, fibroblasts, human tracheal epithelial cells, chicken embryo lens cells, and many more (Thodeti et al., 2001, Goerke et al., 2002, Meller et al., 1996, Goodnight et al., 1995, Liedtke et al., 1997, Berthoud et al., 2000). Two very different compounds targeting PKCs were chosen: 1) A long term acting phorbol ester, TPA, known to deplete PKCs from the cytosol binding to their C1 domains (Brose et al., 2002) and induce their redistribution to the membrane (Issandou et al., 1988); and 2) CET displacing ATP at their C3 ATP binding site domain (Herbert et al., 1990) thus interfering with substrate transphosphorylation.

Selecting four PKC isoforms for their proclaimed pro- and anti-apoptotic properties (Gutcher et al., 2003), this study addressed their presence in FHL-124 cells, their TPA-induced distribution from the cytosol to the membrane, whether treatment with TPA and CET affected membrane transport, especially cell K loss
and K influx measured with the K congener Rb, whether or not these interventions led to the development of apoptosis, and how short term exposure to CET changed channel K efflux, and the Na/K pump and NKCC influx activities. The key findings in this study were that either of two PKC inhibitors had distinct effects on K efflux and major effects on Rb influxes and its subcomponents, specifically the Na-K-pump and NKCC. In the end, these observations made here address the fundamental question whether pro-apoptotic PKC inhibitors exert their effect to lower cellular K required for caspase activation and apoptosis first at the K channel level or at the Na/K pump and NKCC1 level, or independently at both levels.

5.1. PKC isoforms in FHL-124 cells

This study verifies the presence of PKCα, ε, δ, and ζ in a human LEC line (FHL-124). The two anti-apoptotic isoforms (α, ε) have been implicated in cell shape, growth, and survival (Nakashima 2002, Gutcher et al., 2003), while the two pro-apoptotic isoforms (δ, ζ) have been shown to be involved in cell death (Gutcher et al., 2003). Not an obvious fit with this classification, PKCδ, acting upstream, phosphorylates SPAK (Smith et al., 2008), another serine kinase which by phosphorylation regulates NKCC through interaction with WNK4 and thus regulates cell volume (Gagnon et al., 2007). PKCζ participates in the T-lymphocyte signalling cascades (Thuille et al., 2005). All four isoforms were present in the FHL-124 cells with PAGEL protein bands at ~78 kDa eradicated with the blocking peptides (Figure 1) and with PKCα and PKCε IF-positive staining more spread throughout the cytoplasm, and PKCδ and PKCζ more
punctuate (Figure 2). Whether the difference in staining, diffuse vs. punctuate, may be related to the established anti-apoptotic and pro-apoptotic PKC isoform properties and indicate how these PKC isoforms interact with different signalling cascades controlling either cell survival or cell death, remains to be seen.

5.2. TPA and its effect on ion transport in FHL-124 cells

It is well known that long term TPA exposure causes the re-location of PKCs from the cytosol to the membrane (Issandou et al., 1988). Thodeti et al. (2001) showed in human intestinal cells a down regulation of PKCε after just 2 h of TPA exposure while PKCα and PKCδ remained with strong band intensity. The present study was designed to see how each isoform responded to treatment with TPA in FHL124 cells. Figures 4 and 5 show that PKCε, like in the human intestinal cells, was depleted after 2 h. However, PKCα, δ, and θ were also down regulated after 2 h, thus distinguishing the FHL124 cell line from others. Commensurate with observations by others (Thodeti et al., 2001, Muscella et al., 2008, Crabos et al., 1991, Issandou et al., 1988), loss of PKCs in the cytosol is accompanied by their increased presence in the membrane. This may be explained by assuming that the phorbol ester, by binding to the C1 domain of these PKCs, attracts the enzymes preferentially to the plasma membrane (Brose et al., 2002). This conclusion is consistent with the redistribution of the isoforms from the cytosol to the membrane seen by Issandou et al. (1988). It is likely that, once in the membrane, these PKCs are removed by another process, possibly by endocytosis, hydrolyzed by lysosomes, or externalized by exocytosis. PKCα seemed to be the most abundant of the 4
isoforms since it gave the strongest band signal. The high levels of PKCα in the membrane may mean it is also in the FHL124 cell line involved in the regulation of the Na-K- pump through phosphorylation of Ser-18, which was proposed by Feschenko et al. (2000). PKCα and ε are more cytosolically based, as seen in the IF studies, while PKCδ and θ may be more membrane based. In the WB results (Figure 4) PKCδ- and PKCθ-positive protein bands are also seen near the top of the gels. One reason for this may be that these 2 isoforms aggregate with themselves or other proteins to form homo- or hetero-polymers. PKCδ is such a candidate and may combine with the NKCC1 isoform and therefore is in control of this transporter through SPAK, as previously shown (Smith et al. 2008, Gagnon et al., 2007).

