REGULATION OF CLC-2 CHLORIDE CHANNEL BY PROTEIN KINASE C PHOSPHORYLATION

by

Simeon Wong

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Physiology

University of Toronto

© Copyright by Simeon Wong 2000
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-54189-4
ABSTRACT

CIC-2 is an inward-rectifying chloride channel that is expressed ubiquitously in the body. It is found present in the apical membrane of respiratory epithelial cells, where the chloride channel defective in cystic fibrosis (CFTR) is also present, providing a possible target for cystic fibrosis therapy. CIC-2 channel has been shown to be down-regulated by phorbol esters, although the mechanisms are not known. In this study, we show that the phorbol ester phorbol 12,13 di-butyrate (PDBu) regulates CIC-2 via the protein kinase C (PKC) phosphorylation pathway. In addition, we report that activation of PKC phosphorylation by PDBu mediates a decrease in cell-surface expression of CIC-2. Therefore, membrane trafficking plays an important role in the regulation of CIC-2 activity by PKC phosphorylation.
ACKNOWLEDGMENTS

First, and foremost, I would like to thank my supervisor Dr. Christine Bear for her unending support and invaluable advice throughout my enrollment in the M.Sc. program at the University of Toronto. Her graciousness and generosity have made my experience in graduate research truly a rewarding one. I would also like to thank Dr. Bill Trimble and Dr. Mike Salter for their dedicated service as members of my supervisory committee. I am indebted to several members of Dr. Bear’s lab for various types of support (technical assistance, scientific discussion, nice long lunches) over the past two years along with many good laughs. Thank you Elizabeth Garami, Yanchun Wan, Dr. Mohabir Ramjeesingh, Dr. Canhui Li, Dr. Najma Ahmed, Dr. Raha Mohammad-Panah.

Throughout my course of study at the University of Toronto, I always received complete and unconditional support from my family and friends. I am very grateful to my mother (Rosaline) and father (Simon), my sister (Rosinni) and my brother (Seanon); you are always a deep source of inspiration. Thank you dearly to my true love Nancy Quon for all her support and encouragement, and to her mother (Jean) for feeding this starving grad student!

Most importantly, I would like to give thanks to my Lord and Saviour Jesus Christ, who’s gift of salvation is the greatest reward a man could ever desire. To God be the Glory!

Simeon Wong

September 16, 2000
TABLE OF CONTENTS

List of Figures and Tables  v

CHAPTER I: INTRODUCTION

Cystic Fibrosis: Introduction

I.A. Cystic Fibrosis Pathology and Physiology  1
I.B. CFTR: Structure and Function of an Epithelial Chloride Channel  2
I.C. CFTR: An Important Pathway for Chloride Transport  3
I.D. Cystic Fibrosis Etiology: High Salt or Low Volume?  5
   i. The High-salt Hypothesis  6
   ii. The Low-volume Hypothesis  7

ClC Chloride Channels

I.E. The Family of ClC Chloride Channels  8
   i. Functions of ClC Chloride Channels and Disease-causing Defects  8
I.F. Structural and Functional Properties of ClC channels  11
   i. Topology  11
   ii. Ion Selectivity  12
   iii. Gating Mechanisms  14
   iv. Heteromeric Structure of ClC Proteins  17
I.G. ClC-2: The Molecular Profile of an Ubiquitous Protein  19
   i. Localization and Function  19
   ii. Structure and Topology  20
   iii. Channel Gating Properties  22
iv. Regulation of ClC-2 by Protein Kinase C

The Protein Kinase C Phosphorylation Pathway

I.H. Regulation of ClC Chloride Channels by PKC Phosphorylation
i. Regulation of activity via direct channel phosphorylation
ii. Regulation of channel activity via phosphorylation of accessory proteins
iii. Regulation of channel activity via membrane trafficking
iv. Establishing the involvement and role of PKC in ClC-2 regulation

CHAPTER II: MATERIALS AND METHODOLOGY

II.A. Expression and Functional Study of ClC-2 in Xenopus laevis Oocytes
i. Capacitance Measurements

II.B. Expression of ClC-2 in Sf9 cells using the baculovirus expression system

II.C. In vitro PKC Phosphorylation of Purified ClC-2

II.D. Expression of ClC-2 protein in Sf9 insect cells
i. Immunolocalization of ClC-2 and Confocal Microscopy
ii. Cell-surface Biotinylation and Immunoprecipitation of ClC-2

CHAPTER III: EXPERIMENTAL RESULTS

III.A. Phorbol Ester PDBu Down-regulates ClC-2 Activity in Xenopus Oocytes

III.B. Myristolated PKC Inhibitor 19-27 upregulates ClC-2 Currents

III.C. PDBu exerts its effects via the PKC Phosphorylation Pathway

III.D. Purified ClC-2 can be Phosphorylated by PKC in vitro

III.E. PKC Inhibition is not sufficient in ClC-2 gating
III.F. Modulation of PKC phosphorylation status does not affect intrinsic channel characteristics 54

III.G. PDBu Down-regulates Membrane Capacitance in ClC-2 Xenopus oocytes 57

III.H. Down-regulatory Effect of PDBu is Temperature-Dependent 61

III.I. Phorbol ester Causes Re-distribution of ClC-2 in Transfected Sf9 Cells 62

III.J. Activation of PKC Reduces the Amount of Detectable ClC-2 on the Cell Surface 67

CHAPTER IV: DISCUSSION

IV.A. ClC-2: A Prototype for Widely-expressed Chloride Channels 69

IV.B. ClC-2 Activity is Modulated by PKC Phosphorylation in Xenopus Oocytes 70

   i. PDBu inhibits ClC-2 activity via the PKC phosphorylation pathway 71

IV.C. The PKC Inhibitor Peptide – 19-27 72

   i. PKC inhibition up-regulates ClC-2 mediated currents 73

IV.D. Is PKC Involved in Direct Modulation of ClC-2 Activity? 74

IV.E. Modulation of PKC Does Not Affect Important ClC-2 Channel Properties 75

   i. Ion selectivity 75

   ii. Voltage dependence 76

   iii. Channel activation potential 77

   iv. Channel gating 78

   v. Single Channel Conductance 79

   vi. A hyperpolarization-induced ‘Priming’ effect 79
IV.F. PKC Activation Induces Redistribution of ClC-2
   i. Low temperature inhibits down-regulation of ClC-2 by PDBu
   ii. Redistribution of ClC-2 in Sf9 cells
   iii. How does PKC inhibition regulate ClC-2?
IV.G. Is the Phorbol Ester Response Protein-specific?
IV.H. Conclusion and Future Direction

CHAPTER V: REFERENCES
LIST OF FIGURES AND TABLES

CHAPTER I

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CFTR’s multiple roles in fluid and electrolyte transport</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>Hypothesized transmembrane topology of CIC chloride channels</td>
<td>13</td>
</tr>
<tr>
<td>C</td>
<td>Proposed double-pore gating mechanism of CIC-0</td>
<td>18</td>
</tr>
<tr>
<td>D</td>
<td>Putative transmembrane topology of human CIC-2 chloride channel</td>
<td>21</td>
</tr>
<tr>
<td>E</td>
<td>Example of a PKC-activating receptor pathway</td>
<td>24</td>
</tr>
<tr>
<td>F</td>
<td>Structure of various PKC isozymes</td>
<td>26</td>
</tr>
<tr>
<td>Table 1</td>
<td>The known CIC channels, their functions and disease etiology</td>
<td>9</td>
</tr>
</tbody>
</table>

CHAPTER II

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Cell-surface biotinylation protocol</td>
<td>38</td>
</tr>
</tbody>
</table>

CHAPTER III

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PDBu inhibits CIC-2 mediated current in <em>Xenopus</em> oocytes</td>
<td>40,42</td>
</tr>
<tr>
<td>2</td>
<td>PKC inhibitor 19-27 up-regulates CIC-2 currents in <em>Xenopus</em> oocytes</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>19-27 blocks the inhibitory effects of PDBu on CIC-2</td>
<td>47</td>
</tr>
<tr>
<td>4</td>
<td><em>In Vitro</em> PKC phosphorylation of purified CIC-2</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>PKC inhibition by 19-27 is not sufficient for channel gating</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>Effect of PKC phosphorylation on CIC-2 voltage dependence</td>
<td>55</td>
</tr>
<tr>
<td>7</td>
<td>PDBu induces a decrease in cell membrane capacitance</td>
<td>58-9</td>
</tr>
<tr>
<td>8</td>
<td>Low temperatures block inhibition of CIC-2 currents by PDBu</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>Immunolocalization of CIC-2 in Sf9 insect cells</td>
<td>65-6</td>
</tr>
<tr>
<td>10</td>
<td>Cell-surface biotinylation of CIC-2 in Sf9 insect cells</td>
<td>68</td>
</tr>
</tbody>
</table>
CHAPTER I: INTRODUCTION

A. Cystic Fibrosis Pathology and Physiology

Cystic Fibrosis (CF) is an autosomal recessive disease that has an incidence in the Caucasian population of approximately 1 in 2500 live births. Major phenotypic manifestations of CF include elevated sweat chloride levels, obstructive respiratory disease, and severely diminished exocrine pancreatic function. In the airways and gastro-intestinal tracts of CF patients, there is often accumulation of thick mucous in these cavities, chronic inflammation and infection, leading to fatal hypoxia and malnutrition. A typical characteristic of pulmonary disease in CF patients is the chronic occurrence of respiratory infections, as a result of hypersecretion of mucous, and inability of microvilli in the airways to clear the pathogens (reviewed in reference 4).

The diverse clinical pathophysiology of cystic fibrosis has historically made isolating the disease etiology extremely difficult. Nevertheless, investigations have narrowed the cause of CF to a basic defect in the electrophysiological properties of the epithelial cell layer lining ductal, airway and intestinal lumens. Investigations of electrolyte transport properties of CF epithelia lining the lumen of affected organs showed perturbations of Na⁺ and Cl⁻ transport.

A turning point in CF research occurred in 1989 with the significant discovery of the gene mutated in the disease by Collins, Riordan, Tsui and colleagues. Further analysis of the predicted translation protein product of the gene revealed a transmembrane protein, called the Cystic Fibrosis transmembrane conductance regulator (CFTR).
B. CFTR: Structure and Function of an Epithelial Chloride Channel

CFTR is a cAMP-dependent transmembrane chloride channel that is essential in the chloride transport systems of epithelial cells in many organs, including the intestines, lungs, sweat glands and kidneys. It is a member of the ATP-binding cassette (ABC) membrane transporter gene family, which include proteins such as the multi-drug resistant protein (MDR). As the name of the superfamily suggests, activity of CFTR is dependent upon ATP hydrolysis, making CFTR a unique energy-driven ion channel.

CFTR possesses several distinct regions, including a cytoplasmic regulatory domain (R-domain) that contains a significant number of consensus phosphorylation sites. Phosphorylation, in particular via the protein kinase A and C pathways, is a vital step in the activation of CFTR and, along with ATP hydrolysis, contribute cooperatively to regulate CFTR chloride transport (reviewed in reference 9). While phosphorylation occurs in the regulatory domain, ATP hydrolysis takes place in the 2 cytoplasmic nucleotide binding domains (NBD), which contain the highly conserved Walker A and B motifs found in other ATPases (8, reviewed in reference 10).

A current prevailing hypothesis for CFTR gating suggests that, in the presence of R-domain phosphorylation, ATP hydrolysis at NBD-1 opens the channel while ATP hydrolysis at NBD-2 causes channel inactivation. This theory is supported by a number of functional studies involving mutations in the two nucleotide-binding domains (reviewed in reference 1).
In addition to being a chloride channel, CFTR also acts to regulate the activity and function of other channels and transporters, including the amiloride-sensitive epithelial Na\(^+\) channel (ENaC – reference 12) and the outward rectifying chloride channel \(^{13}\). However, whether these regulatory functions result from direct or indirect interactions between CFTR and the channels and transporters remains to be determined. EnaC is thought by some investigators to play a major role in the development of key phenotypical manifestations of CF \(^{14}\).

C. CFTR: An Important Pathway for Chloride Transport

In normal intestinal epithelium, chloride ions enter the cells basolaterally via a Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporter and exit through CFTR in the apical membrane; water follows across the apical membrane osmotically \((15; \text{see Figure } A)\). Absorptive epithelial cells possess similar transport mechanisms, but channel distribution is thought to be slightly different. For instance, in absorptive epithelial cells, the sodium channel ENaC is present on the apical surface, while non-CFTR chloride channels are distributed along the basolateral surface (figure AII). Defects in the gene encoding the CFTR protein or lowered levels of the protein itself, resulting in a decrease in trans-epithelial Cl\(^-\) transport, are the primary cause of cystic fibrosis \((16, 17; \text{reviewed in } 18)\).

More than 800 mutations in the CFTR gene have been identified in CF patients \(^{19}\). These mutations can be broadly grouped into four classes: I) defective protein synthesis, II) defective protein processing, III) abnormalities in ion conduction, and IV) defective regulation of channel gating (reviewed in 20). The
Figure A: CFTR's multiple roles in fluid and electrolyte transport. Above represents epithelial cells. ENaC - epithelial Na⁺ channel; AQP - aquaporin; Na/K/Cl - Na⁺/K⁺/2Cl⁻ co-transporter; TJ - tight junction.
most common form of disease-causing defect is abnormal protein folding of CFTR, leading to inactive channels or channels not being inserted properly into the plasma membrane.

A number of pharmacological therapies and drugs have been in development over the years in attempts to rectify these abnormalities of CFTR synthesis, processing or activity. However, the majority of these trials have proven to be unsuccessful and costly (reviewed in 21). One reason for this is that CF is a complex systemic inherited disorder with a wide variety of severities. Because a single mutation can affect both protein function and location, combination therapies may be needed. More importantly, however, is the inability of scientists to understand conclusively the cause for CF progression. As discussed in the preceding section, there have been several theories put forth to explain the patho-physiology of the disease.

D. Cystic Fibrosis Etiology: High Salt or Low Volume?

Until recently, it was generally thought that defective chloride secretion into the affected lumens prevents water secretion across the apical membrane into organ cavities, resulting in dehydration and mucous accumulation. This theory was favored because under non-pathological conditions, secretion of water, in intestine, is driven by active transport of chloride ions through the epithelial cell apical membranes.

Movement of chloride ions into the lumen results in passive diffusion of Na⁺ into the lumenal fluid through a predominantly paracellular route. The resulting ionic gradient was thought to drive secretion of water into the lumen by osmosis. Thus,
defective regulation of transepithelial movement of these ions was proposed to alter properties of luminal mucous composition, resulting in accumulation of dehydrated and sticky mucous and bacterial growth, leading to the symptoms of CF.

Historically, it was thought that apical CFTR is responsible for chloride secretion, and that water followed into the lumen down its osmotic gradient. Defective CFTR activity reduced chloride secretion and prevented water permeation as well, leading to dehydration of airway mucous.

