Aldosterone regulation of protein kinase D and sodium reabsorption in the renal cortical collecting duct

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Aldosterone regulation of protein kinase D and sodium reabsorption in the renal cortical collecting duct

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A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of

Doctor of Philosophy

Supervisors:

Prof. Brian Harvey

Dr. Warren Thomas
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Student Number ______________________________________________

Date _____________________________________________________
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<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ADPKD</td>
<td>autosomal dominant polycystic kidney disease</td>
</tr>
<tr>
<td>AQP2</td>
<td>aquaporin 2</td>
</tr>
<tr>
<td>ASDN</td>
<td>aldosterone sensitive distal nephron</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>BAR</td>
<td>Bin/Amphiphysin/Rvs domain Bio-Rad Bio-Rad Laboratories Inc.</td>
</tr>
<tr>
<td>BK</td>
<td>Large-conductance K⁺ channel</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumen</td>
</tr>
<tr>
<td>CCD</td>
<td>cortical collecting duct</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>CER</td>
<td>ceramide</td>
</tr>
<tr>
<td>CERT</td>
<td>ceramide transport protein</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovarian cells</td>
</tr>
<tr>
<td>CNT</td>
<td>connecting tubule</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>Cy3</td>
<td>Cyanine</td>
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<tr>
<td>δ</td>
<td>delta</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>2-(4-amidinophenyl)-1H -indole-6-carboxamidine</td>
</tr>
<tr>
<td>DCT</td>
<td>distal convoluted tubule</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMEM F:12</td>
<td>Dulbecco's modified Eagle’s medium and Ham's F-12 medium</td>
</tr>
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<td>Term</td>
<td>Full Name</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------------------</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
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<td>DNA</td>
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<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
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<td>ε</td>
<td>epsilon</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>eCFP</td>
<td>enhanced cyan fluorescent protein</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
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<td>ENaC</td>
<td>epithelial sodium channel</td>
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<td>ENaCα</td>
<td>epithelial sodium channel alpha subunit</td>
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<tr>
<td>ENaCβ</td>
<td>epithelial sodium channel beta subunit</td>
</tr>
<tr>
<td>ENaCγ</td>
<td>epithelial sodium channel gamma subunit</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular stimulus regulated kinase</td>
</tr>
<tr>
<td>ESRD</td>
<td>end stage renal disease</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>η</td>
<td>eta</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>Hsp90</td>
<td>heat shock protein 90</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>JNK</td>
<td>cJun N-terminal kinase</td>
</tr>
<tr>
<td>K+</td>
<td>potassium</td>
</tr>
<tr>
<td>kAE</td>
<td>kidney anion exchanger</td>
</tr>
<tr>
<td>K_{ATP}</td>
<td>ATP-sensitive K+ channels</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM kinase</td>
</tr>
<tr>
<td>M</td>
<td>mole</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>PKD2</td>
<td>protein kinase D2</td>
</tr>
<tr>
<td>PKD3/PKCν</td>
<td>protein kinase D3/protein kinase C nu</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>RAAS</td>
<td>renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROK</td>
<td>Rho activated kinase</td>
</tr>
<tr>
<td>ROMK</td>
<td>renal outer medullary K⁺ channel</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SGK</td>
<td>serum and glucocorticoid regulated kinase</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SM</td>
<td>sphingomyelin</td>
</tr>
<tr>
<td>TAL</td>
<td>thick ascending limb</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>TER</td>
<td>trans-epithelial resistance</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>T75</td>
<td>75cm² tissue culture flask</td>
</tr>
<tr>
<td>WNK</td>
<td>with no lysine family kinases</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>WT 9-12</td>
<td>ADPKD cyst-derived cell line</td>
</tr>
<tr>
<td>µg</td>
<td>micrograms</td>
</tr>
<tr>
<td>µL</td>
<td>microliters</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
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Abstract