Based on the observed PKC redistribution from the cytosol to the membrane after 2 h of TPA exposure, the transport functional properties of FHL124 cells were studied when introduced to TPA for long periods of time. Flux studies were designed to measure the K content and Rb uptake 6h after the addition of the phorbol ester. Six hours after exposure to TPA, there was no significant cell loss since both the total protein and K content principally remained unchanged, however, Rb influx was significantly altered, specifically the activity of the NKCC. NKCC activity was inhibited by ~75% when compared to the 6 h control while the Na/K pump wasn't affected. Cl dependent studies indicate that TPA caused, although not significant, inhibition of KCC by ~40%. This finding needs to be followed up further because earlier work on red blood cells from this laboratory implicated a yet unknown PKC isoform to inhibit KCC in long term
experiments (Ferrell et al., 2000). Thus if KCC is regulated similarly across mammalian cells, TPA should have released this inhibition by PKC and caused a KCC activation, which did not happen (Figure 11).

Prior to discussing the long term TPA action on cation transport, it is important to note that just long term incubation in the absence of TPA led to a loss of Na/K pump activity and a gain of NKCC function in the controls (Figures 7 and 9). There is no ready explanation for this effect other than to assume that perhaps some "basal" phosphorylation of these systems is altered by mere prolonged incubation of the cells. This is not obvious for the Na/K pump, but with respect to the NKCC1 it could have been elicited by volume and pH changes that may have occurred unnoticed, certainly an issue to be followed up in future studies.

The time-dependent studies with TPA (Figure 11) revealed an early TPA-sensitive inhibitory effect on the NKCC and a later TPA-insensitive recovery phase. This novel finding of a TPA-sensitive >70% inactivation of NKCC is consistent with the immuno-fluorescence reports of others that NKCC in T84 cells is retrieved from the membrane only minutes after TPA addition (Mykoniotakis et al., 2010). The loss of NKCC function could be due to PKC activation leading to retrieval of the co-transporter from the membrane to the cytosol through a clathrin-mediated endocytotic pathway (Mykoniotakis et al., 2010). Alternatively or concomitantly, TPA may induce PKCδ inhibition and hence dephosphorylation and inhibition of NKCC (Liedtke et al., 1997, Smith et al., 2008). Another
possibility is that TPA binds non-specifically to and causes conformational changes of the NKCC interfering with its activity.

The second TPA-insensitive recovery phase, where NKCC activity was gradually increased, may be explained by the fact the cells were dividing and escaping the effect of TPA regenerating cells with a normal complement of NKCC molecules in their membranes. Protein synthesis and reinserting of NKCC molecules may also be occurring since no protein degradation was seen over the course of the TPA experiments. A comparison of Figure 5C and 10B reveals similarities between the two. Figure 5C, like 10B, shows a TPA-sensitive from 0-2 h and TPA-insensitive phase from 2-4 h in the membrane fraction. This would fit the notion that PKCδ is in control of NKCC1. PKCδ may also be internalized by endocytosis since highest levels of the isoform were observed in the cytosol at 2 h when the membrane PKCδ is decreased.

Cells regulate their volume by opposing salt transporters, especially by inward transport of Na, K and Cl via the NKCC, called RVI, and outward transport of K and Cl, called RVD (Hoffman et al., 2008, Lauf and Adragna, 2000). This process is inversely controlled by Cl-sensitive protein kinases and by phosphatases (Gillen and Forbush 1999). KCC was also inhibited by TPA (Figure 10C). This was not expected since the two co-transporters (NKCC and KCC) are working in a "yin-yang" manner (as one is inhibited the other is activated) (Gagnon et al., 2005). This result may be cell-dependent, or both NKCC and KCC actually operate inversely but through different mechanisms not involving PKC regulation of KCC.
5.3. The action of CET on K loss and Rb influxes

CET is known to be a selective inhibitor of PKC (Herbert 1990, Chmura 2000). Thus CET was used to study the involvement of PKCs in the regulation of cation transport in FHL-124 cells, especially of their pre-apoptotic K fluxes through K channels and KCC, and K influx components, the Na/K pump and NKCC. CET was first used in combination with TPA to enhance further the PKC modulation of cation transport. CET was added for only 20 min in these long term studies because previous work from this laboratory (not shown here) demonstrated that cells treated with CET for long periods of time became detached from the culture plates thus making it impossible to quantify K content and Rb uptake. Initial results indicated that CET had some effect, although not always significant, on the Rb uptake and its subcomponents Na/K pump, NKCC, and KCC. These experiments were directional in that they provided, though small, first indications of CET-inhibited K influx components. Thus a separation of the CET effect from the K loss and Rb influx measurements was imperative.

The results of the time and dose dependent studies with separate CET pre-incubation and subsequent K efflux and Rb influx regimes (Figures 13-15) demonstrate a complex relationship between K loss and Rb influx as function of the time and dose of CET applied. In comparison to the control, CET reduced the cell K content (Figure 13B) significantly at 10 and 20 min but only by ~10%. Simultaneously measured protein showed a small statistically significant reduction of similar magnitude (~10%) at 20 min (Figure 13D). The small loss of both K and protein could be explained by ~10% loss of cells. If so, then at this
highest CET concentration used, little activation of a K channel occurred, a finding at variance with the STP effect reported by Chimote et al. (2010) where both K and cell water were lost. In sharp contrast, 50 µM CET led within 20 min to a full inhibition of both Na/K pump and NKCC1 (Figure 14D), with both parameters not altered in the control (Figure 14C). Clearly, these data appear to dissociate any channel-mediated K efflux events from the K influx systems, the Na/K pump and the NKCC. Thus were apoptosis to occur, it would be primarily caused by a reduction of cellular K due to CET inhibition of the influx pathways.