In recent years, this hypothesis has been heavily challenged. In its place, there have been two convincing, yet apparently contradictory hypotheses put forth to explain how defective CFTR protein causes symptoms associated with defective absorption in cystic fibrosis, particularly as manifested in the lungs. Both theories are supported with strong experimental data (reviewed in 18). This dilemma has arisen mainly because of a difference in emphasis on two major functions of CFTR: its ability to act as a chloride channel versus its regulatory effects on the ENaC sodium channel.

i. **The High-salt Hypothesis**

The "high salt" hypothesis highlights the function of CFTR as an anion channel. Proponents of this model suggest that missing or defective CFTR causes reduced trans-epithelial chloride conductance, allowing salt levels in the airway mucous to be maintained at high levels. Because there is a lack of osmotic gradient for water permeation, and a lack of alternate chloride pathways, the high salt
environment then interferes with natural antibiotics such as defensins and lysozyme, allowing rapid proliferation of bacterial growth.  

ii. **The Low-volume Hypothesis**

In contrast is the "low volume" hypothesis, first put forth by Matsui et al. in 1998. This theory is based upon the function of CFTR as a regulator of ion channels, in particular the ENaC channel. In non-disease epithelial cells, CFTR normally inhibits the activity of ENaC. CFTR mutations eliminate this inhibition, upregulating Na⁺ transport, but because there are other available chloride pathways proposed, increased Na⁺ transport drives increased absorption of Cl⁻ and water. Thus, the tonicity of the airway fluids remains normal, but its volume decreases significantly, dehydrating the mucus and leading to obstruction and infection.

The discrepancies in findings have been mainly attributed to variations in properties of cell cultures used in experiments supporting either hypothesis. Nevertheless, it is apparent from all groups that CFTR plays a major role in maintaining the healthy function of epithelial cells which play either an absorptive or a secretory role in related organs.

It has been observed that CF progression in animal models vary in severity, even when CFTR function is completely absent (Gyomorey et al., accepted in *Pediatric Research*). This has led to the theory that there might be alternate epithelial chloride pathways compensating for the absence of CFTR-mediated chloride transport. Indeed, there are other chloride channels known to be present along the apical membrane that may contribute to compensation for decreased CFTR chloride.
secretion, including the Ca\(^{2+}\)-activated chloride channel and the ClC family of voltage-gated chloride channels. Their similar localization has made these chloride channels a possible target for CF treatment.

E. The Family of ClC Chloride Channels

The ClC channels are a family of voltage-sensitive chloride channels, of which there are at least nine known members. The first of the ClC channels (ClC-0) was expression cloned from the electric organ of *Torpedo marmorata* in 1990. It was quickly shown to possess a distinctly different structure from other chloride channels such as CFTR. Subsequently, the remaining known members of the ClC family were cloned, and have been the center of great attention, mainly because of their ubiquitous pattern of expression and regulatory effects on various important cell processes.

i. Functions of ClC Chloride Channels and Disease-causing Defects

The ClC channels are known to be involved in various aspects of cell homeostasis and development, such as volume-regulation, acidification of vesicles in endocytotic pathways, synaptic transmission, maintenance of chloride concentration, and vascular smooth muscle response. See Table 1 for a summary of known ClC channels and their functions. A recently cloned ClC-like channel (*clh-1*) has also been shown to contribute to osmotic regulation of the body morphology in nematodes.
**Table 1:** The known CIC channels, their functions and disease etiology. The human CIC channels (in bold) are grouped into three branches with less than 30% identity between them. Adapted from Jentsch et al., 1999 (reference 34)
ClC channels play such a vital role in cell maintenance, in fact, that defects in several members of the ClC family have been directly correlated with pathological diseases (reviewed in 34). For example, Dent’s disease, a disorder characterized by low-molecular-weight proteinuria, hypercalciuria and renal failure has been mapped to the renal chloride gene ClC-5. The mechanism for proteinuria has been proposed to be due to the role of ClC-5 in providing the electrical shunt necessary for the acidification of vesicles in the endocytotic pathway. Interference with apical membrane transport protein recycling and endocytosis would lead to defective reabsorption of glucose, amino acids, phosphate, uric acid and low-molecular weight proteins in various nephron segments.

RT-PCR has revealed the presence of mRNA for a number of different ClC channels (namely, ClC-2, ClC-3, ClC-5 and ClC-6) in vesicles isolated from the human colon cancer cell line, HT29. However, the relative contribution of each channel to chloride conductance in these cells remains to be determined.

ClC-1: The physiological function and disease-causing defects of this channel have been thoroughly characterized. ClC-1 is activated by depolarization, and has been shown to be essential for the electrical stability of the muscle plasma membrane. ClC-1 is expressed almost exclusively in adult skeletal muscle fibers, except the recent finding by RT-PCR of ClC-1 expression in the outer hair cells of rat cochlea. ClC-1 has been linked to both the dominant and recessive (Thomsen’s) form of the muscular disease, myotonia congenita.

In skeletal muscle, mutations in the channel lead to a partial to total loss of ClC-1 function. In myotonia congenita, the loss of ClC-1 mediated chloride...
conductance impairs the repolarization of action potentials. Nearly all mutations also affect intrinsic channel properties, such as shifting the voltage dependence (described later) of ClC-1 to very positive potentials where the chloride channel can no longer contribute to the repolarization process. As a result, sodium channels recover from their inactivation while the membrane potential is still slightly depolarized. This causes the characteristic train of action potentials seen in myotonia congenita upon a single stimulus, preventing muscle relaxation.

ClC-2: ClC-2 is a broadly expressed member of the ClC family that can be activated by strong hyperpolarization, cell swelling, and acidic extracellular pH (discussed later). ClC-2 has not been implicated in inherited disease, but has been shown to be expressed along the apical membranes of lung epithelia where CFTR is also present. Thus, it may be feasible to develop drugs that activate ClC-2, thereby compensating for the defective CFTR channel. Hence, we were prompted to study possible regulation pathways for the ClC-2 channel to elucidate ways in which ClC-2 activity can be controlled within the cell.

F. Structural and Functional Properties of ClC channels

i. Topology

Most available methods for determining the transmembrane topology of proteins with multiple transmembrane domains have certain disadvantages. Therefore, a combination of glycosylation scanning, protease protection assays, and cysteine scanning mutagenesis has been used in generating a model of ClC proteins.
The hypothesized topology of CIC channels consists of at least 12-13 spans of largely hydrophobic amino acids, most of which are transmembrane domains (43, see figure B). Because there are a number of regions of the protein with intermediate hydrophobicity, the exact number of membrane spanning domains is uncertain.

Detection methods have also determined that the N and C- termini of CIC channels face the intracellular environment. In addition, except for some prokaryotic CICs, all known CIC proteins have two cystathionine beta synthase (CBS) domains at the carboxyl terminus 44.

ii. Ion Selectivity

Channel selectivity for ion permeation is largely similar between members in the family. CIC channels have an anion preference of Cl$^-$ > Br$^-$ > I$^-$, and are proposed to select between ions by binding to several anion-selective binding sites within the channel 38,45. This was demonstrated in human CIC-1 channels by anion mixture voltage-clamp experiments in CLC-1 expressing HEK cells. Fahlke et al. observed that external iodide blocks chloride current over a range of voltages by binding to a site within the ion conduction pathway that possessed a higher affinity for I$^-$ than Cl$^-$ 46.

Indeed, site-directed mutagenesis studies in CIC channels have demonstrated that specific residues and protein regions are vital in anion selectivity (CIC-1 47,48; CIC-0 49,50,51. The rate-limiting step for ion permeation in CIC channels can either be the dissociation of an ion already bound to these selectivity regions, or the association
Figure B: Hypothesized transmembrane topology of CIC chloride channels
rate of the permeating ion to the site. As such, little more is known about the detailed mechanisms of ion selection and anion binding kinetics in CIC channels.

iii. Gating Mechanisms

The voltage-dependent gating mechanisms of CIC channels likely differ greatly from that of other known proteins, such as voltage-dependent Na⁺ and K⁺ channels. CIC channels do not possess the typical S4 voltage-sensing region found in many cationic channels, which gate via physical movement of the positive gating charges in these regions as a result of changes in membrane potential.

There are a number of proposed mechanisms for gating in CIC channels. The probable gating scenario becomes even more complicated, however, when the two gating components observed in many CIC current recordings are taken into consideration. These are the "slow" and "fast" gating components such as those observed and studied in recordings of CIC-0 ⁵²,⁵³ and CIC-1 ⁵⁴ mediated currents.

CIC-0: CIC-0 was one of the first channels in the CIC family to be studied for gating characteristics, mainly because of its relatively large single-channel conductance (about 10 pS) and its rather simple gating relaxation.

In a much-simplified model, the "fast" gating of CIC-0 is independent of any intrinsic voltage sensor and depends on the permeating anion as the gating charge ⁴⁵. In this gating picture, the rate constants of gating are influenced by the presence of chloride ions at the inner end of the pore. Gating becomes voltage-dependent as chloride moves along the electric field in order to reach its binding site. As such, gating of CIC-0 also appears to depend upon chloride concentration ⁴⁵.
Other groups have suggested that more a complicated scenario may be warranted for ClC-0 gating. In subsequent studies, Pusch et al. discovered that their model did not satisfactorily account for gating patterns observed at broader chloride concentrations, and thus proposed that ClC-0 may contain a multi-ion pore that could account for these patterns. Chen et al. (1996) have suggested that chloride binds to an external binding site, and that the movement of this binding site together with the chloride ion in the electric field constitutes the voltage-sensitive step.

In addition to anion-dependent gating, functional studies have also shown the C-terminal portion in ClC-0 channels is vital in channel function. The protein becomes non-functional when the cytoplasmic C-terminal portion is missing, while channel function can be restored when the C-terminal portion is co-expressed. Furthermore, slow-gating properties of the channel seem to rely on structures at the C-terminus of this protein.

ClC-1: In contrast to the model for ClC-0, the voltage sensor in ClC-1 was originally proposed to be a single negatively charged residue near the inner region of the protein. However, later experiments revealed that involvement of this residue in channel gating was minimal, and raised doubts that this residue acted as a specific voltage sensor in ClC-1.

At the same time, the gating of human ClC-1 was proposed by another group to be largely dependent on anion occupation of external and internal binding sites of the protein. Although similar to the proposed gating scenario for ClC-0, this gating model for ClC-1 is more complicated, although it also accounts for the inward-rectifying properties of ClC-1. In the proposed model, the two binding sites are
thought to have different anion dissociation constants ($K_d$) at any given membrane potential.

As the dissociation constant of the external site is much smaller than external chloride concentration, the occupation of the external binding site saturates at positive membrane potentials (the relatively positively charged internal environment electrostatically attracts and holds Cl$^-$ bound to the external site. In contrast, anion binding to the internal site is very unlikely at depolarized voltages. Because of these relative binding affinities, anion currents are saturated at positive potentials.

Conversely, at more negative membrane potentials, the intracellular binding site is proposed to have an increased, voltage-dependent probability of being occupied. Fahlke et al. suggest that occupation of the internal binding site destabilizes the external one due to the presence of electrostatic interactions between the two bound anions. This leads to an increased conductance of ClC-1 channels at more negative potentials.

Single channel recordings of ClC-1 have been documented by Saviane et al. (1999; reference 54). In part, the complexity of ClC-1 gating can be attributed to the fact that the “fast” and “slow” gates cannot be as easily separated as in ClC-0. In addition, the slow gating properties among ClC channels are, in general, less well understood. In ClC-0, several mutations along the protein have been shown to affect slow gating. As mentioned earlier, slow gating in ClC-0 seems to also depend on the C-terminal end of the protein.

ClC-2: ClC-2 opens in response to hyperpolarization, among other stimuli, and is thought to depend on an N-terminal “ball” domain that can be transplanted to
the C-terminus without apparent loss of function. Further studies have revealed a possible binding site, or “receptor” for this gating “ball” along a highly charged intracellular loop. ClC-2 gating properties are reviewed in more detail in following sections.

Furthermore, the type of anion-dependent gating implicated in ClC-0 and ClC-1 is thought to be common among other ClC channels, and has even been recently suggested as the gating mechanism for ClC-2, although this is not well documented.

iv. **Heteromeric Structure of ClC Proteins**

ClC channels are believed to exist and function as dimers, and this theory has been supported indirectly for several members of this chloride channel family, including ClC-0 and ClC-1. Concatamer and wild-type/mutant co-expressions studies have suggested that the double-barrel conductance may be attributed to two separate, yet equal subunits forming one functional protein unit.

Single-channel recordings of these channels have revealed two distinct and equal conductance levels contributing to current recordings of single proteins, suggesting a “double-barreled” structure for these channels. In this model, the channel has two identical pores that can gate independently of each other (proposed to be the ‘fast gate’), in addition to a common ‘slow gate’, which can close both pores together.

Importantly, the properties of the pores formed by each subunit (whether wild-type or mutant) were shown to be independent of whether the second, attached pore is
wild-type or mutant. Each pore appeared to retain its ion selectivity, single-channel conductance and gating properties. In relation to the "fast" and "slow" gating mechanisms in ClC channels, the fast gate is thought to act upon the individual pores within a channel, while the slow gate is involved in gating of both pores together (see figure C).

Figure C: Proposed double-pore gating mechanism of ClC-0 (simplified)

For instance, in the ClC-0 channel, the simplest picture of gating consists of a 'fast' gating which acts on single pores of the double-barreled channel. This gating is independent of any intrinsic voltage sensor and depends on the permeating anion as the gating charge. The slow gate is involved in modulating the conductance of
both pores together, and needs to be open in order for chloride current to pass through the channel through either or both pores. The mechanisms of slow gating are less clear, although it is also thought that it may also be dependent on chloride concentration. *(see next sub-section for ClC-2 gating).*

G. ClC-2: The Molecular Profile of an Ubiquitous Protein

i. *Localization and Function*

The base sequence of ClC-2 predicts a protein composed of approximately 907 amino acids with a calculated molecular mass of around 97 kD \(^{65,66}\) although ClC-2 has been reported to have a lower molecular weight in other cell lines, possibly due to varying degrees of glycosylation \(^{67}\).

Of all the members of the ClC family, the ClC-2 chloride channel is most ubiquitously expressed, having been shown to be widely expressed in both epithelial and non-epithelial cells \(^{68}\). Functionally, ClC-2 has been linked mainly to cell-volume regulation \(^{25,26,69}\). Both ClC-2 mRNA and protein have also been shown to be expressed at similar levels to those of the chloride channel defective in cystic fibrosis (CFTR) in intestinal epithelial \(^{70,71,72}\), as well as airway epithelial cells \(^{73}\).

Immunocytochemistry has revealed that one of the areas in which ClC-2 is heavily expressed is along the apical membranes of respiratory epithelial cells \(^{132}\), the very site where CFTR is also found. Thus, ClC-2 is a prime target for therapy in cystic fibrosis, as activation of this channel could provide an alternative pathway of chloride secretion defective in that disease.
ii. **Structure and Topology**

Topologically, as in other CIC channels, CIC-2 has been predicted to have up to 13 hydrophobic regions, most of which may be transmembrane segments. At least 2 of these hydrophobic regions may not be embedded in the plasma membrane \(^{43}\), although this is still mostly speculation.

CIC-2 is known to be a type I protein, with both amino and carboxy-termini facing intracellularly. According to its primary sequence of amino acids, there are a number of significant, reactive domains along the intracellular face of the protein. However, the precise protein structure of CIC-2 is heavily species dependent and varies greatly between cell lines.

The molecular make-up of human cloned CIC-2 includes two CBS (cystathionine beta synthase) domains and three putative protein kinase A phosphorylation sites. There are also at least four putative protein kinase C (PKC – a family of serine/threonine kinases) phosphorylation sites, two along the N-terminus and two near the C-terminus (see figure D).