Aldosterone is the key regulating hormone of whole body fluid and electrolyte balance. Perturbations in aldosterone synthesis and activation of its receptor, the mineralocorticoid receptor (MR), can lead to hypertension. Consequences of impaired sodium handling, for example hypertension, are a key risk factor in both renal and cardiovascular disease and indicative of impaired electrolyte homeostasis in the body. Renal sodium homeostasis can be modulated in several ways for example, by increasing aldosterone synthesis and activation of MR. Hypertension can also occur due to a dysregulation of aldosterone signalling which can often result in chronic pathologies of the kidney such as nephropathy and renal fibrosis. Aldosterone regulates sodium reabsorption in the kidney through its actions on the epithelial sodium channel (ENaC). ENaC abundance and activity are the major determinants of the rate of sodium reabsorption within the renal cortical collecting duct. We have previously reported a new and important role for the protein kinase D isoform PKD1 in regulating renal sodium reabsorption and here we report novel mechanisms by which aldosterone regulates the subcellular trafficking of ENaC subunits through the activation of protein kinase D2 (PKD2). Aldosterone (10nM) produced a rapid phosphorylation of PKD2 (within 10 min) which was sustained over several hours in M1-CCD cells. This rapid response was accompanied by the subcellular redistribution of PKD2 from the apical membrane into the cytosol. The activation of PKD2 was correlated with an increased abundance and stability of ENaC subunits at the apical membrane, an increase in the phosphorylation of the E3 ubiquitin ligase Nedd4-2 and a 3-fold stimulation of the amiloride-sensitive short circuit current (I\text{SC}). Suppression of PKD2 expression in M1-CCD cells using shRNA resulted in an increased expression of ENaC at the apical membrane and an 8-fold stimulation of the I\text{SC}. Conversely, aldosterone treatment resulted in a paradoxical 4-fold decrease in I\text{SC} in the M1-CCD PKD2 knock-down epithelium. In the case of the third isoform, protein kinase D3 (PKD3), aldosterone did not alter the subcellular localisation of this kinase. Additionally, increasing levels of aldosterone in Sprague-Dawley rats did not affect the abundance of PKD3. CRISPR knock-out of PKD3 in M1-CCD cells was ineffective in altering the abundance or the trafficking of ENaC either under basal conditions or in response to aldosterone. Aldosterone signalling has been implicated in autosomal...
dominant polycystic kidney disease (ADPKD) signalling. WT 9-12 cyst derived cells were used as a model for ADPKD and this work has determined that these cells express the PKD family isoforms, ENaC and MR. In conclusion, our results indicate that PKD2 has two opposing actions on ENaC activity. PKD2, in its basal state, normally suppresses ENaC activity and aldosterone releases this tonic inhibition by phosphorylating and removing PKD2 from the apical membrane. The removal of PKD2 from the membrane activates ENaC possibly by de-ubiquitination via PKD2-dependent phosphorylation of Nedd4-2. Knocking down PKD2 releases the basal inhibitory effect of PKD2 on ENaC, which in turn, revealed an inhibitory action of aldosterone on sodium reabsorption, exposing a previously unknown pleiotropic effect of aldosterone on ENaC membrane stability or channel conductance. We propose that protein kinase D isoforms, particularly PKD1 and PKD2, are important signalling molecules controlling the membrane localisation, stability and activity of ENaC and modulate the aldosterone-stimulated regulation of renal sodium reabsorption.
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Publications, Presentations and Awards

Publications


Quinn S, Dooely R, Yusef YR, Harvey BJ, Thomas W. Protein kinase D2 knock-down promotes ENaC trafficking and function in renal epithelial cells. In preparation.

Poster presentations

Sheppard Prize Meeting 2014, Beaumont Hospital, Beaumont, Dublin 9, Ireland. 18th February 2014.

Title: Protein kinase D2 and the regulation of sodium reabsorption by the distal nephron

Research Day 2014, Royal College of Surgeons in Ireland, Dublin, Ireland. 20th March 2014.