However, a very different picture arises when comparing K loss and K influx responses to different CET doses. The Figure 15 independent experiment confirms what has been seen in Figure 13 and 14 that high CET concentrations dissociate between K loss and K influx. Whereas cellular K did not significantly change as compared to zero-CET controls, both Na/K pump and NKCC were completely inhibited >90% and >70%, respectively by 50 µM CET at 20 min and there was no effect on the (ouabain + bumetanide)-insensitive leak flux that, by definition, also contained the KCC activity. In contrast, the dose response study of Figure 15, shows a small but statistically significant protein (13%) and statistically highly significant cell K reduction by 35% at 10, 20 and 30 µM both returning to near zero-time levels at 40 and 50 µM CET (Figure 15A and B). This finding is first commensurate with some 13% cell loss due to removal of anoikis-derived cells during the washing procedure, with anoikis (ανοικίς = without a home) being the early lifting of cells from the incubation wells, a sign of cell damage, and second with CET-induced K loss. In fact, the ratio of K/protein loss...
is 2.3 suggesting that the majority of K loss must have occurred through K channels that are opened by CET treatment, a finding entirely consistent with the STP effect observed by Chimote et al. (2010). The data tell us for the first time that PKCs may actually regulate such a K channel. Future work needs to establish whether this process is inhibited by 4-aminopyridine and hence mediated by the K_v 1.4 channel. The failure of higher 40-50 µM CET concentrations to have a similar effect on K loss as the lower concentrations then remains to be explained; perhaps higher CET concentrations override the low concentration effects reversing protein and K losses by targeting signalling pathways that cause inactivation of K efflux mechanisms, clearly an issue to be studied further in the future.

The second most significant results were the effects of CET on the K influx components measured by Rb uptake (Figure 15D). In Figure 15D, the CET dose dependence of NKCC appears to precede that of the Na/K pump, however, the IC_{50} values for both are not far apart from each other, being some 30 and 35 µM CET, respectively, however, the slopes of Na/K pump and NKCC inactivation are different suggesting a mechanism of inactivation not common to both. It is well known that, without Na/K pump activity, the trans-membrane gradients of K and Na collapse and thus impair the thermodynamic requirements for the two ions to be moved out of or into the cell respectively by K efflux (channels, KCC) and Na influx mechanisms (NKCC and the Na/H exchanger), respectively. In addition, simultaneous changes in cellular Cl functionally affect through Cl-dependent phosphorylation steps for example the KCC and NKCC mechanisms.
The findings in Figures 13-15 suggest several probable independent mechanisms of CET action summarized in Figure 21 which are physiologically significant in regard to the processes that initiate K changes preceding PKC inhibitor induced apoptosis. Comparison of the experiments in Figure 13/14 with that in Figure 15 suggest that CET, dependent on the concentration chosen, acts independently on a K channel, perhaps the K_v1.4 channel reported earlier (Chimote et al., 2010), and on both the Na/K pump and the NKCC1. With respect to the Na/K pump, it is conceivable that CET inhibits a putative PKCα-dependent phosphorylation of the Na/K pump causing dephosphorylation at its 3 phosphorylation consensus sites, amongst them the serine 11 and 18 residues, which may be needed for activation, or after PKC phosphorylation has been transferred to another protein (Feschenko et al., 1995, 1997, 2000). This means that the Na/K pump, once inserted into the membrane must be phosphorylated in order to perform its catalytic activity as a Na plus K-dependent ATP-hydrolase (Skou 1992, Lauf and Adragna review 2011). The difference in the slopes of CET inactivation (Figure 15D) then suggest that the NKCC1 inactivation is different from that of the Na/K pump, most likely involving the PKCδ isoform proposed by Liedtke's group (Smith et al., 2008) that phosphorylates SPAK, the enzyme that according to Delpire (Piechotta et al. 2002) is the key regulator of KCC1 phosphorylation and activity. It is also possible that the dephosphorylation-induced deactivation of the Na/K pump causes the Na plus K gradients to collapse and thus indirectly or secondarily inhibits NKCC. However, this appears to be less likely since at 50 µM CET and 20 min incubation time, there was no
equivalent change in cellular K (compare Figure 13B and 15B). Its chemical composition allows for CET to diffuse across the membrane and inhibit the Na/K pump directly at the ATP-binding and phosphorylation site on Asp 369. Unlike how PKC transfers its phosphorylation to other proteins, Na-K-ATPase is able to phosphorylate itself.

The effect of CET on the Na/K pump might mimic that of the selective Na/K pump inhibitor ouabain. Figure 21 reveals that the structures of the two alkaloids share only remote similarities. Both drugs have heterocyclic phenolic rings: the hydrophilic ouabain, MW 584 daltons, with a cyclo-perhydrophenanthrine ring with a 5 carbon 3, 4, 5-hydroxylated rhamnose and a lactone ring with a ketose group, and the hydrophobic chelerythrine, MW 386 daltons, a benzophenanthridine alkaloid with a two-oxygenated ring. Whether the 5 member ring of CET has any affinity for the Na/K ATPase site to which the lactone moiety of ouabain binds, remains to be tested in experiments with tritiated ouabain, as shown earlier in red blood cells (Joiner and Lauf 1978).