In the CIC-2 channel, the N-terminus is thought to be involved in channel gating, and is proposed to act as an inactivation domain \(^{60}\), the deletion of which leads to a constitutive activation with a complete loss of swelling and voltage-sensitivity \(^{60}\). Even when transplanted to the carboxy-terminus of the protein, this domain retained its effects on gating, indirectly suggesting a “ball-and-chain”-type gating mechanism \(^{60}\). In this model, binding of an intracellular ‘ball’ (inactivation) domain to a ‘receptor’ on the channel backbone leads to its inactivation.
*Figure D*: Putative membrane topology of human CIC-2 chloride channel
Further studies by Jordt and Jentsch (1997), involving systematic mutagenesis of residues in the highly charged loop between D7 and D8, abolished this type of gating. This suggests that this region of the ClC-2 protein may be involved as some sort of receptor for the N-terminal portion in this ball-and-chain mechanism. Mutations in this cytoplasmic loop led to a constitutively open channel.

iii. Channel Gating Properties

As mentioned earlier, ClC-2 is ubiquitously expressed and remains in its closed state at resting membrane potentials, but can be activated slowly and time-dependently by strong hyperpolarization more negative than −90mV. ClC-2 can also be activated by cell swelling, and this appears to account for the volume regulatory effects of the channel in cells. Channel gating is also affected by extracellular pH: low pH increases the activity of ClC-2.

In all forms of activation, there appears to be at least two time components characteristic of ClC-2 mediated currents: a fast and a slow component. As seen in the ClC-2 raw current traces in this study, (see Results sections) the fast gating component is detected as the near instantaneous rise in inward currents at hyperpolarized potentials. This is followed by the slow gating, an obvious gradual increase in the inward currents that persists throughout the duration of the voltage step.

This supports the suggestion that there are two gating mechanisms, a slow and fast gate, both of which need to be open in order for the channel to conduct current. There is, however, no conclusive evidence describing the mechanisms for these
gating processes. ClC-2 channels possess inactivation-gating characteristics similar to those of ClC-0 (described previously).

Recent studies have suggested that gating in ClC-2 channels may also be dependent on external anion concentration \(^{61}\). The proposed model suggests that there may be a 'high-affinity' anion binding site that is more readily accessible from the extracellular side of the protein. Changing the halide contents of the extracellular solution, Pusch et al. were able to show that ClC-2 gating seems to depend on the concentration of extracellular chloride. In fact, their experiments suggested that extracellular chloride closes ClC-2 channels. The proper interpretation of their findings, however, is complicated. In addition, their data is not sufficiently convincing to prove the necessary involvement of permeating anions in ClC-2 gating.

iv. Regulation of ClC-2 Activity by Protein Kinase C

As already mentioned, ClC-2 has been localized to the apical surface of airway and intestinal epithelial cells, among many other cells. Because of this convenient location, ClC-2 has been highly favored to be a candidate to act as an alternate chloride secretion pathway in these cells, particularly in people afflicted with cystic fibrosis.

Not much is known about regulation of ClC-2 activity. The channel opens in response to hyperpolarization \(^{68}\), and this can be modulated by cell swelling \(^{60}\) or by acidic extracellular pH \(^{43,65}\). In addition, ClC-2 activity has been shown to be down-regulated by protein kinase C activation \(^{74}\), although the mechanisms for this regulation are unknown. The purpose of this study is to elucidate the role of the PKC
pathway in regulation of the CIC-2 channel, as a means of modulating the activity of such a vital chloride channel.

Understanding regulation of CIC-2 by PKC both provides another clue in the development of combative technology against CF, and further helps clarify the basic science of membrane protein regulation.

H. The Protein Kinase C Phosphorylation Pathway

Protein kinase C (PKC) is a family of related serine-threonine kinases. It is a key player in the cellular responses mediated by the ubiquitous lipid secondary messenger diacylglycerol (DAG). Since membrane receptors and channels coupled to phospholipase C (PLC) cause a transient elevation in DAG levels, the PKC family members are key enzymes in the signaling mechanisms linked to these membrane proteins (figure E).

![Diagram of PKC-activating receptor pathway. Phorbol esters mimic DAG in the PKC phosphorylation pathway, acting to up-regulate protein kinase C.](image)
Initial purification experiments and subsequent molecular cloning studies revealed that PKC is a family of lipid-regulated serine-threonine kinases that phosphorylates a variety of cellular proteins and plays an essential role in signal transduction mechanisms. To date, at least 11 different PKC isoforms have been identified. Each PKC isozyme consists of a single polypeptide chain having two structurally well-defined domains: the amino-terminal regulatory domain and the carboxyl-terminal catalytic domain. The carboxyl-terminal region is the active kinase domain and includes motifs involved in ATP and substrate binding. The regulatory region possesses the motifs involved in the binding of the phospholipid cofactors and Ca\(^{2+}\) ions and participates in protein-protein interactions that regulate PKC activity and localization. The regulatory and catalytic domains are connected by a “hinge region” that is highly sensitive to proteolytic cleavage by cellular proteases (see 77 and 78 for review – figure F).

The regulatory region includes a ‘C1’ domain, which contains an autoinhibitory domain, or pseudosubstrate, that binds to the PKC active site in the catalytic domain and keeps the enzyme in an inactive state in the absence of cofactors and activators. Certain isoforms of PKC are regulated by Ca\(^{2+}\) ions, diacylglycerol and phorbol esters. Phorbol esters essentially mimic the regulatory effects of DAG by binding onto specific sites on the regulatory domain of PKC kinases. One important site for phorbol esters and DAG is a cysteine-rich domain in the C1 region of the regulatory portion of PKC. The accepted model of activation of PKC is that on binding of DAG or phorbol ester in the presence of phospholipid cofactors,
**Figure F:** Structure of various PKC isozymes. PKC kinases are divided into three subclasses according to their regulatory properties. 'Conventional' PKCs (cPKC) can be activated by Ca\(^{2+}\) and/or by DAG and phorbol esters. 'Novel' PKCs (nPKC) are similar to cPKCs, but are insensitive to Ca\(^{2+}\). 'Atypical' PKCs (aPKC) are unresponsive to all stimulus. PS, pseudosubstrate; CR, cysteine-rich domain; C#, conserved regions; V#, variable regions. (adapted from Ron & Kazanietz, 1999)
a conformational change in PKC results in the removal of the pseudosubstrate from its binding site and in the activation of the enzyme.

It is proposed that ligand binding of phorbol ester or DAG to PKC has another important function. Ligand binding might also serve to 'cap' a hydrophilic site at the top of the structure forming a 'hydrophobic' region that promotes insertion of the domain into the lipid bilayer, enhancing the activity of PKC.

In addition to binding to lipids, PKC can also interact with proteins via protein-protein interactions. These interactions play an important role in the localization and function of PKC isozymes. Additionally, proteins associated with PKC in such a manner serve many functions, which include localizing inactive or active PKC isozymes to specific intracellular sites or serving as substrates, shuttling proteins, PKC activators or PKC inhibitors (see 77 for review).

Once activated, PKC facilitates a hydrolysis reaction in which the phosphate in the $\gamma$ position of ATP is cleaved off and covalently bound to the oxidized $-\mathrm{OH}$ side chain of the serine or threonine residue. Phosphorylation by PKC can exert a number of effects, such as inducing conformational change in the target protein that leads to functional change, or modifying interactions between the phosphorylated protein and other associated proteins.

I. Regulation of CIC Chloride Channels by PKC Phosphorylation

i. Regulation of activity via direct channel phosphorylation

Regulation of membrane proteins by PKC phosphorylation may occur via one or a combination of several pathways. Firstly, the phosphorylation status of the
protein itself may be regulated, inducing a direct change in the protein properties (Figure 2A). For instance, phosphorylation or de-phosphorylation of serine/threonine residues on an ion channel might alter its single channel conductance or voltage-dependence. There are relatively few reports documenting this type of channel regulation, however.

One good example of direct modulation of channel conductance by phosphorylation is in the chloride channel, CFTR. As mentioned at the beginning of this introduction, the more extensive the phosphorylation of the CFTR R-domain, the greater the channel open probability. There have also been reports that PKC phosphorylation of CFTR may be a prerequisite for channel activation by the protein kinase A pathway.

Another example of direct regulation by PKC phosphorylation has been shown in the study of human CIC-1 chloride channels. Exposure of the channel to a phorbol ester resulted in diminished whole-cell currents, but more importantly, apparent changes in ion transfer and gating processes. Rosenbohm et al. suggest that an important PKC phosphorylation site may be located at the cytoplasmic vestibule face of the pore.

ii. Regulation of channel activity via phosphorylation of accessory proteins

Secondly, the PKC pathway may affect the phosphorylation status of proteins associated with the studied membrane protein (Figure 2B). Such 'accessory' proteins may include cytoskeletal and structural proteins such as actin or dynein. Since CIC-2
is known to be activated by cell swelling, it would not be surprising if CIC-2 activity was intimately regulated by nearby proteins involved in a membrane-stretch response.

**iii. Regulation of channel activity via membrane trafficking**

Thirdly, changes in cell PKC phosphorylation status may regulate other intracellular proteins, such as those involved in vesicle trafficking to and from the plasma membrane (Figure 2C). Thus, PKC would directly affect the number of ion channels (or other proteins) being expressed on the cell surface. This type of regulation by PKC has been shown for a number of different proteins, such as the type II Na⁺-phosphate cotransporter[^84], and the renal Na⁺/dicarboxylate cotransporter, NaDC-1[^85], both proteins expressed and studied in *Xenopus laevis* oocytes.

**iv. Establishing the involvement and role of PKC in CIC-2 regulation**

As already mentioned, it has been shown that exposure of CIC-2 expressing cells to a phorbol ester causes down-regulation of CIC-2 mediated currents[^74]. Since phorbol esters are known activators of the PKC pathway, this finding highly implicates the involvement of PKC in CIC-2 regulation. Since CIC-2 is known to possess at least four putative PKC phosphorylation sites, it might be possible that direct protein phosphorylation is responsible for the changes in CIC-2 mediated currents. The general goal of this study was to establish the involvement of PKC in CIC-2 regulation, and to elucidate to mechanism(s) via which PKC phosphorylation modulates CIC-2 activity.
CHAPTER II: MATERIALS AND METHODOLOGY

A. Expression and Functional Study of CiC-2 in *Xenopus laevis* Oocytes

*Xenopus laevis* toads were acquired from *Xenopus* One (Michigan, USA). Prior to oocyte extraction, animals were anaesthetized by immersion in 0.17% tricaine solution (3-amino acid ethyl ester methanesulfonate salt, Sigma) for approximately 15 – 20 minutes. Oocytes were then surgically removed from the abdominal region of the toads, manually dissected, and incubated in collagenase (2 mg/ml; Sigma) for 30 minutes to further separate the cells. Stage VI mature oocytes were manually selected out for micro-injection with cRNA or ddH2O, as control.

Oocytes were then placed in a de-folliculating solution (0.1M potassium phosphate, pH 6.5) for 10 minutes, washed with standard oocyte solution (SOS – 100mM NaCl, 2mM KCl, 1.8mM CaCl2, 1mM MgCl2, and 5mM HEPES, pH 7.6) and stored in pyruvate (2.5mM) and gentamycin (50mg/L) supplemented SOS (sSOS). No manual de-folliculation was involved. The vegetal (non-nuclear) poles of oocytes were injected with 50nl of de-ionized distilled water (ddH2O) as control or 50nl ddH2O containing cRNA encoding wild-type CiC-2 (approximately 1.56ng/ml).

During initial studies, non-injected oocytes were used to assess the presence of leak currents. CiC-2 channel activity was assessed between 18 – 32 hours after cRNA injection using two-electrode voltage clamp technique. CiC-2 was activated using a voltage step protocol outlined in the Results section.

The stock solution of the myristolated PKC inhibitor peptide used, 19-27, was dissolved and stored in ddH2O. Stock solutions for both the active phorbol 12,13 di-butyrate (PDBu) and the inactive 4α-phorbol were first dissolved in DMSO, then
Further diluted and stored in ddH₂O. Each drug was further diluted into SOS to the desired concentration, and perfused into the oocyte chamber for subsequent experiments. All drugs were purchased from Calbiochem (California, USA).

Whole-cell current measurements were acquired using the Geneclamp 500 amplifier (Axon Instruments, Inc.) and the Digidata 1300a interface (Axon Instruments, Inc.). The software pClamp 8.0 (Axon instruments, Inc.) was used in all data acquisition and recording. All electrodes used were pulled to have resistances between 1 and 5 MΩ, and were filled with a 3M KCl solution to ensure a complete circuit for accurate current measurement. The voltage-clamp setup was placed in an encompassing wire-mesh cage to minimize noise and interference.

The volume of the oocyte perfusion chamber was approximately 0.5ml, and perfusion was maintained at approximately 1 ml/min in all experiments. With the exception of the endocytosis experiments, all electrophysiological studies in Xenopus oocytes were carried out at room temperature (23°C). In the endocytosis experiments, the temperature of the perfusion buffer was maintained at approximately between 1°C and 4°C by running a coiled section of the tubing through an icebox. Several trial runs were performed to ensure the constant temperature of the buffer in the oocyte bath.

i. Capacitance Measurements

CIC-2 injected oocytes were voltage-clamped and subjected to short potential steps of 5 mV (V) from a holding potential of -40mV, each step lasting several milliseconds to initiate capacitative currents. Pclamp data acquisition software (version 8.0 from Axon Instruments, Inc.) was utilized to plot the resulting currents,
and to calculate the area underneath the current curves to determine the absolute trans-membrane charge movement, in coulombs (Q). The software then used the formula relationship $Q = C \% V$ to compute the capacitance (C), which was plotted and recorded over time.

**B. Expression of CIC-2 in Sf9 cells using the baculovirus expression system**

Expression of CIC-2 protein in Sf9 cells was done by Yanchun Wang, a research technician in our lab. Clone B12-2, encoding the full length cDNA of rat CIC-2 was received from T. Jentsch. The open reading frame (ORF) was amplified using the oligonucleotides 5'- GCTAGATCAGATGGCGGCCGCAAC - 3' and 5' – GGAATTCACTGGCACTTGTCATCA - 3', which overlap the start and stop codon respectively, of rat CIC-2 and contain BamHI and EcoRI sites respectively, for cloning purposes.

The resulting amplified fragment was ligated first into pBlueBacHisB, a baculoviral vector which incorporates an N-terminal, polyhistidine tag (Invitrogen, California, USA). The first 50 amino acids of the expected protein are as follow: [MRGSHHHHHHGMASTMGQQMGRDLYDDDKDPSSRSEMMAATAAAATVA] (CIC-2 protein is indicated in bold). Finally the entire tagged CIC-2 ORF introduced either into the pBlueBac4 vector for high-level expression in the baculoviral system, or into a high efficiency oocyte expression vector (described by Lorenz et al.).

The bulk of the ORF was then replaced with unamplified DNA from the original B12-2 clone, using the restriction sites NotI and BspEI. Regions not replaced
by this procedure were sequenced to ensure that no errors were introduced during amplification. The resulting clones encode an unambiguous ORF representing rat ClC-2 with an N'-terminus hexa-histidine tag driven from either the baculovirus polyhedrin promoter for Sf9 cell expression or Xenopus beta-globin promoter for Xenopus oocyte expression studies respectively.