Title: Protein kinase D2 and the regulation of sodium reabsorption by the distal nephron

European Co-Operation in Science and Technology (COST) network on Aldosterone and Mineralocorticoid REceptor: COST-ADMIRE: 1st European Meeting, University of Padua, Padua, Italy. 16th-18th October 2014.

Title: Aldosterone-induced activation of protein kinase D2 in the distal nephron and its impact on renal health and disease

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Title: Aldosterone-induced activation of protein kinase D2 in the distal nephron and its impact on renal health and disease

Title: Aldosterone-induced activation of protein kinase D2 in the distal nephron and its impact on renal health and disease

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Title: Protein kinase D2 modulation of aldosterone-sensitive ENaC activity in renal cortical collecting duct cells

Physiology 2015, Motorpoint Arena, Cardiff, UK

Title: Protein kinase D2 modulation of aldosterone-sensitive ENaC activity in renal cortical collecting duct cells

8th International Symposium and Annual ADMIRE Cost Meeting, Zermatt, Switzerland. 7th – 11th October 2015.

Title: Modulation of aldosterone-sensitive ENaC activity in the renal cortical collecting duct by protein kinase D


Title: Modulation of aldosterone-sensitive ENaC activity in the renal cortical collecting duct by protein kinase D


Title: Novel mechanisms of aldosterone-regulated ENaC activity by protein kinase D in the renal cortical collecting duct


Title: Modulation of aldosterone-sensitive ENaC trafficking in the renal cortical collecting duct by protein kinase D
Oral Communications

ESH-ISH Renin-Angiotensin-Aldosterone System: Putting the A back into RAAS (Official Satellite to the ESH/ISH meeting), Majestic Hotel, Santorini, Greece. 10th-12th June 2014.

Title: Protein kinase D2: A newly identified aldosterone regulator of renal Na+ reabsorption

Royal Academy of Medicine in Ireland, Section of Biomedical Sciences, Annual Meeting 2014, University College Dublin, Dublin, Ireland. 19th June 2014.

Title: PROTEIN KINASE D2, A NOVEL REGULATOR OF ALDOSTERONE AND SODIUM REABSORPTION IN THE RENAL SYSTEM


Title: Protein kinase D2 modulates ENaC activity and aldosterone-sensitive sodium reabsorption in the kidney cortical collecting duct

European Co-Operation in Science and Technology (COST) network on Aldosterone and Mineralocorticoid REceptor: COST-ADMIRE: 1st European Meeting, University of Padua, Padua, Italy. 16th-18th October 2014.

Title: Aldosterone-induced activation of protein kinase D2 in the distal nephron and its impact on renal health and disease

The 7th Annual Meeting of the Irish Epithelial Physiology Group, Kilkenny, Ireland. 23rd-24th October 2014.

Title: Aldosterone-induced protein kinase D activation in the distal nephron, and its contribution to health and disease

European Co-Operation in Science and Technology (COST) network on Aldosterone and Mineralocorticoid REceptor: COST-ADMIRE: ESR Symposium, IRCCS San Raffaele Pisana, Rome, Italy. 22nd April 2016.

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Title: Modulation of aldosterone-sensitive ENaC activity in the renal cortical collecting duct by protein kinase D and its impact on renal health and disease

Joint meeting of the Federation of the European Physiological Societies & the French Physiological Society Congress, Cordeliers Research Centre, Paris, France. 29th June to 1st July 2016.

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STSM Placements

STSM Topic 1: Evaluating Protein Kinase D Isoform Expression along the Distal Nephron

STSM Number: COST-STSM-BM1301-17463

STSM Applicant: Sinéad Quinn MSc.

Home Institution: Royal College of Surgeons in Ireland, Dublin, Ireland

Host Name: Prof. Dr. Christoph Korbmacher

Host Institution: Institut für Zelluläre und Molekulare Physiologie, Friedrich-Alexander Universität Erlangen-Nürnberg (FAU), Erlangen, Germany

Period: 7th July to 18th July 2014

STSM Topic 2: Development of a non-invasive electrophysiological set-up for measuring rectal PD in vivo

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STSM Applicant: Sinéad Quinn MSc.