Chelerythrine is known to induce apoptosis in a variety of cell lines and tissues tested; murine embryonic fibroblasts (Wan et al., 2008), human
neuroblastoma cells (Chan et al., 2003), rat cardiac myocytes (Yamamoto et al., 2001). In most cases apoptosis develops over longer periods (hours) exposure to CET (Chan et al., 2003). Apoptosis occurs by either the intrinsic or extrinsic pathways (Fulda et al., 2006). The intrinsic or mitochondrial pathway involves mitochondrial release of cytochrome c (Fulda et al., 2006) a process inhibited by anti-apoptotic proteins of the Bcl-2 family. Chelerythrine targets the BclXL-Bak Bcl-2 protein family by displacing Bax a protein of said complex (Chan et al., 2003) and thus triggers cytochrome c release and breakdown of the mitochondrial membrane potential with subsequent failure of aerobic ATP production, one of the main mitochondrial functions. Thus, using this mechanism, CET should have lowered ATP, the fuel of the Na/K pump. Figure 16 refutes this hypothesis: ATP was present at full levels during 30 min after CET addition. Thus it is of little surprise that application of the membrane potential monitoring mitotracker dye for testing mitochondrial integrity did not show significant differences from the control (Figure 17).

The apparent lack of apoptosis 3 h after CET treatment contrasts with the evidence of Chimote et al. (2010) of DNA fragmentation after 3 h of STP treatment. However, one has to bear in mind that the methodology chosen in the present study was different from that of Chimote et al. (2010). Because it is well known that trypsinization causes major membrane cation transport changes leading to a temporal but recoverable collapse of ion gradients (Adragna 1988), the ELISA method was modified to collect the cells with a scraper from the wells without trypsinization and re-suspending the cells in the ELISA reagents as
prescribed by the manufacturer. This lead to an enhanced but identical background in both controls and CET-treated cells, and therefore Figure 18 displays the ratios between the two. Therefore, it is not possible to compare the data obtained here with those published by Chimote et al. (2010).

Given the fact that neither ATP nor mitochondrial function were altered and, in our opinion, apoptosis was not detectable, the question arose whether CET treatment would diminish the membrane localization of the Na/K pump in analogy to the reported phorbol ester effect to retrieve NKCC1 within minutes from the plasma membrane (Mykoniatis et al., 2010). In situ biotinylation was the method of choice to address this point and Figure 19 A shows beyond doubt that the Na/K ATPase α-1 isoform as tested by its respective antibody was present in the plasma membrane prior to and somewhat attenuated after CET treatment. However, the attenuation of the Na/K ATPase α-1 isoform protein band did not at all correspond to the near 100% loss of the Na/K pump activity measured at 30 min after CET treatment. This suggests that any retrieval of the Na/K pump was functionally irrelevant. In addition, Figure 19C shows no difference in the expression of NKCC1 before and after exposure to CET. Thus neither the Na/K pump nor the NKCC1 proteins were retrieved during 30 min exposure to CET. Unlike in the TPA experiments of Mykoniatis et al. (2010) the NKCC remained in the membrane of CET-treated FHL124 cells, together with the Na/K pump.

Based on the previous experiments and the scheme developed in Figure 22, it was predicted that if CET inhibits through a PKC controlling the Na/K pump activity through serine/threonine phosphorylation, there should be a difference in
**Figure 22: Proposed CET action mechanisms.** CET may exert direct and independent inhibition of the PKC isoforms controlling by phosphorylation various membrane transport systems important for cellular K homeostasis. Inhibition of an unknown PKC isoform causes dephosphorylation and activation of $K_v$ 1.4 potassium channels shown earlier to be STP-sensitive (Chimote et al., 2010). CET- inactivation of the Na/K pump and NKCC1 may be through PKC$\alpha$ or PKC$\delta$, respectively, the latter acting at the level of SPAK (Smith et al., 2008) or, alternatively (dotted lines), through direct interaction with the $\alpha$, $\beta$, $\gamma$ subunits of the Na/K pump and/or directly with the SPAK-NKCC1 regulation.

**Possible Chelerythrine Action Mechanisms**

![Diagram showing possible mechanisms of CET action](image-url)
the phosphorylation levels of the Na/K pump ATPase before and after 50 µM CET. This "null hypothesis" was tested in the Na/K ATPase preparation collected through biotinylation and disproven by the finding of Figure 19 B: There was no difference in the expression of phosphoserine levels in the biotinylated plasma membrane protein fraction. If confirmed by mass spectrometry, this finding then appears to disprove at least the hypothesis that the PKCs cannot be required for phosphorylation of the Na/K pump at its tentative serine residues in lens epithelial cells. It is important to note that biotinylation cannot be performed on the PKCs since PKC is cytosolic and biotinylation is used to pick up surface membrane proteins. PKC is only brought to the membrane through recruitment by DAG or TPA. However, the results in Figure 20 rule out that CET failed to alter the membrane/cytosol distribution of the 4 PKC isoforms tested in non-biotinylated FHL124 cells, suggesting that the drug apparently did not modify the interaction with its putatively phosphorylating PKC isoform. Since CET was used at concentrations (50 µM) much exceeding those required for biochemically isolated PKCs (Kᵢ =0.7 µM) and thus must have completely inhibited intracellular PKC activity, the tentative conclusion is reached that CET directly interacts with the heterotrimeric Na/K pump complex consisting of the catalytic α1, and the supportive β and γ subunits (dotted lines in Figure 22).