C. In vitro PKC Phosphorylation of Purified ClC-2

ClC-2 protein was purified and reconstituted by Dr. Mohabir Ramjeesingh, a research associate in our lab (methods in press, Biochemistry Journal). Briefly, Sf9 insect cells expressing recombinant ClC-2 (see section B) were harvested from 1 litre of medium by centrifugation at 2000g at 4°C and the cell pellet washed once in PBS. The cells were lysed in a French Press (Spectronic Inst. NY, USA), the nuclei and cell debris pellet and the supernatant was centrifuged at 100,000g for 90 minutes to pellet a crude membrane preparation. Peripheral membrane proteins were extracted from this membrane pellet using 25 volumes of ice-cold 10mM sodium hydroxide and 0.5mM EDTA and the stripped membranes pelleted by centrifugation at 100,000g for 2 hours.

Integral membrane proteins were solubilized using a detergent solution 8% pentadecafluorooctanoic acid (PFO, Oakwood Products Inc., SC, USA) in 25 mM phosphate, pH 8.0 and stirred with a magnetic stirrer overnight at room temperature. The solubilized protein sample was then applied to a nickel column, washed at pH 8.0, and eluted with a pH gradient. The proteins were then reconstituted in a micelle and liposome mixture, and dialyzed to remove the detergent.
The purified CIC-2 was incubated with the various reagents in 1.5 ml Eppendorf tubes for each study. 20 pM of protein substrate was added to 0.05 mU PKC (Boehringer Mannheim, Germany) and PKA reaction buffer (20 mM Tris-HCl, pH 7.5; 10 mM MgCl₂, 500 μM CaCl₂, 0.25% BSA, 100 μg/mL phosphatidylserine (PS) and 20 μg/mL diacylglycerol (DAG)) for a final volume of 200 μl. The Diacyl glycerol and phosphatidyl serine were included to mimic the lipid environment of plasma membranes. Several control experiments were investigated, including 1) absence of CIC-2 protein, and 3) absence of the 50mU PKC.

The dissolved lipids were then mixed in chloroform, which was then evaporated with N₂ gas. The residue was dissolved in 10 mM CHAPS and 10 mM Tris-HCl, pH 7.5. The phosphorylation reaction was started by the addition of 3 μCi of γ - P₃₂ ATP and 100 μM cold ATP. The mixture was incubated at 30°C. After 3 minutes, the reaction was stopped by pipetting 50 μl of Loading Sample Buffer (LSB) into the reaction tube, and pipetting up and down several times to mix.

Samples were then placed on ice and then briefly spun in a low-speed centrifuge for several seconds. 50 μl of each sample was run on an 8% SDS gel, and protein bands were detected by direct exposure to film.

D. Expression of CIC-2 protein in sf9 insect cells

Sf9 cells derived from ovaries of fall army worm (Smith & Cherry, 1983), were transfected with CIC-2 protein by Yanchun Wang, a technician in our lab (see section B). In both immuno-labeling and cell-surface biotinylation studies, un-transfected cells were used as control. Transfected and control cells were incubated at 37°C on 35 mm circular glass cover slips, and 60 mm circular petri dishes in the
immuno-labeling and biotinylation studies, respectively. In each case, cells were incubated for 24 – 48 hours to enable adequate protein expression.

i. Immunolocalization of CIC-2 and Confocal Microscopy

Cells on the glass cover slips were rinsed several times with phosphate buffer solution (PBS), and then treated in PBS containing PDBu, 19-27, or 4α-phorbol for 35 – 45 minutes. Two sets of control cells were run concurrently: un-transfected and CIC-2 transfected cells incubated in PBS. The cover slips were then washed several times again with PBS, followed by incubation in paraformaldehyde AM (4% in ddH₂O) for 10 minutes to fix the cells. Sf9 cells were then permeabilized with 0.5% Triton diluted into PBS for 30 minutes.

Cells were washed several times with 0.05% Triton in TBS (solution A), blocked with 5% goat serum in solution A for 30 minutes, and then incubated with an affinity-purified polyclonal antibody against the N-terminus of CIC-2 (using a dilution of 1/100) for 2 ½ - 3 hours. Following a thorough wash with solution A, samples were incubated with a goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC – 0.02mg/ml; Molecular Probes, Inc., Eugene, OR, US) for 1 hour.

The samples were then rinsed thoroughly with solution A, then with TBS to minimize non-specific signal. Two control experiments in CIC-2 transfected cells were included in these studies. A competition control was run, in which a 100-fold excess of CIC-2 antigenic fusion peptide was incubated with the CIC-2 primary
antibody for 2 ½ - 3 hours at room temperature to confirm specificity of ClC-2 protein labeling.

The second control involved omission of the ClC-2 primary antibody to detect non-specific binding of the secondary antibody. Cover slips were mounted onto microscope slides with Dako mounting medium (California, USA) and left overnight to allow the cover slips to be steadily mounted.

For confocal microscopy, cells were viewed with a 100x objective on a Zeiss Axiovert 100M, with a Zeiss LSM 510 confocal system using the Zeiss LSM imaging program (Zeiss, Jena, Germany). In order to ensure an accurate representation of protein distribution, cross-sectional pictures were taken at or near the mid-line section of each of the cells.

ii. Cell-surface Biotinylation and Immunoprecipitation of ClC-2

Transfected and control sf9 cells were transferred from the 60 mm petri dishes to 1.5ml Ependorff tubes after the 24 – 48 hour incubation period. The cells were rinsed several times with PBS, spun down, and then were treated with PDBu, 4α-inactive phorbol, or 19-27 in PBS for 45 minutes. The samples were then washed in PBS, and incubated PBS containing 2.5mM sulfo-NHS-LC-Biotin (Pierce) for 20 minutes in order to label protein at the cell surface.

The biotin was washed out, and cells were then homogenized manually in a cocktail of protease inhibitors in PBS: aprotin (10 g/ml), leupeptin (10 g/ml), benzamidine (1mM) and E64 (10μM). The crude membrane preparation was pelleted
by centrifugation at 20,000 g for 15 minutes at 4°C and solublized by nutation at 4°C in 2% Triton-X in PBS.

The solubilized membrane proteins were then spun down at 50,000 g for 2 hours at 4°C, and the supernatant retained for immunoprecipititation of ClC-2 using the same polyclonal antibody as employed in the immunofluorescence studies. A pre-clearing step was done by adding approximately 40μl of protein G sepharose gel beads (Sigma) to the supernatant, and nutating at 4°C for 30 minutes. This step was to remove any proteins that were non-specifically bound by the sepharose beads.

The beads were removed by low-speed centrifugation, and 50μg of an affinity-purified polyclonal antibody against the amino-terminus of ClC-2 was added for overnight incubation at 4°C. Antibody complexes were then precipitated by interaction with protein G sepharose beads (nutated for 2 hours at room temperature) and low speed centrifugation at 1,000 g. After three washes with buffer containing (mM): NaHCO₃ (100) and NaCl (200), immunoprecipitated protein complexes were solubilized in a SDS sample buffer containing 10 mM mercaptoethanol. The mixture incubated in a 40°C water bath for approximately 15 minutes to resolublize the proteins.

The quantity of immunoprecipitated protein was determined by Lowry assay and then 10μg of protein analyzed by SDS-PAGE (8%) and silver stained or transferred on to nitrocellulose paper. Silver staining confirmed equal protein loading in each lane that was run. Extravidin-peroxidase (1.25 g/ml – Sigma) was used to detect biotinylated proteins (see figure G).
**Figure G: Cell-surface Biotinylation Protocol**

1. **CIC-2 transfected Sf9** + drug (45 minutes)
2. + 2.5mM LC-Biotin in PBS (20-30 minutes)
3. Membrane Homogenization
4. **PBS + protease inhibitors**
5. **Nutate @ 4°C (1/2 - 1 hour)**
6. **Membrane pellet in PBS + 2% Triton-X**

**Centrifugation Steps**

**Nutate @ 4°C overnight**

**Protein G beads (binds CIC-2 1° Ab)**

1) **+ Protein G beads**

2) **nutate for 2 hours**

3) **spin & remove supernatant**

**Membrane pellet in PBS**

**Incubate (40°C) - 15 min**

**Resuspend beads in fresh loading buffer (dye/m mercaptoethanol/PBS)**

**Protein Assay**

**Run Western Blot**

**Probe with Avidin**

To detect biotinylated proteins
CHAPTER III: RESULTS

A. Phorbol Ester PDBu Down-regulates ClC-2 Activity in Xenopus Oocyte

ClC-2 expressing Xenopus oocytes were manipulated using two-electrode voltage clamp techniques to observe the hyperpolarization-dependent activity of whole-cell ClC-2 mediated chloride currents. The same protocol was used in all of the whole-cell current measurements: oocytes potentials were held at -30 mV for several seconds, then stepped to a desired potential (V2) for a 12 second duration, and returned to the -30 mV holding potential. V2 varied between -140 and +20 mV in increments of 40 mV. The cycle was repeated until all stepping potentials (V2) had been completed.

ClC-2 raw currents were recorded before and 25 minutes after addition of 0.5 \( \mu \)M phorbol 12,13 di-butyrate (PDBu) phorbol ester to the perfusion chamber (Figure 1A). In each of the raw tracings, the dotted line represents the zero-current baseline. In figure 1A, the lowest trace corresponds to the -140 mV stepping potential, while the highest trace to the +20 mV potential. No similar pattern of current was seen in non-injected and ddH2O injected oocytes (data not shown).

Several characteristic features of the ClC-2 current can be observed from these raw currents, e.g. the pattern of inward rectification, and time-dependent activation with hyperpolarization. As seen from the whole cell recordings, ClC-2 mediated chloride currents decrease significantly (to approximately 30-55% of pre-drug magnitude – \( p < 0.05 \)) after 25 minutes exposure of the cell to 0.5 \( \mu \)M phorbol ester.

As the stock solution of PDBu was dissolved in DMSO and then diluted in
Figure 1A - CIC-2 mediated currents in *Xenopus* oocytes. Typical raw CIC-2 currents, before and after phorbol treatment. 1B - Averaged I-V plot of CIC-2 currents before (squares) and 25 min after (circles) 0.5µM PDBu treatment. Oocytes were held at -30 mV, then stepped to desired potentials (V2) for 12 seconds, and returned to -30 mV. V2 varied between -140mV and +20mV in 40mV increments. **Solid:** CIC-2 injected oocytes (n=4). **Open:** ddH₂O injected oocytes (n=4).
SOS, control experiments were performed adding a 1:100 dilution of pure DMSO in SOS to the oocyte perfusion chamber to account for any effects of DMSO on channel activity. No change in whole-cell current was observed under these conditions (data not shown).

The voltage-current relationships of the ClC-2 currents in oocytes prior to (square icons), and after 25 minutes of PDBu treatment (n = 4; circle icons) are summarized in figure 1B. The solid icons represent ClC-2 injected oocytes, while the hollow ones represent water injected cells. In this I-V plot, the inward rectification of anionic currents typical of ClC-2 can be clearly seen. It is important to note that decrease in current magnitude, as a result of phorbol treatment, occurs throughout all stepping potentials, not limited to the more hyperpolarized, channel-activating potentials. Furthermore, the reversal potential (E rev) for the ClC-2 mediated currents, as indicated from the graph, is -40.4 ± 3.14 mV, and did not change after exposure to PDBu.

Figure 1C represents the normalized bar graph of steady-state currents at the -140mV stepping potential. The conditions included are: control, 0.05 μM PDBu (n = 6), 0.5 μM PDBu (n = 4), 2 μM PDBu (n = 4), and 0.5 μM 4α-phorbol (an inactive phorbol analogue, n = 6) treated oocytes. In addition to this, the same experiment was performed on water-injected oocytes in the presence of 0.5 μM PDBu, and the -140 mV currents recorded and normalized (figure 1D).

The down-regulating effect of the phorbol ester on ClC-2 mediated current appears to be dose-dependent. After 25 minutes, there was a 24.3 ± 12.94 %, 49.87 ± 11.21 % and 69.5 ± 5.73 % reduction in steady state hyperpolarization-induced
25 minutes of PDBu treatment. Phorbol ester exhibits specificity for CIC-2 over leak currents.

- Normalized currents at the -140 mV step-up potential in CIC-2 (n=4) and water (n=4) injected oocytes after omission of PDBu is dose-dependent. Inactive phorbol causes no significant change in chloride currents (P > 0.05).

**Figure 1C** - Normalized averaged inward CIC-2 mediated currents after 25 minutes of drug treatment. Each
currents in 0.05 μM, 0.5 μM and 2 μM PDBu treated oocytes, respectively.

For all three concentrations of PDBu, these current changes were considered significant (p values < 0.05). On the contrary, there was no significant change in hyperpolarization-induced currents in either water-injected oocytes exposed to 0.5 μM PDBu, nor ClC-2 expressing oocytes exposed to 0.5 μM inactive phorbol (p values both > 0.05). Thus, the phorbol appears to exhibit some sort of specificity to down-regulating ClC-2, as it does not affect the magnitude of leak currents in water-injected oocytes to the same extent.

In further experiments, higher phorbol concentrations seemed to affect the integrity of the cell membrane, preventing proper clamping and current recordings. As such, it is known that at high concentrations, the effects of phorbol esters become less specific, affecting other phosphorylation pathways (e.g. protein kinase A) as well. In some studies, PKC has also been shown to regulate membrane proteins cooperatively with tyrosine kinase. Introduction of a similar concentration of inactive phorbol did not cause a significant decrease in normalized whole cell currents (P > 0.05).

This suggests that the down-regulatory effects the phorbol ester PDBu are via the PKC pathway, as treatment with an equivalent inactive phorbol analogue does not exert the same effects on whole cell current. The effect of the phorbol also appears to display preferential regulation of ClC-2, as it does not cause the same significant change in non-ClC-2 oocytes.
B. Myrisolated PKC Inhibitor 19-27 upregulates CIC-2 Currents

Figure 2A shows equivalent raw CIC-2 current traces before and 25 minutes after perfusion of 4 μM 19-27 myristolated PKC inhibitor peptide in SOS buffer through the oocyte perfusion chamber. Interestingly, the inhibitor 19-27 appears to have an opposite effect on CIC-2 currents compared to the PKC activator PDBu, increasing the current magnitude at all stepping potentials, but more noticeably at the hyperpolarized voltages.

The averaged voltage-current relationship of CIC-2 currents before (square icon) and 25 minutes after 4 μM 19-27 PKC inhibitor treatment (n = 8; circle icon) are plotted in figure 2B. As can be seen, properties such as channel rectification are maintained; only the magnitude of the currents increase at each voltage step. As in the phorbol ester experiments, there is no noticeable difference in reversal potential \( (E_{rev}) \) of the channel after exposure to the PKC inhibitor drug, indicating maintenance of ion selectivity (see Discussion).

As in figure 1C, the normalized steady-state currents at -140 mV for control, 2 μM 19-27 treated (n = 6), 4 μM 19-27 (n = 8) and 6 μM 19-27 (n = 6) oocytes are averaged and plotted in figure 2C. The up-regulation by the inhibitor appears to be dose-dependent. Oocyte membranes were found to lose their integrity easily at concentrations of 19-27 higher than 6-8 μM, and usually were not able to survive for an entire experiment. These cells were not included in our data analysis. After 25 minutes of drug perfusion, there was a 29.74 ± 11.02 %, 126.79 ± 48.83 % and 198.33 ± 53.12 % increase in hyperpolarization-induced current in 2 μM, 4 μM and 6
Figure 2A - Typical CIC-2 mediated currents before and 25 minutes after 19-27 treatment. 2B - Averaged V-I plots before and 25 minutes after 19-27 treatment (n=8) in CIC-2 injected oocytes. 2C - Normalized CIC-2 currents after 25 minutes of 19-27 treatment. Effect of 19-27 is dose-dependent.
$\mu$M 19-27 treated oocytes, respectively. These represented a significant change in currents for each inhibitor concentration ($p < 0.05$).