Home Institution: Royal College of Surgeons in Ireland, Dublin, Ireland

Host Name: Prof. Frédéric Jaisser
Host Institution: Cordeliers Research Centre, INSERM U1138 Team 1
Mineralocorticoid receptor: pathophysiology and therapeutic innovations

Period: 6th to 18th March 2016

**Training Schools**

COST action BM1301 Tenerife Training School

Topic: Mineralocorticoid receptor cell biology and pharmacology

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Title: Protein kinase D2: A newly identified aldosterone regulator of renal Na+ reabsorption

**Royal Academy of Medicine in Ireland, Section of Biomedical Sciences, Annual Meeting 2014**, University College Dublin, Dublin, Ireland. 19th June 2014.

Title: PROTEIN KINASE D2, A NOVEL REGULATOR OF ALDOSTERONE AND SODIUM REABSORPTION IN THE RENAL SYSTEM

Title: Protein kinase D2 modulates ENaC activity and aldosterone-sensitive sodium reabsorption in the kidney cortical collecting duct

European Co-Operation in Science and Technology (COST) network on Aldosterone and Mineralocorticoid Receptor: COST-ADMIRE: 1st European Meeting, University of Padua, Padua, Italy. 16th-18th October 2014.

Title: Aldosterone-induced activation of protein kinase D2 in the distal nephron and its impact on renal health and disease

Royal Academy of Medicine in Ireland, Section of Biomedical Sciences, Annual Meeting 2015, Royal College of Surgeons in Ireland, Dublin, Ireland. 18th June 2015.

Title: Protein kinase D2 modulation of aldosterone-sensitive ENaC activity in renal cortical collecting duct cells

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Title: Protein kinase D2 modulation of aldosterone-sensitive ENaC activity in renal cortical collecting duct cells

Awards

Donegan Medal, Best oral communication, Royal Academy of Medicine in Ireland, Section of Biomedical Sciences, Annual Meeting 2014, University College Dublin, Dublin, Ireland. 19th June 2014.

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**Best oral presentation**, European Co-Operation in Science and Technology (COST) network on Aldosterone and Mineralocorticoid Receptor: COST-ADMIRE: 1st European Meeting, University of Padua, Padua, Italy. 16th-18th October 2014.

Title: Aldosterone-induced activation of protein kinase D2 in the distal nephron and its impact on renal health and disease

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Title: Aldosterone-induced protein kinase D activation in the distal nephron, and its contribution to health and disease


Title: Aldosterone-induced activation of protein kinase D2 in the distal nephron and its impact on renal health and disease.


Title: Modulation of aldosterone-sensitive ENaC activity in the renal cortical collecting duct by protein kinase D and its impact on renal health and disease


Title: Protein kinase D, a novel regulator of aldosterone action on sodium transport in the kidney
Chapter 1

General Introduction
1. Introduction

The principal site for regulated salt conservation in the body is the distal nephron of the kidney. Dysregulation of membrane transporters that are involved in sodium homeostasis makes a significant contribution to the development of hypertension. The progression of other chronic conditions such as polycystic kidney disease and cardiovascular disease can also be influenced by hypertension of renal origin.