Another explanation would be that the CET effect is not only working on the PKCs but working on another kinase-dependent pathway like the extracellular signal-related kinase (ERK) pathway (Mohammadi et al., 2003) and therefore leading to the observation that PKC is not directly involved in the
phosphorylation of the Na/K pump. Khundmiri et al. 2004 previously demonstrated that parathyroid hormone (PTH) causes Na-K-ATPase inhibition and concluded that Na-K-ATPase regulation by PTH requires phosphorylation of PKC and ERK (Khundmiri et al., 2004). This would explain why CET does not lower phosphorylation in the biotinylated Na/K ATPase subunit, as the CET may initially inhibit ERK during the early time frame of 30 min and then PKC thereafter. Another possibility is pSer antibody cross reaction with tyrosine phosphates effected by tyrosine kinase. Tyrosine kinase has been proposed to be in regulation of Na-K-ATPase through a Src tyrosine kinase-dependent pathway (Tamiya et al., 2007). Finally, a remote possibility is phosphorylation of NKCC1 and not Na-K-ATPase. Figure 19 shows NKCC1 staining at 175 kDa and degradation of the cotransporter at 95 kDa. The pSer antibody shows phosphorylation at both of these molecular weights. If indeed these bands are due to NKCC1 phosphorylation, the initial concept that dephosphorylation of the Na/K pump leads to its inactivation is still very much at play.

5.4. Summary

This study has offered some important and intriguing insight into possible development of cataract by taking a closer look at how PKC modulation by pro-apoptotic agents affect cation transport in human LECs. In particular these studies shed light on the sequential or simultaneous effects these agents exert on cation transport and hence cellular K homeostasis. This study may be a model start for other naturally occurring PKC modifiers such as UV light and glycosylation due to diabetes. There are 4 PKC isoforms (α, ε, δ, θ) present in
the FHL-124 cell line. Their post-translational modifications by a tumor promoting phorbol ester, TPA, and a selective inhibitor of PKC, CET, are important when trying to understand the kinetics and mechanism of ion transport in these cells. TPA was shown to have a prolonged effect while CET had a short term effect. In our cell line, TPA was shown to redistribute the PKC isoforms from the cytosol to the membrane, while CET held the isoforms in the membrane. Functional studies with pharmacological intervention demonstrated that both TPA and CET play an important role in the inhibition of the Rb influx into cells. Long term TPA exposure exerted its effect on the NKCC either through endocytosis (Mykoniatis et al., 2010) or through PKCδ down regulation (Liedtke et al., 2002) without causing major K loss. However, short term pre-incubation with CET increased K loss by 33% most likely via K channels earlier proposed preceding apoptosis. Apparently independent from the action on K channels, CET had a major effect on the Na/K pump and NKCC with a slight effect on the KCC. The significant inhibition of the Na/K pump and cation cotransporters occurred without any early signs of apoptosis, which was shown through ATP assays, mitotrackers dyes, cell death detection ELISA, and protein measurements. Also shown for the first time in FHL124 cell line is the presence of Na-K-ATPase α1 subunit. Biotinylation revealed the presence of Na-K-ATPase, NKCC1, and pSer with and without 30 min CET treatment. Taken together, our results suggest that PKC modulation through the use of pro-apoptotic agents, and dependent on their chemical nature, causes major early membrane changes independently affecting K channels, the Na/K pump and NKCC1 function preceding the onset of apoptosis.
5.5. Future Studies

1. There was a CET-concentration dependent K loss through the opening of K channels, similar to the observation from this laboratory of a STP-sensitive $K_v1.4$ channel that is 4-AP sensitive. In order to confirm the K efflux was through the $K_v 1.4$ channel, 4-AP needs to be added along with the CET. A positive result would guide research to identify the PKC isoform that controls this $K_v1.4$ channel.

2. The structure of CET shares similarities to ouabain and hence CET could be another direct inhibitor of the Na/K pump. To test this hypothesis competition experiments are indicated in which increasing doses of CET would displace a fixed dose of tritiated ouabain. If the result is positive, one could postulate binding of CET to the ouabain binding site on the pump. This could be proven on a stoichiometric basis with radio-labelled CET.

3. Treatment of LECs with CET causes Na/K pump inhibition and NKCC inhibition. Older LFCs are deficient of the Na/K pumps needed to obtain nutrients. Instead, they rely on anion currents to get nourishment. CET appears to be causing LECs to behave as if they are LFCs. This means that CET could serve as a model substance to explore the mechanism by which Na/K pumps are inactivated and remain non-functionally present throughout the LEC-LFC transdifferentiation. Obviously, this mechanism would contribute to understanding mechanisms underlying cataract formation either due to aging or diabetes.