C. PDBu exerts its effects via the PKC Phosphorylation Pathway

Since we observed that the phorbol ester PDBu down-regulates CIC-2 mediated currents, we were prompted to design experiments to confirm that PDBu was indeed exerting its effects via the PKC phosphorylation pathway. It was possible that PDBu had some sort of a direct effect on the channel, perhaps blocking the pore, or directly affecting its gating mechanisms.

To confirm that the PDBu effect is mediated by PKC, we assessed its sensitivity to PKC inhibitors. The PKC inhibitor peptide 19-27 we used is known to irreversibly bind the PKC enzyme at its active site (reference 90 – see Discussion section). Thus, pre-treatment of oocytes with the membrane-permeant inhibitor would be expected to block the down-regulating effects of phorbol ester on CIC-2 currents if PDBu was exerting its effects by up-regulation of the PKC pathway.

CIC-2 injected oocytes were pre-treated with 2 $\mu$M 19-27 and voltage-clamp protocols previously described were performed once every 5 minutes ($n = 5$). After 30 minutes, the oocyte chamber was perfused with 0.5 $\mu$M PDBu in SOS containing 2 $\mu$M 19-27, and current recordings were continued further for 25 minutes. The averaged and normalized currents at $-140\text{mV}$ are plotted in figure 3A.

The normalized steady-state currents at $-140\text{mV}$ increased as expected when the oocytes were exposed to the PKC inhibitor, but further introduction of PDBu to the bath in the presence of 19-27 did not result in significant change in CIC-2 currents
Figure 3A - Normalized ClC-2 currents at -140mV in oocytes. **Solid squares:** oocytes were pre-treated with 19-27, and then treated with PDBu (n=5). **Open triangles:** control study in non-pretreated cells (n=4). The same protocol was used as in Figure 1.

Figure 3B - Normalized currents 25 minutes after PDBu treatment in 19-27 pretreated (n=5) and non-pretreated (n=4) cells. 19-27 pretreatment inhibits the down-regulatory effects of phorbol.
even after 25 minutes ($p > 0.05$).

The lower dotted curve represents normalized current measurements of non-19-27 pretreated ClC-2 expressing oocytes after perfusion of 0.5 $\mu$M PDBu alone ($n = 4$). Note that there is a significant reduction in normalized current magnitudes over time ($P < 0.05$), and the effects of phorbol can be seen almost immediately.

Figure 3B is a bar graph of the normalized ClC-2 currents 25 minutes after exposure of cells to 0.5$\mu$M PDBu in 19-27 pre-treated and non-pre-treated cells. As seen in figure 3A, there is a significant difference in the down-regulatory effects of phorbol on ClC-2 in the presence of the PKC inhibitor.

These results strongly suggest that down-regulation of ClC-2 activity by phorbol ester is mediated by the PKC phosphorylation pathway, because its activity can be sufficiently hindered in the presence of an irreversible PKC substrate analog. In the presence of both drugs, the 19-27 peptide likely binds PKC in a covalent manner via a crucial cysteine residue found in the active site of the PKC enzyme.

Phorbol esters are known to mimic diacyl glycerol (DAG) in the PKC pathway, and induce an increase in the concentration of active PKC enzymes in the cell. It is highly plausible that the amount of active PKC produced by PDBu will eventually saturate the available inhibitor peptide, and the excess PKC then begins to exert its effects on whole-cell current. However, oocytes rarely lasted long enough to observe the long-term effects of the PKC inhibitor.
D. Purified ClC-2 can be Phosphorylated by PKC in vitro

Our previous experiments have shown that whole-cell ClC-2-mediated currents can be modulated via manipulation the PKC phosphorylation pathway. The next logical step was to determine 1) whether the ClC-2 protein itself is capable of being phosphorylated by PKC, 2) whether direct phosphorylation of the protein plays any part in its regulation, and 3) whether ClC-2 activity was being modulated by other pathways of regulation involving PKC phosphorylation.

In order to address the first question, ClC-2 protein was affinity-purified from sf9 cells and reconstituted in phospholipid liposomes (see methods section). A series of in vitro protein phosphorylation studies were performed using radiolabelled γ-P\textsuperscript{32}ATP to label phosphorylated protein. Two proteins, myelin basic protein and histone IIIa, were used as positive phosphorylation controls, as they are known to possess a significant number of PKC phosphorylation consensus sites \(93, 94, 111, 112\). Both were shown to be phosphorylated in autoradiography analysis (data not shown).

Several conditions were investigated, in the presence of 3 \(\mu\)Ci γ-P\textsuperscript{32} ATP, DAG (20 \(\mu\)g/ml), 100 \(\mu\)M non-labelled ATP, phosphatidyl serine (100 \(\mu\)g/ml), made up in de-ionized distilled water. The conditions were 1) ClC-2 protein + 50 mU PKC, 2) ClC-2 protein alone, and 3) 50 mU PKC alone. These conditions are shown in the autoradiograph in figure 4.

Diacyl glycerol and phosphatidyl serine were included to mimic the lipid environment of plasma membranes. On autoradiography analysis, a major band was observed near the 97 kD marker (the predicted molecular mass of ClC-2) in condition 1, which was absent in both conditions 2 and 3.
control experiments in the absence of PKC, and CIC-2 protein, respectively.

**Figure 4** - In vitro PKC phosphorylation of purified CIC-2. Lanes B and C are

| 50 μg PKC: | + | + | - | + |
| 3μCi γ-P32-ATP: | + | + | + | + |
| CIC-2 Protein: | + | + | - | + |

A → B → C → 97 kD
One other minor band was seen in the lane of condition 1, of slightly higher molecular weight than ClC-2. No bands were seen at all in the absence of PKC (condition #2), whereas condition 3 yielded one (or several) non-97 kD bands. Protein Kinase C is known to undergo auto-phosphorylation \textsuperscript{77}, which likely accounts for the presence of other observed bands. Another possibility is that the other protein bands detected are protein fragments or aggregations of ClC-2 or protein kinase C. This is likely, because the incubation step at 30\degree C may cause protein break-up and/or aggregate formation.

**E. PKC Inhibition is not sufficient in ClC-2 gating**

The in vitro radioactive phosphorylation studies indicated that purified ClC-2 protein could indeed be phosphorylated. Since we observed earlier that the PKC inhibitor peptide 19-27 up-regulates ClC-2 current in *Xenopus* oocytes, and the phorbol ester PDBu vice versa, the next question we wished to address was whether the regulatory mechanism was due to direct phosphorylation of ClC-2.

As in the experiments described in figures 1 and 2, two-electrode voltage clamp technique was used to assess ClC-2 activity via electrophysiology. ClC-2 expressing oocytes were voltage-clamped at -60 mV for the duration of these studies (refer to methods section for further details). After several minutes of current recording at -60 mV, 8 \mu M 19-27 inhibitor was continuously perfused into the oocyte chamber for 30 minutes, followed by hypotonic SOS perfusion for an additional 20 minutes. Figure 5A is a representative current tracing of the described protocol. At selected time points, currents were averaged and normalized against initial currents,
**Figure 5A** - Typical raw current trace of CIC-2 mediated chloride current at a -60mV holding potential. 8μM 19-27 is introduced to the oocyte at the beginning of the experiment. **5B** - Normalized averaged currents at -60mV holding potential. After 30 minutes of drug incubation, a hypotonic solution is perfused into the oocyte chamber to elicit cell-swelling activation of CIC-2.
and plotted on the bar graph in figure 5B.

The holding potential, -60 mV, was selected as it was sub-threshold to the activating potential of ClC-2 (approximately -80 mV – see figure 6), but provided a significant driving force for chloride currents that could be detected and measured across the oocyte membrane. Thus, any changes in ClC-2 mediated currents due to direct protein phosphorylation could be detected if this was the preferred pathway of PKC regulation.

On perfusion of the inhibitor peptide, there was no significant (p > 0.05) increase in the amount of current measured after 30 minutes. Hypotonic SOS was then perfused into the oocyte chamber, resulting in a significant increase (p < 0.05) in measured outward chloride flux (implicated by the large measured increase in inward current). This large increase in current confirmed the presence of ClC-2 expression on the oocyte membrane and is summarized in the bar graph in figure 5B. Control oocytes placed in a hypotonic environment did not exhibit such increases in whole cell current (data not shown).

The inability of the PKC inhibitor peptide 19-27, even at high a concentration, to upregulate current in the absence of hyperpolarization suggests that down-regulating the cell PKC phosphorylation status alone is not sufficient to cause the changes ClC-2 mediated currents observed in figure 2. Thus, up-regulation of ClC-2 activity by PKC down-regulation likely does not act via mechanisms directly affecting channel gating, but perhaps through other pathways.
F. Modulation of PKC phosphorylation status does not affect intrinsic channel characteristics

In some species of ion channels, phosphorylation of certain residues along or near the pore region of the channel alters different characteristics and properties of the channel, such as ion permeability, gating, and voltage-dependence. As mentioned earlier, however, this is uncommon. We have observed that changes in PKC phosphorylation status of the oocyte does not significantly alter ClC-2 ion permeability (because reversal potential is not affected by either drug).

Figure 6 represents the averaged activation curves of ClC-2 mediated currents before and after exposure to drug. From the plot, it is clear that ClC-2 only begins to be opened at potentials more hyperpolarized than ~80 mV. Channel rectification is not affected by PKC phosphorylation status, as one would expect to observe variations in slope and shape of the activation curves seen in figure 6. As such, there is no difference between the control and drug-treated oocytes in ClC-2 activity.

Voltage dependence of an ion channel is defined as the activity of the channel associated with any given membrane potential; thus, a channel may operate at 75% of maximal activity at -100 mV whereas a phosphorylated form of the same channel may only operate at 50% of maximal activity at -100 mV. Channel phosphorylation could then be indirectly detected by the change in current magnitude measured at a holding potential such as -100 mV. The $V_{1/2}$ is the membrane potential that elicits half maximal response in steady-state currents, and is a useful index of voltage dependence.

In a number of ion channels, direct phosphorylation of the protein affects its
Figure 6 - Activation curve of CIC-2 in *Xenopus* oocytes. Three conditions are represented; control, PDBu treated and 19-27 treated. In each case, currents were measured after 30 minutes. Table on the right lists the voltage-dependence ($V_{1/2}$) of CIC-2 under each condition.

- Control (n=4)
- 1μM PDBu - 30 min (n=4)
- 4μM 19-27 - 30 min (n=4)

<table>
<thead>
<tr>
<th></th>
<th>$V_{1/2}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-124.92 ± 1.77</td>
</tr>
<tr>
<td>PDBu</td>
<td>-123.88 ± 1.70</td>
</tr>
<tr>
<td>19-27</td>
<td>-132.43 ± 3.09</td>
</tr>
</tbody>
</table>
voltage dependence noticeably. Thus, our rationale was if PKC phosphorylation was exerting its effects on ClC-2 via direct phosphorylation, then perhaps this might produce changes in channel properties that could be detected. From figure 5, the average $V_{1/2}$ for untreated oocytes ($n = 4$) was $-124.92 \pm 1.77$ mV. Voltage dependence of ClC-2 did not change significantly in cells treated with 1μM PDBu, with an average $V_{1/2} = -123.88 \pm 1.70$ mV ($n = 4; p > 0.05$).

However, ClC-2 expressing oocytes treated with 4 μM 19-27 ($n = 4$) exhibited a small, but significant shift (approximately 8 mV) in the voltage dependence in the negative direction (average $V_{1/2} = -132.43 \pm 3.09$ mV; $p = 0.00557$). We propose that ClC-2 may be tonically phosphorylated, and addition of a PKC inhibitor results in a shift in balance towards the dephosphorylated state of the protein that possesses a different voltage-dependence.

The effect of PKC phosphorylation on ClC-2 single channel conductance was not investigated, largely because single channel currents are too small to be detected and studied by conventional patch-clamp techniques. However, other studies are being done in our laboratory, utilizing artificial lipid-bilayer models and reconstituted ClC-2 to look at single channel activity in vitro.

In general, despite the slight shift in voltage dependence due to 19-27, PKC drugs do not significantly affect many intrinsic properties of the ClC-2 channel. This does not prove that ClC-2 is not regulated by direct phosphorylation in some way, but merely shows that any phosphorylation present does not affect these tested properties.

However, it is likely that the regulatory effects of PKC activating and inhibiting drugs do not operate via direct PKC phosphorylation of the ClC-2 channel.
itself, although phosphorylation of this protein is possible in vitro, as shown in figure 4. It seems, therefore, that the changes in whole-cell current observed in Xenopus oocytes (figures 1 and 2) may not be attributed to changes in channel gating, which would increase or decrease the current activity per channel.

Perhaps, then, modulation of cell PKC phosphorylation status somehow regulates the number of ClC-2 proteins being expressed on the membrane surface, which would result in the changes in currents seen. We addressed this question next by using several different techniques and methods to study the role of PKC phosphorylation in mediating cell-surface expression ClC-2.

G. PDBu Down-regulates Membrane Capacitance in ClC-2 Xenopus Oocytes

Cell membrane capacitance is defined as the ability of the membrane to store charge and, among other factors, correlates inversely with the thickness of the plasma-lipid bilayer, and directly with the surface area of the membrane (see Discussion section). Thus, cellular processes that result in changes in membrane surface area (such as endocytosis, exocytosis, and protein insertion) can be detected by measuring changes in membrane capacitance in the oocyte. (Refer to methods section for capacitance protocol.)

The membrane capacitance of ClC-2 expressing oocytes was measured every two minutes for a duration of 35 minutes. In figure 7A, the capacitance values were averaged, normalized to the initial capacitance and plotted over time. Two conditions are shown in this graph: cells exposed to 0.5 \( \mu \)M 4\( \alpha \)-inactive phorbol (open circles), and cells exposed to 0.05 \( \mu \)M PDBu (solid squares).
Figure 7A - Capacitance measurements taken from CIC-2 expressing oocytes. Open circles: capacitance of cells treated with 0.5μM inactive phorbol. Solid squares: cells treated with 0.05μM PDBu.
Figure 7B - Normalized averaged capacitance values in CIC-2 expressing Xenopus oocytes. X-axis represents time elapsed after introduction of drug to the cell.
There is no significant change in the membrane capacitance for cells exposed to the inactive phorbol (p > 0.05; n = 4) even after 35 minutes of drug exposure. This is in contrast to the near 20% decrease in measured cell capacitance in oocytes exposed to 0.05 μM PDBu (p < 0.05; n = 4).

Capacitance data for all concentrations of each drug protocol are tabulated in the bar graph in figure 7B. Three time points were taken for each condition: 0 minutes (prior to drug treatment), 25 and 35 minutes following drug perfusion in SOS. The capacitance measurements at time 0 in each case were taken to be the control, and all subsequent membrane capacitance recordings were normalized to the control values.

In non-treated CIC-2 expressing oocytes (n = 4), average membrane capacitance did not change significantly after 25 and 35 minutes (p > 0.05 for both time points). The effect of PDBu on oocyte membrane capacitance appeared to be dose-dependent: in 0.05μM PDBu (n = 4), average capacitance decreased by 11.85 ± 1.2 % and 19.41 ± 0.5 % after 25 and 35 minutes of incubation, respectively (p < 0.05 for both values). In oocytes treated with 0.5μM PDBu (n = 4), average capacitance decreased by 26.34 ± 2.84 % and 39.82 ± 1.9 % at the same two time points (p < 0.05 for both).