Steroid hormones such as oestrogen, glucocorticoids and aldosterone are influential in maintaining electrolyte balance within the body and in part achieve this by regulating renal function. Aldosterone stimulates physiological responses in the distal nephron through multiple mechanisms including gene expression, modulating protein stability and subcellular trafficking. The actions of aldosterone on the nephron and cardiovascular system (1) can lead to the development of hypertension leading to cardiovascular disease and stroke (2). Hypertension may lead to the aggravation of genetic pathologies such as autosomal dominant polycystic kidney disease (ADPKD) and additionally the signalling cascades activated by aldosterone may impact directly on the growth and pathophysiology of the renal cysts. Rare genetic disorders underlying salt-induced hypertension such as pseudohypoaldosteronism type I (3) and Liddle’s Syndrome (4) have provided great insights into the importance of the Renin-Angiotensin-Aldosterone system (RAAS) in regulating normal renal function. A common feature of these pathologies is an increase in the activity of the epithelial sodium channel (ENaC) which subsequently drives the excessive conservation of Na\(^+\). Na\(^+\) in the renal ultra-filtrate is reabsorbed at the apical or the luminal surface of the aldosterone sensitive distal nephron (ASDN) principal epithelial cells through the ENaC and Na\(^+\): Cl\(^-\) co-transporter (NCC) under the influence of circulatory levels of aldosterone (5). The ASDN, is comprised of the distal convoluted tube (DCT), the connecting tubule (CNT), the thick ascending limb (TAL) of the loop of Henle and the cortical collecting duct (CCD) which all show mineralocorticoid receptor (MR) expression (6).
1.1. Basic structure and function of the renal excretory system

Water is the major component of the body’s fluid where it is distributed between the intracellular and extracellular compartments. Cells contain the intracellular fluid which consists of approximately 60 – 65% of the body’s water, while the remainder is extracellular (Lote, 1987. Principles of renal physiology: 2nd edition, London, UK, Chapman and Hall). This extracellular fluid is sub-divided into: blood, interstitial fluid and transcellular fluid. The excretory system drives the movement of solutes between the external environment and the internal fluid. The excretory system has four functions in mammals: filtration; reabsorption; secretion and excretion. In mammals, the excretory system is centred on the kidneys which are the principle site for electrolyte and water balance in the body.

The kidneys are located behind the peritoneum on each side of the lower vertebral column and they make up <1% of the total body weight. The medial segment of each kidney surrounds a cavity through which the renal artery and vein pass through along with nerves and the renal pelvis (7). There are two main regions in the kidney: an outer region known as the cortex and an inner region called the medulla. Both regions are comprised of nephrons, blood vessels and nerves. The medulla is subdivided into renal pyramids of which the corticomedullary border orginates from the base. The apex then terminates at the papilla which rests within the calyx.

The nephrons are the fundamental functional units of the kidney and each human kidney is comprised of approximately 1 – 1.5 million nephrons. The renal corpuscle is located at the head of each nephron and it consists of a single glomerulus that is enclosed by a Bowman’s capsule. It is here that glomerular filtration occurs (8). The glomerular filtrate is processed to produce urine due to the flow of filtered tubular fluid from the renal corpuscles to the intermediary regions of the nephron and on to the collecting duct. On the border of the corpuscle is the proximal convoluted tubule which firstly forms numerous coils that precede a straight section that moves down towards the medulla (9).

The loop of Henle connects the proximal and distal tubule and it consists of a straight section of the proximal tubule, the descending thin limb, the ascending thin limb and the thick ascending limb. The final section of the nephron, the distal nephron, is divided
into four segments: the distal convoluted tubule (DCT), the connecting tubule (CNT), the cortical collecting duct (CCD) and the medullary collecting duct (MCD) (figure 1.1). The DCT is adjacent to the thick ascending limb of the loop of Henle and is followed by the CNT which then delivers fluid into the CCD. The CCD then receives the renal filtrate from the CNT and passes it into the renal medulla resulting in the formation of the MCD.