4. If the CET effect to inhibit both Na/K pump and NKCC can be shown to occur in other normal tissues and in the development of cancer, the findings
would be of general importance in cell physiology and pharmacology and contribute to the development of therapeutic measures.

5. Another experiment that needs future work is long term CET treatment in regards to apoptosis, since there was no significant apoptosis after 3 h. One possibility is that CET blocks the apoptotic process in LECs by favouring blockage of the pro-apoptotic PKCs, thus actually preventing the onset of apoptosis.
6. References


reveal a complete phosphatidylinositol cycle in low K sheep erythrocytes. *The Journal of Membrane Biology, 177*(1), 81-93.


Table 1: Statistical significance of Figure 6 & 7 data.
- Each value compared to control (10 min control, 6 hr control)

None

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$Rb_i$</th>
<th>Pump</th>
<th>NKCC</th>
<th>Leak</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA CET</td>
<td>0.0422 *</td>
<td>0.1324</td>
<td>0.0037 **</td>
<td>0.0837</td>
<td>0.0188 *</td>
<td>0.0058 **</td>
</tr>
<tr>
<td>Cont CET</td>
<td>0.9652</td>
<td>0.1200</td>
<td>0.0028 **</td>
<td>0.2502</td>
<td>0.0004 ***</td>
<td>0.0124 *</td>
</tr>
<tr>
<td>TPA</td>
<td>0.7733</td>
<td>0.4820</td>
<td>0.0035 **</td>
<td>0.3873</td>
<td>&lt; 0.0001 ***</td>
<td>0.2562</td>
</tr>
<tr>
<td>TPA CET</td>
<td>0.5013</td>
<td>0.0070 **</td>
<td>0.0004 ***</td>
<td>0.2235</td>
<td>&lt; 0.0001 ***</td>
<td>0.0354 *</td>
</tr>
</tbody>
</table>

Ouabain

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$Rb_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA CET</td>
<td>0.3204</td>
<td>0.3143</td>
<td>0.0086 **</td>
</tr>
<tr>
<td>Cont CET</td>
<td>0.4332</td>
<td>0.2448</td>
<td>0.0003 ***</td>
</tr>
<tr>
<td>TPA</td>
<td>0.7984</td>
<td>0.0240 *</td>
<td>&lt; 0.0001 ***</td>
</tr>
<tr>
<td>TPA CET</td>
<td>0.1043</td>
<td>0.0072 **</td>
<td>0.0001 ***</td>
</tr>
</tbody>
</table>

Oua Bum

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$Rb_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA CET</td>
<td>0.1847</td>
<td>0.0085 **</td>
<td>0.0058 **</td>
</tr>
<tr>
<td>Cont CET</td>
<td>0.9729</td>
<td>0.0265 *</td>
<td>0.0124 *</td>
</tr>
<tr>
<td>TPA</td>
<td>0.3633</td>
<td>0.0206 *</td>
<td>0.2562</td>
</tr>
<tr>
<td>TPA CET</td>
<td>0.8844</td>
<td>0.0052 **</td>
<td>0.0354 *</td>
</tr>
</tbody>
</table>
Table 2: Statistical significance of Figure 8 & 9 data.
- Each value compared to control (10 min control, 6 hr control)

None

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$Rb_i$</th>
<th>Pump</th>
<th>NKCC</th>
<th>Leak</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS CET</td>
<td>0.2670</td>
<td>0.0026</td>
<td>0.0005</td>
<td>0.0073</td>
<td>0.7205</td>
<td>0.0425*</td>
</tr>
<tr>
<td>Cont CET</td>
<td>0.1988</td>
<td>0.0112</td>
<td>0.0308</td>
<td>0.2646</td>
<td>0.0031</td>
<td>0.0028**</td>
</tr>
<tr>
<td>TPA</td>
<td>0.1772</td>
<td>0.2383</td>
<td>0.0013</td>
<td>0.8960</td>
<td>&lt; 0.0001***</td>
<td>0.0774</td>
</tr>
<tr>
<td>TPA CET</td>
<td>0.4416</td>
<td>0.0091</td>
<td>0.0021</td>
<td>0.2361</td>
<td>&lt; 0.0001***</td>
<td>0.0008***</td>
</tr>
</tbody>
</table>

Ouabain

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$Rb_i$</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS CET</td>
<td>0.3958</td>
<td>0.0456</td>
<td>0.4795</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cont CET</td>
<td>0.9628</td>
<td>0.0062</td>
<td>0.0028</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>0.1483</td>
<td>0.7950</td>
<td>&lt; 0.0001***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA CET</td>
<td>0.0985</td>
<td>0.0021</td>
<td>&lt; 0.0001***</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Oua Bum

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$Rb_i$</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS CET</td>
<td>0.5919</td>
<td>0.0992</td>
<td>0.0425*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cont CET</td>
<td>1.0000</td>
<td>0.0097</td>
<td>0.0028**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>0.8145</td>
<td>0.7515</td>
<td>0.0774</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA CET</td>
<td>0.1496</td>
<td>0.0056</td>
<td>0.0008***</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Statistical Significance for the time dependent CET studies of Figures 13 and 14.