In oocytes treated with 0.5 μM inactive 4α-phorbol (n = 4), average membrane capacitance showed no significant change over time (1.21 ± 0.3 % and 1.25 ± 0.3% decrease after 25 and 35 minutes, respectively; p > 0.05). Interestingly, however, cells treated with 6 μM 19-27 (n = 4) showed no significant change in average capacitance either, even after a prolonged incubation time beyond the 35 minute time point (6.04 ± 1.8 % and 7.19 ± 1.5% decrease after 25 and 35 minutes,
These results seem to indicate that PKC activation by PDBu may induce membrane retrieval that results in a decrease in oocyte surface area and membrane capacitance over time. This might account for the down-regulating effects of PDBu on whole-cell ClC-2 current in these cells. This is not a novel finding, as phorbol esters have been shown to cause membrane retrieval in studies of other proteins in oocyte 83-5 and in various cell lines 102-3.

The capacitance changes due to phorbol treatment were accompanied by slight, but noticeable changes in the size and shape of the oocytes, although data for this was not tabulated. After each experiment involving PDBu incubation, oocytes showed indications of shrinkage and often lost their rigid spherical shape. It is important to note, however, that some of these changes may have been due to physical depreciation of the cell after the long duration of each protocol.

Down-regulation of PKC phosphorylation, by the PKC inhibitor peptide 19-27, did not cause an observable increase in membrane capacitance, however, as one might expect to see if up-regulation of ClC-2 currents was due to cell-surface protein insertion. The possible rationale behind this is looked at in the proceeding Discussion section.

**H. Down-regulatory effect of PDBu is temperature-dependent**

The possibility that PKC activation by PDBu down-regulates ClC-2 currents by enhancing membrane retrieval was further investigated in a temperature-dependence study. The experimental rationale behind this was that since low ambient
temperatures have been shown to inhibit endocytosis\textsuperscript{98,113,114}, placing cells in a cold environment would ideally down-regulate membrane retrieval. Therefore, if the phorbol ester effect on ClC-2 current were due to this pathway, current down-regulation would be expected to be hindered or blocked if cells were placed in a cold bath.

We investigated this phenomenon by perfusing ClC-2 expressing oocytes with 4°C SOS, and subsequently 4°C SOS containing 0.5 μM PDBu ($n = 5$) for approximately 30 minutes. Then, SOS containing 0.5 μM PDBu at room temperature (23°C) was introduced into the oocyte chamber for a further 25 minutes (solid squares – figure 8). Normalized currents showed no significant change in a 4°C bath in the presence of phorbol ester, but immediately began to decrease when the 4°C SOS was replaced with room temperature SOS containing PDBu.

In a separate set of control experiments, ClC-2 mediated currents in oocytes decreased significantly after introduction of SOS containing 0.5 μM PDBu at room temperature (open triangles).

I. **Phorbol ester causes re-distribution of ClC-2 in transfected Sf9 cells**

From the data collected from the PKC inhibitor and temperature-sensitive endocytosis studies, we were prompted next to visualize the effect of the PKC drugs on ClC-2 distribution in the cell. The Sf9 cell line was favored because of its capability to express large amounts of protein, which would improve the resolution of labeled ClC-2 under confocal microscopy.

Sf9 insect cells were transfected with baculovirus containing ClC-2 ORF (see
Figure 8 - Inhibitory effect of PDBu on CIC-2 current is blocked at low temperatures and restored at room temperature. 0.5 μM PDBu was used throughout the experiment. Temperature was kept below 4°C.
Several different control experiments were concurrently run to isolate possible non-specific binding. 1) Sf9 cells were treated in the absence of ClC-2 primary antibody to observe non-specific binding of the FITC-labeled anti-rabbit secondary antibody (figure 9C). Non-specific binding observed in the cells treated without primary antibody may account for some of the labeling observed in drug-treated cells, particularly within the cytoplasmic and nuclear regions.

Cells were also treated with a ClC-2 N-peptide fragment, the antigenic peptide, to determine specificity of ClC-2 antibody binding (figure 9B). Most of the signal was competed out by the peptide added at a 100x concentration. Figure 9A shows transfected cells incubated with ClC-2 primary and secondary antibodies, in the absence of the competitive N-peptide.

Figures 9D through 9G show immunolabeling of drug pre-treated ClC-2 transfected Sf9 cells. Cells were either untreated (figure 9D), or pre-treated with 5 \( \mu M \) PDBu (figure 9E), 8 \( \mu M \) 19-27 (figure 9F), or 5 \( \mu M \) 4\( \alpha \)-phorbol (figure 9G) for 45 minutes prior to immunolabeling. Untreated cells exhibit a homogenous, distinct band of protein at or near the plasma membrane surface, illustrating cell surface expression of ClC-2. Cells treated with PDBu have less continuity in the band of immunolabeled proteins on cell surface likely because of the endocytotic effects of phorbol ester on ClC-2.

There is no noticeable difference in membrane protein distribution in PKC inhibitor treated cells, except that there seems to be less labeling in the cytoplasmic regions of the cell. Cells treated with the inactive 4\( \alpha \)-phorbol do not show significant change in ClC-2 distribution from the untreated cells (see Discussion section).
Figure 9A - C - Immunolocalization of CIC-2 in Sf9 insect cells. Panels A-C represent control protocols. (A: Control conditions; B: CIC-2 N-peptide competition; C: Only FITC 2° Antibody present.)
Figure 9D - G - Immunolocalization of CIC-2 in Sf9 insect cells. Panels D-G represent cells treated with drug for 30-40 minutes (D: untreated; E: 2µM PDBu; F: 8µM 19-27; G: 2µM 4α-inactive phorbol)
J. Activation of PKC reduces the amount of detectable ClC-2 on the cell surface

ClC-2 transfected sf9 cells were treated as in the immunolocalization experiments in figure 9 and further processed for cell-surface biotinylation, as described in the methods section. Cells were treated with the either 5 μM PDBu, 5 μM 4α-inactive phorbol, or untreated. After 35-40 minutes, cells were then incubated in sulfo-NHS-LC-Biotin in PBS for 20 minutes before being manually homogenized in PBS/protease inhibitor cocktail (see methods section). ClC-2 protein was then immuno-precipitated from the transfected sf9 cells, protein samples run on a western blot, and extravidin peroxidase was used to detect biotinylated proteins.

Figure 10A shows a western blot of the samples run; the lanes of untreated, 5 μM PDBu and 5 μM 4α-phorbol treated Sf9 cells are indicated. For each lane, a distinct bands near the 97 kD marker can be detected, indicating the presence of ClC-2. However, it is clear that there is less protein detected in the phorbol treated lane than in the control or the inactive phorbol treated cells. However, this method does not provide for an accurate measure of quantifying the differences in amount of between PDBu treated and control cells.

In order to rule out unequal protein lane loading as the cause for less detected ClC-2 in lane 2, the run protein samples were silver-stained (figure 10B). In all three lanes, the amount of silver-stained protein immunoprecipitate was equal, indicating that the same amount of protein was loaded in each case. Therefore, less membrane ClC-2 is seen in PDBu treated cells (figure 10A, lane 2) because there is less biotinylated ClC-2 present that can be detected by the extravidin, and not because of a general decrease in protein concentration in lane 2.
Figure 10 - Cell-surface biotinylation of CIC-2 in Sf9 insect cells. **A:** Western blot of protein samples, probed with extravidin to bind biotinylated proteins. **B:** Silver staining of protein samples. Cells were either untreated (lane A), or treated with 2μM PDBu (lane B) or 2μM 4α-inactive phorbol (lane C).

**A** Immuno-precipitated CIC-2
(from transfected Sf9 cells)

**B** Silver Staining

---

- PDBu 4αPDBu

- biotinylated CIC-2

- silver-stained immunoppt
CHAPTER IV: DISCUSSION

A. ClC-2: A Prototype for Widely-expressed Chloride Channels

ClC-2 is likely to be crucial in normal cell functioning and development. The significance of this channel is reflected in the wide range of cell species that possess ClC-2 currents and chloride currents with properties resembling those of expressed, recombinant ClC-2.

In 1992, Thiemann and Jentsch were the first to clone the ClC-2 protein from rat. From this template, the cDNA of the human isoform of ClC-2 was cloned by Gary Cutting and colleagues in 1995. Since the identification of ClC-2 in 1992, the protein has been found to be ubiquitously expressed in the body. Furthermore, chloride currents resembling those of ClC-2 have been reported in many different cell lines, including parotid rat cochlea, acinar cells, sympathetic neurons, and cortical astrocytes, pig pancreatic acinar cells, mouse and guinea pig cardiac myocytes, human intestinal T84 cells, and in yeast cells.

Until recently, little has been understood about regulation of ClC-2 and its related channels, although all characterized channels were shown to be activated by cell-swelling (reviewed in references 128-131). Several groups report chloride channels that display similarities to ClC-2, including inward-rectification, ion selectivity and gating mechanisms. It is likely that these currents represent ClC-2 mediated ones, although most of these channel proteins have not yet been cloned and identified.

Because ClC-2 mediated chloride currents play such a major role in cell homeostasis, understanding ways in which it is regulated can provide much insight.
into basic cellular processes, as well as possible compensatory treatments for cystic fibrosis.

B. CIC-2 Activity is Modulated by PKC Phosphorylation in *Xenopus* oocytes

In this study, we have investigated the effects of modulation of PKC phosphorylation on CIC-2 chloride channel activity and localization. From our studies, we have shown that CIC-2 activity is regulated by protein kinase C phosphorylation.

As we observed, exposure of oocytes to phorbol 12,13 di-butyrate (PDBu), a phorbol ester that upregulates the PKC pathway, significantly decreased the magnitude of CIC-2 currents at all stepping potentials. On the other hand, the specific PKC inhibitor 19-27 significantly increased CIC-2 currents at all stepping potentials.

Although the inhibitory effects of PKC activation on CIC-2 channel activity have been documented previously in non-pigmented ciliary epithelial (NPE) cells, our study is the first to characterize and quantify the changes in channel properties, as a result of phorbol activation. In addition, this study presents data on the effect of PKC inhibition on CIC-2 activity, which was previously not reported. Since PKC inhibition causes a clear up-regulation of CIC-2 currents, these experiments strengthen the claim that the chloride channel can indeed be regulated by the protein kinase C pathway.

It is interesting to note that phorbol esters have also been reported to regulate the outwardly rectifying chloride channel CIC-3. In this CIC channel, Duan et al. report that a certain serine residue in CIC-3 links PKC phosphorylation-
dephosphorylation to regulation of the channel by cell volume. Other CIC channels have not been studied with regards to phorbol regulation.

i. **PDBu inhibits CIC-2 activity via the PKC phosphorylation pathway**

Phorbol esters are thought to modulate the PKC pathway by mimicking the intrinsic secondary messenger diacyl glycerol (DAG) in directly binding to the PKC enzyme and causing upregulation of the catalytic domain, leading to an increase in the phosphorylation status of proteins in the cell (see figure E).

Since the PKC phosphorylation pathway is an important secondary messenger system in the cell, activation of PKC by PDBu likely causes a number of different cellular changes in its regulation of CIC-2 activity. For instance, the inhibition of CIC-2 currents by PDBu could be due a decrease in channel open probability, a decrease in channel conductance, a decrease in the number of channels able to be activated on the cell surface, or a combination of these. The general purpose of this study was to elucidate the pathway more likely to be involved in the modulation of CIC-2 activity by PKC phosphorylation.

Our first goal was to establish the mechanism through which PDBu was exerting its effects on CIC-2. It is possible that the phorbol ester itself was directly modifying currents, rather than acting upon the PKC phosphorylation pathway. Also, it is known that at higher concentrations, phorbol esters have an effect on a large range of other pathways, including the PKA phosphorylation pathway. Thus, we were prompted to design experiments that would determine the specificity of PDBu on the PKC pathway at the concentrations we adopted in our studies. The
concentrations of phorbol ester selected were modestly low in order to lessen the likelihood of cross reactivity.

In oocytes treated with the inactive 4α-phorbol, CIC-2 mediated currents were not significantly affected, providing partial evidence that PDBu was acting via a cellular pathway to affect CIC-2 current, and that addition of the drug itself was not responsible for the changes in current seen. Furthermore, subsequent experiments involving drug wash-out failed to reverse the inhibitory effects of the phorbol ester. This strongly suggests that the drug permeates the cell membrane, and exerts its effects by modulating intra-cellular processes.

In subsequent studies involving the PKC inhibitor peptide, up-regulation of CIC-2 activity by PDBu could be blocked when oocytes were pre-treated with the inhibitor. Taking all our data into consideration, this is convincing evidence that PDBu acts via the PKC phosphorylation pathway to regulate CIC-2 activity.

C. The PKC Inhibitor Peptide – 19-27

In our study of CIC-2 channel activity, we utilized the aforementioned PKC inhibitor peptide, 19-27. This drug has not been widely documented in previous studies, and represents a new group of PKC inhibitors different from more traditional inhibitors such as chelerythrine chloride. The PKC inhibitor we used in our studies was a myristylated peptide fragment, consisting of nine amino acids (figure H):
The peptide is myristylated (N-Myr) at the amino-terminus to allow membrane permeability, but the myristyl group did not interfere with the peptide inhibitory activity. The inhibitor competes against PKC substrates in cells for the active site along the PKC catalytic domain. Unlike inhibitors such as chelerythrine chloride, 19-27 forms an irreversible, most likely covalent, bond with at least one cysteine residue in the PKC active site.

The 19-27 inhibitor is thus very specific for the PKC phosphorylation pathway, and provides a useful method of determining the specificity of PKC activators such as phorbol esters in regulation of the PKC pathway.

i. **PKC inhibition up-regulates CIC-2 mediated currents**

Introduction of CIC-2 expressing oocytes to the specific PKC inhibitor peptide 19-27, increased the magnitude of CIC-2 mediated currents at all stepping potentials in the oocytes. As in the phorbol experiments, the PKC inhibitor does not appear to affect channel gating, and both fast and slow gating mechanisms are maintained. As seen in figure 2C, 19-27 acts on CIC-2 activity in a dose-dependent manner.

The mechanism for this up-regulation of CIC-2 current is not known. As discussed later, the PKC inhibitor by itself is not sufficient to cause channel gating. Although the drug causes a slight shift in voltage-dependence, 19-27 does not appear to affect other channel properties such as rectification and ion permeability.
Moreover, neither capacitance measurements nor Sf9 studies indicated clear increase in membrane expression of the protein. Thus, further studies will need to be conducted to better understand the effect of PKC inhibition on CIC-2 activity.

D. Is PKC involved in direct modulation of CIC-2 activity?

Regulation of ion channel activity by phosphorylation has always been a subject of great interest among physiologists. Because there are many potential target proteins for PKC phosphorylation within the cell, isolating the proteins responsible for affecting CIC-2 gating is a challenge.

As mentioned in the introduction, one of the possible mechanisms of channel regulation is by direct phosphorylation of CIC-2. Human CIC-2 has at least 4 putative PKC phosphorylation sites, as shown by its amino acid sequence. Our in vitro radiophosphorylation studies indicate that the protein itself is, indeed, capable of being PKC phosphorylated under artificial conditions (figure 4).