There are two classes of nephrons: juxtamedullary (approximately 15% of nephrons) and cortical (make up the remaining 85%). Each nephron has the renal corpuscle positioned in the outer cortex region. The cortical nephron is almost entirely located within the cortex with the exception of a small part of the loop of Henle entering into the outer medulla (figure 1.1). In the case of the juxtamedullary nephrons, the renal corpuscle is generally larger and is situated within the internal region of the medulla. The efferent arteriole forms the vasa recta that slope into the medulla and form capillaries that surround the ascending limb of the loop of Henle and the collecting duct. This network of capillaries transports oxygen and nutrients to the tubular segments and also facilitate secretion of substances from the blood into the tubular fluid. This network also allows for the return of reabsorbed solutes and water back into the circulatory system (8).
Figure 1.1. Anatomy and function of the kidney. The renal cortex consists primarily of cortical nephrons with the juxtamedullary nephrons extending into the renal medulla. Numerous nephrons continue into each collecting duct and subsequently enter into the renal pelvis. Each nephron consists of a Bowman’s capsule that encapsulates the glomerulus, a proximal tubule, a loop of Henle and a distal tubule. Urine is formed from water and solutes that are forced into the Bowman’s capsule from the glomerulus by the nephrons, collecting duct and their associated blood vessels. The resulting filtrate from water and solutes travels from the Bowman’s capsule into the collecting duct. The proximal convoluted tubule is responsible for 65% of Na\(^+\) reabsorption with 25% occurring in the thick ascending limb of the loop of Henle. In the distal nephron, the distal convoluted tubule and the collecting duct segments account for 8 – 10% of the total Na\(^+\) absorption and it is these segments that are under tight hormonal regulation. Modified from (10).
1.2. Physiology of the kidney

The kidney plays a critical role in electrolyte homeostasis which is achieved through the regulation of water and ion reabsorption from ultra-filtrate. There are two primary processes that are involved in renal homeostasis: tubular transport and glomerular filtration. The glomeruli filter approximately 180 litres of water and a large quantity of ions (for example, 720 mM of K\textsuperscript{+} and 25,000 mM of Na\textsuperscript{+} pass through the kidney daily) (11). The renal tubules also reabsorb useful substances that cross the filtration barrier of the glomerulus in order to prevent them from leaving the body in the urine. A small proportion of the total number of ions and water filtered by the kidney, that equal the daily metabolic and dietary input are also excreted. Metabolic waste by-products such as hormone metabolites, urea (from amino acids) and creatinine (from the muscle metabolite creatine) are also excreted by the kidney. The kidneys also have an important role in eliminating foreign substances (such as drugs and environmental pollutants) from the body.