– Each value compared to its control at (0 min, 10 min, and 20 min)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$Rb_i$</th>
<th>Transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.4534</td>
<td>0.1207</td>
<td>0.7250</td>
<td></td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.8212</td>
<td>0.2308</td>
<td>0.4177</td>
<td></td>
</tr>
<tr>
<td>OuaBum</td>
<td>0.1116</td>
<td>0.0425 *</td>
<td>0.0005 ***</td>
<td>0.4383</td>
</tr>
<tr>
<td>Pump</td>
<td></td>
<td></td>
<td></td>
<td>0.0005 ***</td>
</tr>
<tr>
<td>NKCC</td>
<td></td>
<td></td>
<td></td>
<td>0.0004 ***</td>
</tr>
<tr>
<td>Leak</td>
<td></td>
<td></td>
<td></td>
<td>0.0005 ***</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$Rb_i$</th>
<th>Transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.0037 **</td>
<td>0.0005 ***</td>
<td>0.0005 ***</td>
<td>0.0034 **</td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.5638</td>
<td>&lt; 0.0001 ***</td>
<td>0.0342 *</td>
<td>0.1491</td>
</tr>
<tr>
<td>OuaBum</td>
<td>0.0817</td>
<td>0.0019 **</td>
<td>0.0057 **</td>
<td>0.0057 **</td>
</tr>
<tr>
<td>Pump</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKCC</td>
<td></td>
<td></td>
<td></td>
<td>0.0004 ***</td>
</tr>
<tr>
<td>Leak</td>
<td></td>
<td></td>
<td></td>
<td>0.0057 **</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$Rb_i$</th>
<th>Transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.0044 **</td>
<td>&lt; 0.0001 ***</td>
<td>&lt; 0.0001 ***</td>
<td></td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.0136 *</td>
<td>&lt; 0.0001 ***</td>
<td>&lt; 0.0001 ***</td>
<td></td>
</tr>
<tr>
<td>OuaBum</td>
<td>0.0026 **</td>
<td>0.0014 **</td>
<td>0.0015 **</td>
<td></td>
</tr>
<tr>
<td>Pump</td>
<td></td>
<td></td>
<td></td>
<td>&lt; 0.0001 ***</td>
</tr>
<tr>
<td>NKCC</td>
<td></td>
<td></td>
<td></td>
<td>0.0001 ***</td>
</tr>
<tr>
<td>Leak</td>
<td></td>
<td></td>
<td></td>
<td>0.0015 **</td>
</tr>
</tbody>
</table>
Table 4: Statistical Significance for Dose Dependent CET Studies of Figure 15