One major drawback of this experimental protocol is the presence of other protein bands detected, likely a result of CIC-2 fragments or aggregates. In addition, the PKC enzyme itself is capable of being auto- or trans-phosphorylated, and could account for the bands as well. Protein kinase C possesses several serine, threonine and tyrosine residues that are putative phosphorylation sites via the PKC or tyrosine kinase pathways. The control conditions were important in confirming the identity of the 97 kD band as CIC-2.

From our data, it is apparent that CIC-2 is phosphorylated in vitro. However, our studies could not conclude the phosphorylation status of the protein in vivo.
Although we did not develop further experiments to study in vivo phosphorylation, we were interested in finding out whether modulation of PKC phosphorylation status in cells could alter certain channel properties of ClC-2. The rationale was that, if ClC-2 was phosphorylated in vivo, phosphorylation of specific residues along the protein might produces changes in channel characteristics, such as gating. As we discuss later, there is precedence for this type of channel regulation.

E. Modulation of PKC does not affect important ClC-2 channel properties

i. Ion selectivity

Selectivity of an ion channel for its transport substrates is determined by intrinsic properties of the protein. For instance, size of the pore, binding affinities and transport kinetics all play major roles in determining the specie(s) of ions that permeate the membrane via a particular channel.

The membrane reversal potential ($E_{\text{rev}}$) is the membrane potential at which net current flux (inward and outward) equals zero, and is determined by the relative permeability of the channel to various ions present in the extracellular and intracellular environment. For instance, if there only two cations present, $A^+$ and $B^+$, of the same valence, the permeability ratio $P_A/P_B$ would be defined by the equation:

\[
E_{\text{rev}} = \frac{RT}{zF} \ln \frac{P_A [A]_o}{P_B [B]_i}
\]

where $E_{\text{rev}}$ is the membrane reversal potential.
PA and PB are the relative permeabilities of the two ions, a measure of the ion selectivity of the channel. The more permeable a channel is to a particular ion, the higher its selectivity is for the ion over others present in the solution. Since the ionic composition of the buffers is constant in the oocyte studies, channel reversal potential and ion permeability are intimately related. Changes in ion permeability would be reflected in changes in the reversal potential \( E_{\text{rev}} \). Since neither PDBu nor 19-27 appears to shift the reversal potential (as determined from the I-V plots in figures 1 and 2), it is safe to assume that modulation of cell phosphorylation status via PKC does not affect the ion permeability (i.e. chloride ion transfer) of CIC-2 channels.

This is contrast to studies of effects PKC phosphorylation on human CIC-1 activity by Rosenbohm et al. (1999). From their experiments, they report that phosphorylation by PKC appears to affect ion transfer and gating processes, and suggest that an important phosphorylation site may be present in or near the channel pore.

\textit{ii. Voltage Dependence}

The \textit{voltage-dependence} \( V_{1/2} \) of an ion channel is the voltage which elicits half-maximal magnitude of currents at a given time point (see Results section). In the determination of the \( V_{1/2} \) of CIC-2, maximal current amplitude was taken as the current measured at the end-point of the \(-140\text{mV}\) stepping potential. All currents at the same time point at other stepping potentials were measured and normalized against the maximal current, and the results were plotted against the stepping potential.
From these data (figure 6), modulation of PKC phosphorylation in the oocytes by PDBu does not appear to affect voltage dependence (which would be indicated by a left-right shift in the curves), nor gating processes of the channel (which would be indicated by changes in the slope of the curve). In human ClC-1, activation of PKC by phorbol esters does not significantly affect voltage-dependence.

However, the PKC inhibitor 19-27 was found to cause a significant shift in voltage-dependence, approximately 8mV in the negative direction (p < 0.05). It is possible that ClC-2 may be tonically phosphorylated, and that addition of 19-27 disturbs the phosphorylation balance in the cell. De-phosphorylation is favored, and proteins such as ClC-2 are affected when the intrinsic phosphate group is cleaved. This may then result in changes in channel properties, such as voltage-dependence.

There is, of course, no clear evidence for this that can be seen from our studies. However, there is precedence for this type of modulation of voltage-gating behavior in studies of other membrane proteins. In these studies, changes in PKC phosphorylation status appear to drastically alter voltage-dependence of the channel (e.g. K⁺ channels; Ca²⁺ channels). For instance, Barros et al. (1998 – reference) report that PKC phosphorylation modulates the gating of the HERG potassium channel by inducing a 20 mV shift in voltage dependence in the depolarizing direction.

iii. Channel Activation Potential

*Activation Potential of ClC-2:* Figure 6 indicates that ClC-2 remains relatively dormant at membrane potentials more positive than approximately −80mV. At
potentials negative to this, however, C1C-2 begins to open, and activity increases near exponentially as the voltage decreases further. We found that -140mV was the optimal stepping potential to elicit the maximal magnitude of current without compromising the integrity of both the oocyte membrane, and the voltage clamp. In general, oocytes do not tolerate potentials much lower than this.

**iv. Channel Gating**

Since the PKC inhibitor peptide 19-27 up-regulated whole-cell CIC-2 mediated current in oocytes that was detected by performing hyperpolarizing voltage-step protocols, we were interested in whether the inhibitor itself was sufficient to cause channel opening in the absence of hyperpolarizing stimulus.

If direct de-phosphorylation of CIC-2 or associated gating proteins was important for up-regulation of channel activity, the effects of the PKC inhibitor could be assessed in the oocytes in the presence of a potential difference sufficient to provide a driving force for chloride ions. The potential difference would need to be sub-threshold (i.e. greater than -70mV), however, so as not to provoke voltage-dependent activity of CIC-2. Thus, any increases in measure currents would be due solely to the direct effects of 19-27.

We chose a holding potential of -60mV that satisfied these criteria, and held the oocyte membrane potential at this voltage throughout the experiments. Exposure of CIC-2 expressing oocytes to a high concentration of the PKC inhibitor did not cause a significant increase in current even after 30 minutes of drug perfusion (figure 5b). This is in contrast to the 250% increase in whole-cell currents seen after 25
minutes of exposure to 19-27 when stimulated by hyperpolarization to -140mV (figure 2b).

v. **Single-channel Conductance**

No experiments were done to study the effect of PKC phosphorylation drugs on CIC-2 single channel conductance. This is mainly because, unlike CIC-0 currents, single channel currents for CIC-2 are too small to be accurately detected and studied in typical patch-clamp set-ups. However, as mentioned earlier, single reconstituted CIC-2 proteins are currently being studied in lipid-bilayer experiments in our lab, and modification of single channel activity by PKC phosphorylation is one of the parameters being investigated. So far, there is little conclusive evidence showing any direct changes in single channel conductance by PKC phosphorylation.

vi. **A Hyperpolarization-induced ‘Priming’ Effect**

At the -60mV stepping potential, however, whole-cell current is seen to increase nearly 200% in hyperpolarization stimulated oocytes (figure 2b). As just mentioned, holding the membrane potential at -60mV, no such increase was detected even after 19-27 treatment. The explanation for this difference is likely due to ‘priming’ effects of hyperpolarization of CIC-2 activity. The protocol that is used in these experiments involve holding the oocyte membranes at a -30mV resting potential, then stepping the potential to -140mV through +20mV for 12 seconds in increments of 40 mV, each time returning to -30mV for several seconds between stepping potentials. It is likely that the first large voltage step down to -140mV may
cause mass opening of CIC-2 channels, and full channel recovery is not attained during the subsequent seconds of 'rest' at −30mV.

Thus, some of the current observed at the remaining stepping potentials are possibly due to channels that either have not completely closed, or are hyper-sensitive to voltage changes as a result of the initial hyperpolarization priming.

In the −60mV holding potential experiments, a hypotonic form of SOS was perfused into the bath after 19-27 treatment, and a typical CIC-2 mediated swelling-activated current was observed (figure 5a). In non-injected or water-injected oocytes, currents at a −60mV holding potential are typically near the zero base line, and do not change noticeably after treatment of either PKC inhibitor or hypotonic SOS (data not shown).

One hypothesis to explain the increase in currents seen in figure 2 might have been that CIC-2 is tonically phosphorylated in the native membrane, and addition of the PKC inhibitor de-phosphorylates the channel, causing it to open or increasing channel conductance via some gating mechanism. Conversely, PKC activation by phorbol esters might increase the phosphorylation status of channels, somehow stabilizing the closed-state of CIC-2, and decreasing its conductance and/or open probability.

However, these studies suggest that the phosphorylation status of CIC-2 itself is likely not crucial in regulation of its activity by the PKC phosphorylation pathway, although there are several available consensus sites along the protein. As mentioned, modulation of the cell PKC phosphorylation status either by phorbol activation or 19-27 inhibition does not alter key channel properties of CIC-2. In addition, the PKC
inhibitor does not exert any significant effect on ClC-2 current in the absence of hyperpolarization. Therefore, PKC seems to moderate ClC-2 activity through a less direct pathway.

This is not a novel idea, and other membrane proteins possessing PKC consensus phosphorylation sites have been shown to be modulated by PKC via other indirect mechanisms (e.g. the GABA_A and GABA_2/ GABA_3 receptor [55]). In their study, Kusama et al. found that mutating the PKC consensus phosphorylation sites on the receptor did not alter its regulation by PKC via the phorbol ester phorbol 12-myristate 13 acetate (PMA). In wild-type and mutant GABA_2/ GABA_3 injected oocytes, down-regulation of the receptor at the plasma membrane occurred when cells were exposed to the phorbol ester, causing decrease in receptor activity. Similar findings were reported in GABA_A receptors by Chapell et al. (1998 – reference 99).

If direct PKC phosphorylation and de-phosphorylation does not play a significant role in ClC-2 regulation, the next question we wished to address was what other pathways might be involved in the patterns of current changes we observed in our previous experiments. Intuitively, there are only several likely possibilities. The first has already been mentioned; that is, direct phosphorylation leading to changes in channel activity.

The second possible mechanism is modulating accessory proteins that are important in channel gating. These proteins might include cytoskeletal proteins such as actin, which might be associated physically with ClC-2 near either of its termini, or elsewhere along the protein. Currently, another member of our lab is investigating the possibility of channel gating by accessory proteins in the lipid bilayer system.
The third possibility, and the pathway we decided to pursue, is that PKC phosphorylation might modulate the activity of intracellular proteins and processes which are involved in cell trafficking of vesicles to and from the plasma membrane. We were inclined to investigate this possibility mainly because of two reasons. Firstly, preliminary membrane fractionation studies in our lab have shown that CIC-2 is present in multiple compartments in the cell, such as endosomal and plasma membranes. Secondly, other membrane proteins have been shown to be regulated by PKC phosphorylation via alterations in cell surface expression, such as the Na⁺/phosphate cotransporter and the Na⁺/dicarboxylate cotransporter.

F. PKC Activation Induces Redistribution of CIC-2

We set about studying cell-surface expression and regulation of CIC-2 by several means. Firstly, in the oocyte system, we investigated the effects of phorbol, 19-27 PKC inhibitor and inactive phorbol on membrane capacitance. This method of detection of changes in cell surface area has been commonly utilized to study the regulation of other membrane proteins in Xenopus oocytes.

The principle behind using capacitance as an indirect measure of changes in cell-surface protein expressions lies in the correlation between the cell membrane capacitance and its surface area. In addition to containing many ion-conducting channels, the lipid bilayer of biological membranes separates internal and external conducting solutions by an extremely thin insulating layer. Such a narrow gap between two conductors resultantly forms a significant electrical capacitor.
Because of the separation of charge between the internal and external environment, a potential difference exists across the cell membrane. Capacitance (C), measured in farads, is a measure of how much charge (Q) needs to be transferred from one conductor to another to set up a given potential (V). The relationship between these values are given by the equation:

\[
C = \frac{Q}{V}
\]

Thus, a one farad capacitor (1-F) will be charged to one volt (1 V) when +1.0 C of charge is on one conductor, and -1.0C on the other. If the potential difference (V) is known, which can be determined arbitrarily on our amplifier (but was set at 5mV), and the total charge transfer (Q) as a result of the voltage step is calculated (which can be found by calculating the area of the current plot), then the membrane capacitance can be deduced (C). The experimental protocol is discussed in the methods section.

The capacity of biological membranes to store charge arises from the mutual attraction between the internal and external environments across the bilayer gap and by the polarization they develop in the insulating medium. The membrane capacitance, therefore, depends on the dielectric constant of the bilayer (an intrinsic property, determined by relative fluidity, conductivity, phospholipid composition, etc.), the distance of separation between the two side (the thickness of the bilayer), and on the area of the conductors, i.e. the membrane surface area.
The relationship between capacitance and these variables can be summed up in the equation:

\[
C = \frac{\varepsilon \varepsilon_0 A}{d}
\]

where \(d\) is membrane thickness, \(A\) is surface area, \(\varepsilon\) is the membrane dielectric constant, and \(\varepsilon_0\) is a universal constant, termed the polarizability of free space.

Thus, we can see that changes in membrane surface area due to membrane insertion or retrieval would be reflected in changes in capacitance values, which can be detected by the method mentioned earlier. It is important to note, however, that this technique is prone to several sources of experimental error.

Firstly, as can be seen from the above equation, the capacitance is also dependent upon the separation between the two faces, i.e. bilayer thickness. Any perturbation that would affect the thickness of the membrane, such as mechanical movement due to buffer perfusion would affect capacitance measurements.

One example of this is during the exchange of perfusion buffers throughout the various protocols. Although every measure is taken to ensure a smooth transition of one buffer to another to the oocyte bath, occasionally minute air bubbles becomes trapped in the tubing that is ejected into the chamber, causing a small disturbance in the oocyte membrane and voltage-clamp set-up. Also, any slight oocyte swelling or shrinking due to a difference in osmolarity of buffer versus cytoplasm may cause an capacitance artifact that is recorded. The probability for this, however, is very low as buffers were carefully prepared and constantly checked for proper osmolarity. Any differences in tonicity were compensated with mannitol.
Secondly, a major assumption is that membrane insertion or retrieval due to cellular processes does not affect membrane thickness, and that vesicular insertion merely increases the surface area of the membrane. Another possible source of experimental error is the potential effects of any of the drugs on the dielectric constant, or even the phospholipid composition of the plasma membrane. Our results assume \( \varepsilon \) as a non-changing value; however, the effects of either PDBu or 19-27 on this property of the membrane over time are not known.

In the absence of drug treatment, oocytes displayed a relatively stable capacitance over a period of 40 minutes. Introduction of PDBu to the cells caused a decrease in capacitance over time, in an apparent dose-dependent manner (figure 1C). Oocytes treated with 0.5\( \mu \)M 4\( \alpha \)-inactive phorbol did not show a significant decrease in capacitance over the same period of time. This suggests that phorbol ester is likely causing an increased rate of endocytosis, leading to decreased cell surface area and number of ClC-2 proteins, and that this may account for the decrease in whole-cell ClC-2 mediated currents observed in oocytes treated with PDBu.

1. **Low Temperature Inhibits Down-Regulation of ClC-2 by PDBu**

As mentioned before, the inhibitory effects of phorbol ester on ClC-2 currents were hindered when oocytes were perfused with a PDBu solution at 4°C (figure 8). When temperature was restored to 23°C, down-regulation of ClC-2 mediated currents was once again observed.