1.2.1 Transport properties of the nephron

The proximal tubule produces and secretes NH\textsubscript{3} while maintaining a stable pH by managing HCO\textsubscript{3}\textsuperscript{-} reabsorption and the secretion of H\textsuperscript{+} from the renal filtrate. Amino acids, glucose and K\textsuperscript{+} are transported to the interstitial fluid from the filtrate and finally into the blood. A primary role of the proximal tubule is the reabsorption of NaCl. Salt is transported with the aid of an electrochemical gradient from the filtrate into the cells via symport and exchanger mechanisms across the apical membrane with the subsequent transport of Na\textsuperscript{+} across the basolateral membrane via the Na\textsuperscript{+}/K\textsuperscript{+} ATPase. The transfer of this positive charge is equalized by the movement of Cl\textsuperscript{-} out of the tubule and into the interstitium. The reabsorption of water occurs via local osmosis. The main reabsorptive processes for salt in the proximal nephron is driven by chemical gradients via electroneutral ion exchangers and cotransporters, and for water via osmotic gradients through transcellular (aquaporins) or intercellular leak pathways.
Figure 1.2. The kidney and control of fluid homeostasis. The kidney is the principle site for the control of water and Na\(^+\) homeostasis. At the proximal tubule (PCT) NH\(_3\) is produced and secreted while a stable pH is maintained by managing HCO\(_3^-\) reabsorption and the secretion of H\(^+\) from the renal filtrate. The renal filtrate then reaches the loop of Henle and travels into the cortex within the thick ascending limb (TAL) of the loop. The descending limb has only limited NaCl transport while the TAL has a high density of Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter 2 (NKCC2) and Na\(^+\)/K\(^+\)-ATPase pumps that actively move salt into the interstitium. The remainder of the NaCl reabsorption takes place in the distal nephron under hormonal control. Approximated 5% to 7% of filtered Na\(^+\) occurs in the distal convoluted tubule (DCT) and this section of the distal nephron plays an important role in the adjustment of urinary Na\(^+,\) K\(^+,\) and Ca\(^{2+}\) excretion. Active Na\(^+\) reabsorption in the DCT and CD is also driven by the Na\(^+\)/K\(^+\) - ATPase pump, and expression of the alpha subunit of the pump together with activity of the epithelial Na\(^+\) channel (ENaC) is regulated by aldosterone.
Reabsorption of water continues to the descending limb of the loop of Henle as the filtrate travels along the length of the tubule. In this segment, the epithelium has little permeability for both Na\(^{+}\) and Cl\(^{-}\) along with a moderate permeability for urea and a high permeability for water due to aquaporin expression. The interstitial fluid that surrounds this segment of the tubule must be hypertonic to the filtrate in order to move water by osmosis. There is a gradual increase in the osmolarity of the interstitial fluid as it moves form the outer cortex to the inner medulla. The renal filtrate then reaches the loop of Henle and travels into the cortex within the ascending limb of the loop. The epithelium in the ascending limb differs from the descending limb in that it is very permeable to salt due to high levels of Na\(^{+}/K^{+}\) ATPase activity but impermeable to the movement water. The ascending limb of the loop of Henle is comprised of two segments: a thin segment at the tip of the loop and a thicker segment that leads into the distal tubule. As the filtrate passes through the thin segment, diffusion of NaCl out of the tubule and into the interstitial fluid occurs. In the TAL, 25% of the filtered NaCl is actively transported into the interstitial fluid. The salt gradient established across the renal cortex and medulla by the counter current transport system facilitates the conservation of water by the distal nephron.

The distal tubule plays a key role in reabsorption and selective secretion. The plasma concentrations of both NaCl and K\(^{+}\) are regulated by the changes in the abundance of transporters that are regulated by aldosterone. Excess K\(^{+}\) is secreted into the filtrate as up to 10% of the filtered NaCl is reabsorbed. The correct extracellular K\(^{+}\) concentration is critical in maintaining normal function of excitatory cells, and hyperkalaemia is highly detrimental to normal cardiac function. In common with the proximal tubule, the distal tubule is also involved in the regulation of pH and does so mainly through the controlled secretion of H\(^{+}\).

Transport processes in the CD determine the composition of the filtrate entering the renal medulla and the renal pelvis. It also provides the final opportunity to conserve NaCl and water before the remainder is excreted from the body. The CD is linked to the DCT via the connecting tubule (CNT) which has a role in the reabsorption of both Na\(^{+}\) and Ca\(^{2+}\) along with the secretion of K\(^{+}\) due to the transitional phenotype of cells in this part of the nephron. The fine-tuning of Na\(^{+}\) reabsorption and K\(^{+}\) secretion occurs in the cortical collecting duct (CCD), the outer medullary collecting duct (OMCD) and the inner medullary collecting duct (IMCD) under the dynamic control of aldosterone.
release in response to dietary intake and blood pressure, while vasopressin released by the posterior pituitary gland regulates the reabsorption of water through the up-regulation of aquaporin expression in response to changes in plasma osmolarity.

The DCT is responsible for reabsorbing approximately 5% to 7% of the filtered load of Na+, and plays an important role in the adjustment of urinary Na+, K+, and Ca2+ excretion. The thiazide-sensitive Na+ - Cl- co-transporter (NCC) facilitates the electroneutral absorption of Na+ and Cl- across the apical membrane of the DCT, driven by the basolateral Na+/K+ ATPase (12, 13) In the rat kidney, NCC localisation is restricted to the apical cell membrane and in sub-apical cytosolic vesicles of DCT1 and DCT2 nephron segments (14). ENaC is also present but is only expressed in the principal cells. Renal ENaC consists of three subunits, α, β and γ which are assembled as a heterotrimer composed by of 1 α, 1 β and 1 γ subunit (15, 16).