- Each dose vs. control at 0 uM

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$R_{bi}$</th>
<th>Transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>10uM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.3101</td>
<td>0.0003 ***</td>
<td>0.0697</td>
<td></td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.8105</td>
<td>&lt; 0.0001 ***</td>
<td>0.0214 *</td>
<td></td>
</tr>
<tr>
<td>OuABum</td>
<td>0.6597</td>
<td>0.0002 ***</td>
<td>0.1509</td>
<td></td>
</tr>
<tr>
<td>Pump</td>
<td></td>
<td></td>
<td>0.7320</td>
<td></td>
</tr>
<tr>
<td>NKCC</td>
<td></td>
<td></td>
<td>0.0440 *</td>
<td></td>
</tr>
<tr>
<td>Leak</td>
<td></td>
<td></td>
<td>0.1509</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$R_{bi}$</th>
<th>Transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>20uM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.1073</td>
<td>&lt; 0.0001 ***</td>
<td>0.0214 *</td>
<td></td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.0210 *</td>
<td>&lt; 0.0001 ***</td>
<td>0.0020 **</td>
<td></td>
</tr>
<tr>
<td>OuABum</td>
<td>0.4445</td>
<td>&lt; 0.0001 ***</td>
<td>0.1783</td>
<td></td>
</tr>
<tr>
<td>Pump</td>
<td></td>
<td></td>
<td>0.9582</td>
<td></td>
</tr>
<tr>
<td>NKCC</td>
<td></td>
<td></td>
<td>0.0051 **</td>
<td></td>
</tr>
<tr>
<td>Leak</td>
<td></td>
<td></td>
<td>0.1783</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$R_{bi}$</th>
<th>Transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>30uM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.0373 *</td>
<td>0.0002 ***</td>
<td>0.0001 ***</td>
<td></td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.3998</td>
<td>&lt; 0.0001 ***</td>
<td>0.0001 ***</td>
<td></td>
</tr>
<tr>
<td>OuABum</td>
<td>0.0883</td>
<td>&lt; 0.0001 ***</td>
<td>&lt; 0.0001 ***</td>
<td></td>
</tr>
<tr>
<td>Pump</td>
<td></td>
<td></td>
<td>0.0023 **</td>
<td></td>
</tr>
<tr>
<td>NKCC</td>
<td></td>
<td></td>
<td>0.0002 ***</td>
<td></td>
</tr>
<tr>
<td>Leak</td>
<td></td>
<td></td>
<td>&lt; 0.0001 ***</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein</th>
<th>$K_i$</th>
<th>$R_{bi}$</th>
<th>Transporters</th>
</tr>
</thead>
<tbody>
<tr>
<td>40uM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.3698</td>
<td>0.0052 **</td>
<td>&lt; 0.0001 ***</td>
<td></td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.7947</td>
<td>0.0254 *</td>
<td>0.0001 ***</td>
<td></td>
</tr>
<tr>
<td>OuABum</td>
<td>0.0512</td>
<td>0.1661</td>
<td>0.0004 ***</td>
<td></td>
</tr>
<tr>
<td>Pump</td>
<td></td>
<td></td>
<td>&lt; 0.0001 ***</td>
<td></td>
</tr>
<tr>
<td>NKCC</td>
<td></td>
<td></td>
<td>0.0003 ***</td>
<td></td>
</tr>
<tr>
<td>Leak</td>
<td></td>
<td></td>
<td>0.0004 ***</td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>Protein</td>
<td>$K_i$</td>
<td>$R_{bi}$</td>
<td>Transporters</td>
</tr>
<tr>
<td>------------</td>
<td>----------</td>
<td>--------</td>
<td>----------</td>
<td>---------------</td>
</tr>
<tr>
<td>50uM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.3064</td>
<td>0.0091</td>
<td>&lt; 0.0001</td>
<td>**</td>
</tr>
<tr>
<td>Ouabain</td>
<td>0.8035</td>
<td>0.0236</td>
<td>&lt; 0.0001</td>
<td>***</td>
</tr>
<tr>
<td>OuaBum</td>
<td>0.9803</td>
<td>0.0002</td>
<td>&lt; 0.0001</td>
<td>***</td>
</tr>
<tr>
<td>Pump</td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
<td>***</td>
</tr>
<tr>
<td>NKCC</td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
<td>***</td>
</tr>
<tr>
<td>Leak</td>
<td></td>
<td></td>
<td>&lt; 0.0001</td>
<td>***</td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Word</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl-phorbol-13-acetate</td>
</tr>
<tr>
<td>CET</td>
<td>Chelerythrine</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>NKCC</td>
<td>Na-K-2Cl cotransporter</td>
</tr>
<tr>
<td>KCC</td>
<td>K-Cl cotransporter</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>Cl</td>
<td>Chloride</td>
</tr>
<tr>
<td>Rb</td>
<td>Rubidium</td>
</tr>
<tr>
<td>pSer</td>
<td>Phosphoserine</td>
</tr>
<tr>
<td>RVD</td>
<td>Regulatory volume decrease</td>
</tr>
<tr>
<td>RVI</td>
<td>Regulatory volume increase</td>
</tr>
<tr>
<td>HLEC</td>
<td>Human lens epithelial cells</td>
</tr>
<tr>
<td>LEC</td>
<td>Lens epithelial cells</td>
</tr>
<tr>
<td>LFC</td>
<td>Lens fibre cells</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>4-AP</td>
<td>4-Aminopyridine</td>
</tr>
<tr>
<td>Cl/HCO3</td>
<td>Chloride-bicarbonate exchanger</td>
</tr>
<tr>
<td>NHE</td>
<td>Sodium proton (hydrogen) exchanger</td>
</tr>
<tr>
<td>Na/H</td>
<td>Sodium proton exchanger</td>
</tr>
<tr>
<td>VSP</td>
<td>Volume set point</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>PP1</td>
<td>Protein phosphatase 1</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>SK</td>
<td>small conductance K channel</td>
</tr>
<tr>
<td>IK</td>
<td>Intermediate conductance K channel</td>
</tr>
<tr>
<td>BK</td>
<td>Big K conductance K channel</td>
</tr>
<tr>
<td>VSOR</td>
<td>Volume-sensitive outwardly rectifying anion channels</td>
</tr>
<tr>
<td>VRAC</td>
<td>Volume-regulated anion current</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>SPAK</td>
<td>Ste20 related proline/alanine rich kinase</td>
</tr>
<tr>
<td>OSR1</td>
<td>Oxidative stress response 1</td>
</tr>
<tr>
<td>WNK4</td>
<td>With no lysine</td>
</tr>
<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PCO</td>
<td>Posterior capsule opacification</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Word</td>
</tr>
<tr>
<td>--------------</td>
<td>------</td>
</tr>
<tr>
<td>PDK-1</td>
<td>3-phosphoinositide-dependent kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4, 5-biphosphate</td>
</tr>
<tr>
<td>RACKs</td>
<td>Receptors for activated C kinases</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>ε</td>
<td>Epsilon</td>
</tr>
<tr>
<td>δ</td>
<td>Delta</td>
</tr>
<tr>
<td>θ</td>
<td>Theta</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>STP</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular potassium content</td>
</tr>
<tr>
<td>Rb&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular rubidium content</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>1&lt;sup&gt;o&lt;/sup&gt;</td>
<td>Primary</td>
</tr>
<tr>
<td>2&lt;sup&gt;o&lt;/sup&gt;</td>
<td>Secondary</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Nitrate</td>
</tr>
<tr>
<td>BSS</td>
<td>Balanced salt solution</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Oua Bum</td>
<td>Ouabain + Bumetanide</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
</tr>
</tbody>
</table>