At low temperatures, cellular processes in general decrease in rate and efficiency, because enzymes perform less optimally in such an environment. Many of
these processes are energy-dependent; higher temperatures provide higher thermal
ergy that can contribute to intracellular pathways and mechanisms. Endocytosis is a
process that has been shown to be hindered by low temperatures in many cell systems
\(^{96-8}\). In our studies, perfusing the cells with 0.5 \( \mu \)M PDBu SOS at 4\(^\circ\)C appears to block
the affect of the phorbol ester on CIC-2 current, most likely because the endocytotic
processes of the oocyte are inhibited by the low temperature of the environment.

It is uncertain whether the low temperature has an effect on the rate of ion
transfer across the CIC-2 chloride channels. For example, it is feasible that the
inhibitory effects of the decreased temperature on PDBu down-regulation of CIC-2 is
due to a direct change in gating kinetics or pore conductance of the channel; i.e. low
temperatures might cause an increase channel activity, thus compensating for the
effects of the phorbol present. This might explain why a sustained CIC-2 mediated
current is observed even in the presence of PDBu.

However, this is highly unlikely, as any effect that low temperature would
have on channel activity would be in the negative direction. Rather than increasing
channel activity, at 4\(^\circ\)C there is much less thermal energy, and thus single channel
conductance would \textit{decrease}. The ion channel can be considered, in many ways, to be
similar to enzymes in their respective binding kinetics. As in enzymatic reactions,
there is a ‘saturation point’ in ion binding to the channel pore before it moves across
the membrane. This is, in essence, what determines the single channel conductance;
i.e. the maximal current conductance a channel can possess.

In the enzymatic model, increasing the ambient temperature increases the rate
of reaction, as the ‘thermal activation barrier’ is lowered, and decreasing temperature
has the opposite effect. Similarly, if low temperatures had an effect on channel activity, it would serve to decrease conductance, which would not explain the sustained currents observed when CIC-2 expressing oocytes are exposed to PDBu SOS at 4°C.

Therefore, it is more probable that the low temperature affects mainly the rate of endocytosis, decreasing the efficiency of this process, and preventing the phorbol ester from down-regulating CIC-2 by membrane retrieval.

ii. Redistribution of CIC-2 in Sf9 cells

In order to strengthen our hypothesis that PKC activation induces membrane retrieval of CIC-2, we sought to visually localize changes in protein distribution in Sf9 insect cells. This cell line was favored because of its ability to express large amounts of protein, which would improve the resolution of labeled CIC-2 under confocal microscopy.

However, it should be noted that the effect of PKC phosphorylation in a CIC-2 over-expression system versus its effect on CIC-2 normally in cells, is unclear. It could be possible that the vast amount of protein being synthesized in sf9 cells induces an effect of PDBu not correlating to its actual activity in cells not over-expressing protein. Therefore, the redistribution of CIC-2 we observe presupposes that protein over-expression is not causing abnormal phorbol regulation of the channel.

From our data, there is clearly redistribution of CIC-2 protein that can be seen in cell treated with phorbol ester, while redistribution is not seen in cells treated with
inactive phorbol. In non-drug treated sf9 cells, there is a clear, distinct band of protein that can be seen around the borders of the plasma membrane, while moderate protein staining can also be observed intracellularly. Perhaps, though, much of this may be accounted for by non-specific binding of the primary and secondary antibody (see panels 10b and 10c).

Sf9 cells treated with phorbol ester exhibit a scattered distribution of protein throughout the cell, with less localization observed around the membrane periphery. As can be seen from the profile plots, there appears to be a relatively higher amount of protein intracellularly in cells treated with phorbol ester than in untreated cells.

Most cells treated with PDBu exhibited this pattern of protein re-distribution (>80%) when studied under confocal microscopy. This correlates well with our previous capacitance data if this phenomenon is explained by an increased endocytosis of CIC-2 from the cell surface due to PDBu.

Cell-surface biotinylation further revealed that less CIC-2 could be detected from the extracellular surface in cells treated with PDBu (figure 9). Untreated CIC-2 transfected cells run on a western blot displayed a clear and distinct band of protein at the 97 kD marker, the molecular mass of CIC-2, while phorbol treated cells showed significantly less protein at that molecular weight. Exposure of cells to similar concentrations of inactive phorbol did not cause a significant change in the amount of protein detected by avidin.

As in the immunolocalization experiments, these results suggest that PDBu decreases the amount of total CIC-2 localized to the cell-surface via membrane
retrieval. This observation further supports the specific effect of the phorbol ester on protein retrieval from the cell surface.

Silver staining experiments were performed as a control for the total amount of protein loaded into each lane (figure 5B). This provided evidence that differences in the intensity of protein bands in the western blot between lanes was not due to discrepancies in protein loading, but that the decrease in CIC-2 detected was indeed due to less cell-surface expression. The band seen in the silver staining is likely fragmented IgG that forms part of the antibodies used in these experiments. Since the total amount of antibodies used was consistent throughout the conditions, these fragments provide a good indicator to compare the relative amounts if protein present in each lane.

One drawback to this method is the presence of many different proteins in the Sf9 cell membrane preparations. In order to remove most of the irrelevant proteins, CIC-2 was immuno-precipitated out of the membrane homogenates with a CIC-2 antibody. Probing the western blots with extravidin, a marker specific for biotinylated proteins, further rectified the problem of other proteins being present.

iii. How does PKC Inhibition Regulate CIC-2?

It is intriguing that PKC inhibition via the 19-27 peptide did not cause a significant change in oocyte capacitance, nor induce redistribution of CIC-2 in the Sf9 experiments. In 19-27 treated oocytes, CIC-2 mediated currents were observed to increase significantly, as shown in earlier experiments. If PKC activation and inhibition were operating via a similar pathway of regulation, we would expect to
observe at least a slight increase in capacitance, possibly due to increased membrane insertion of CIC-2 protein.

A possible explanation for this is that this method of detection may not be sensitive enough to detect small positive changes in capacitance, i.e. increases in surface area. In order for capacitance increases to be detected, there might need to be a significant amount of membrane insertion. Alternatively, membrane insertion may have other effects on the cell surface that prevent accurate detection of changes in membrane area, such as altering bilayer thickness or improperly being inserted, causing infolding of the plasma membrane. Admittedly, however, there is no demonstrable evidence for these effects in our studies.

In both the immuno-localization and surface biotinylation experiments, treatment of CIC-2 transfected sf9 cells with the PKC inhibitor peptide 19-27 did not result in any significant changes in protein distribution or amount of detectable CIC-2 by avidin. In these treated cells, however, there appears to be less protein staining within these cells than in untreated ones, while a similar intense band of protein around the membrane periphery is maintained when confocal immunofluorescent images are compared.

This might suggest that PKC inhibition via the inhibitor peptide is causing increased protein insertion into the membrane, i.e. increased rate of vesicle trafficking to the surface. Although this possibility is feasible, we expect that if this were indeed occurring, the increase in the amount of protein found on the cell surface should be significantly noticeable, seeing as there is, on average, a 125% increase in whole-cell currents after 19-27 treatment measured in earlier experiments (see figure 2).
A major assumption, however, is that the oocyte and the sf9 insect cell systems are similar in their response to PKC activating and inhibiting drugs. This comparison would not hold true if, for instance, sf9 cells were less sensitive to the 19-27 inhibitor than oocytes, resulting in smaller changes in ClC-2 distribution that is difficult to detect via these immuno-localization and biotinylation protocols.

Both of these protein localization techniques were attempted several times in the *Xenopus* oocyte system, but to no avail. The amount of protein produced in this system is not enough to enable clear and accurate localization and quantification.

There is an apparent lack of response to inhibition of the PKC pathway by 19-27 on protein distribution in each of the experiments, as well as capacitance measurements. However, the significant up-regulatory effects of 19-27 on ClC-2 mediated current magnitude in *Xenopus* oocyte indicate that the drug is causing some sort of change in channel function or properties. Taking all into consideration, it may be possible that these methods of protein distribution detection are simply not sensitive enough to detect positive changes in protein expression.

G. Is the phorbol ester response protein-specific?

There is extensive literature reporting redistribution of membrane proteins via the PKC phosphorylation pathway as a result of phorbol treatment involving various other proteins in different cell systems. For instance, Chapell et al. (1998) observed that activation of the PKC pathway by phorbol esters induced internalization of GABA receptors in *Xenopus* oocytes. Similar to our study, functional assays and confocal microscopy were utilized to show the down-regulatory effects of the phorbol...
ester 4β-phorbol 12-myristate 13-acetate (PMA) on cell surface expression of the GABA receptor. Additionally, mutations directed at consensus PKC phosphorylation sites of GABA receptors did not affect the inhibitory effects of PMA.

Similar effects of phorbol activation of the PKC pathway have been shown to down-regulate other membrane proteins such as the human dopamine transporter, the mouse retinal taurine transporter (TAUT), type II Na⁺-phosphate co-transporter, and the human Na⁺/H⁺ exchanger by enhancing the rates of membrane protein retrieval.

In most of these studies, capacitance measurements were also used to help determine the effect of the respective phorbol esters on changes in cell surface area. In these cases, introduction of the cells to phorbol resulted in significant decreases in cell capacitance over time, which correlated with a decrease in the amount of protein detected in the plasma membrane.

In another study by Pajor et al. (1999), they report that both phorbol 12-myristate, 13-acetate (PMA) and sn-1,2-dioctanoylglycerol (DOG – another PKC activator) both decrease cell-surface expression of the sodium/dicarboxylate co-transporter (NaDC-1). They also found that the effect of PMA was dose-dependent, and could be prevented by incubation of cells with staurosporine, a PKC inhibitor. Subsequent mutations performed on the two consensus PKC phosphorylation sites on the protein did not affect inhibition by PMA. Furthermore, Pajor et al. report that the inhibitory effects of PMA could be partially prevented by cytochalasin D, which disrupts microfilaments and endocytosis.
From these studies, it is possible that activation of PKC may have a sort of general up-regulatory effect on the rate of membrane retrieval in cells, as PKC activators such as phorbol esters seem to have a common effect in regulating these proteins. Furthermore, there is evidence that the PKC pathway may likely act via the clathrin-coat mechanism of membrane retrieval in these proteins\textsuperscript{103}.

However, there are various reports suggesting that up-regulation of the PKC pathway varies in its modulation of different membrane proteins. For example, in ClC-3 channels, a serine residue involved in PKC is strongly implicated to be important for volume regulation of the channel in cells\textsuperscript{104}. Another example of direct PKC regulation of a ClC channel is in human ClC-1\textsuperscript{83}. As mentioned before, they found that phosphorylation by PKC appeared to affect channel properties such as ion transfer and gating processes, and postulated that the phosphorylation site may be located near the pore.

Even within one regulatory pathway, PKC has been shown to exert an opposite effect in different proteins. For instance, in their study of the Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel, Ji et al. (1998) found that PKC activation by PMA caused an up-regulation of whole cell currents, while inhibition of PKC by chelerythrine chloride down-regulated currents\textsuperscript{105}. It is intriguing that they adopted the same Xenopus oocyte system as in this study, but their findings on PKC regulation of the Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel were opposite to ours in ClC-2. Thus, the effects of the PKC pathway likely display specificity for different membrane proteins.

Murray et. al (1994) reported that PKC activation by PMA down-regulated human cardiac K\textsuperscript{+} channels expressed in Xenopus oocytes, but that the likely
mechanism for this was not membrane retrieval, but by direct channel phosphorylation \(^{106}\). In fact, they found that capacitance measurements in cells did not change over time after phorbol treatment in \(K^+\) channelexpressing oocytes.

Another point from this study worth noting is that PDBu down-regulation of whole-cell currents in ClC-2 injected oocytes is much more than in water-injected controls (figure 1D). Since the values are normalized to the currents at the beginning of each protocol, this provides a good comparison as to the changes before and after phorbol treatment. PDBu does not down-regulate oocyte leak currents to the same extent as ClC-2 currents. Since the majority of leak currents are via intrinsic membrane channels, it seems that PDBu has more of a specific effect in causing membrane retrieval of ClC-2 than other channels present.

Yet another study was done in cardiac swelling-induced chloride currents (\(I_{Cl,swell}\) channel) in canine atrial cells. The phorbol ester PDBu was reported to concentration-dependently \textit{up-regulate} channel activity \(^{107}\). In their paper, Du & Sorota contrast this finding with PKC regulation of human ClC-3, a putative clone for the currents \(I_{Cl,swell}\) channel, in which PKC activation decreased channel activity (Duan et al., 1999 – reference 104). Du & Sorota suggest that there might be species-dependent variations in the modulation of these channels by PKC.

It seems that PKC modulation of membrane proteins adopts a variety of different possible mechanisms, even within the same cell or of the same protein, but in different species of cells. As we can see, there is evidence for specificity of PKC in regulating the membrane expression of proteins, although there does not seem to be a clear pattern for its preference.
H. Conclusion and Future Direction

From the CIC-2 data presented in this study, it is probable that PKC activation by PDBu largely affects membrane distribution of the protein, and that regulation of its activity is mostly via cell-surface expression. However, we cannot rule out the possible involvement of other pathways, such as direct protein phosphorylation, although our experiments show that activation of PKC phosphorylation does not appear to alter key channel properties (e.g. voltage-dependence and gating processes). As such, further experiments involving single-channel analysis of CIC-2 will be needed to confirm the role of PKC phosphorylation in direct channel modulation.

The mechanism for up-regulation of CIC-2 by the inhibitor peptide 19-27 is less clear. The inhibitor does not seem to be sufficient to trigger channel gating, although it is seen to increase CIC-2 mediated currents in the presence of a hyperpolarizing stimulus (figure 2). Intuitively, this might suggest that PKC inhibition causes increased cell-surface expression of CIC-2, and that this positive change in protein concentration is detected only when channels are activated during hyperpolarization. However, positive changes in amount of membrane CIC-2 could not be seen using the biochemical and electrophysiological techniques we adopted.

Thus, further study needs to be done on elucidating the mechanisms for the involvement of PKC inhibition in regulation of CIC-2. A possible approach might be to investigate the effects of phosphatase and phosphatase inhibiting drugs on channel activity and function. Phosphatase might induce a change in CIC-2 distribution similar to that associated with the PKC inhibitor 19-27, which could be detected more readily by immuno-labeling or other techniques. On the other hand, phosphatase
inhibitors would be predicted to induce similar changes in CIC-2 activity and
distribution as the PKC activator PDBu.

It would also be interesting to study in vivo phosphorylation of CIC-2,
perhaps by utilizing radioactive membrane-permeable ATP. We would then be able to
assess whether the protein can be phosphorylated in a realistic cellular environment,
and whether phosphorylation could be readily controlled by the use of PKC drugs.
This would help in further establishing the role of direct protein phosphorylation in
regulating channel activity and distribution. Other phosphorylation pathways could be
studied as well, including the PKA pathway, which has been implicated in
modulating activity of a chloride channel resembling CIC-2\textsuperscript{115,125}.

Perhaps a more fundamental question that needs to be addressed is the
molecular basis for regulation of cellular proteins by phosphorylation pathways like
protein kinase C. This would involve isolating and studying possible target proteins
involved in the PKC cycle, including cytoskeletal proteins (such as actin) and
enzymes involved in protein synthesis. The use of cytoskeletal-disrupting drugs such
as cytochalasin D in tandem with PKC activators and inhibitors may provide further
insight into the role of membrane trafficking in regulation of CIC-2 by the
phosphorylation pathway.

As mentioned at the beginning of the discussion, understanding how the
ubiquitous CIC-2 can be regulated will provide vital clues to better comprehending
basic cellular processes and hopefully spawn new developments in a host of
innovative treatments for cystic fibrosis.
CHAPTER V: REFERENCES