Intercalated cells of the distal nephron have a significant role in the maintenance of body fluid homeostasis despite the fact that they do not directly reabsorb Na+. Reabsorption of Cl- occurs in type B intercalated cells in exchange for different ions via pendrin (Pds). Pds is a member of the SLC26 anion exchanger super family (17) that is expressed along the lengths of the distal nephron (18). Pds localizes at the apical plasma membrane and near apical cytoplasmic vesicles of type B, non-A and non-B intercalated cells (18) (figure 1.3). The bumetanide-sensitive Na+ - K+ - 2Cl- cotransporter (NKCC1) is also expressed at the basolateral membrane of type A intercalated cells (19) (figure 1.3). The cotransporter is responsible for the movement of 1 Na+, 1 K+ and 2 Cl- ions internally from the basolateral membrane. Secretion of the Cl- ions into the lumen across the apical membrane of these cells occurs as a consequence. It was originally believed that a genetic disruption of NKCC1 would result in a reduction in secretion of Cl- along the collecting duct which could in turn control volume expansion and hypertension. Conversely, in NKCC1 knockout mice, their phenotype was hypotensive rather than hypertensive. The NKCC1 knockout mice resulted in a reduction in the release of aldosterone from the adrenal cortex along with a defected kidney that dulled the renal response to vasopressin and aldosterone, causing an abnormal regulation of Na+, K+, and water excretion (20).
Figure 1.3. Membrane transport proteins in the cortical collecting duct (CCD) cells. The CCD cells play a vital role in electrolyte balance in the body. Under hyperkalemic conditions, K⁺ is secreted by the principal cells via the renal outer medullary K⁺ (ROMK) channel. Reabsorption of Na⁺ through the epithelial Na⁺ channel (ENaC) is driven by basolateral Na⁺/K⁺ ATPase. The recovery of water is by osmosis which occurs either through paracellular transport or via the apical water channel aquaporin 2 (AQP2). The intercalated cells regulate blood acid/base homeostasis. α-type intercalated cells secrete protons via apical H⁺ ATPase and H⁺/K⁺ exchangers. Bicarbonate is reabsorbed by anion exchangers at the apical (Slc26a11) and basolateral (kAE) membranes. The β-type intercalated cells exhibit secretion of bicarbonate through the anion exchangers Slc26a4 (Pendrin) and Slc4a8 and these β-type intercalated cells also reabsorb protons via a basolateral H⁺ ATPase. Figure obtained from (21).
1.3. The Epithelial Sodium Channel

ENaC is primarily located in tight epithelia and as a constitutively active channel, ENaC allows for the flow of Na\(^+\) ions from the lumen, across the apical membrane and through the cell (22-24). ENaC is part of the ENaC/Degenerin superfamily that includes the acid-sensing ion channels (ASICs) (24). ENaC subunits were first sequenced and cloned based on cDNAs that had been isolated from both human and rat tissue (25-31). ENaC paralogue subunits are characterised into two families based on their homology: 1. The proton-gated ASIC family subunits that includes five homologous genes and 2. A family of non-voltage gated Na\(^+\) channel subunits comprised of four genes that encode ENaC homologs i.e. SCNN1A (ENaC\(\alpha\)), SCNN1B (\(\beta\)), SCNN1G (ENaC\(\gamma\)) and SCNN1D (ENaC\(\delta\)) in the case of the human genome, while SCNN1D is not expressed in the mouse (32).

Previous studies suggest that the structure of ENaC is similar to that of the trimeric structure of ASIC1 whereby they have a similar organisation of their transmembrane segments (33). ENaC is assembled from homologous \(\alpha\), \(\beta\) and \(\gamma\) subunits. Each subunit has two transmembrane helices (TM1 and TM2), relatively short amino and carboxyl termini, and a large extracellular region that encompasses several domains (23). Post-translational processing of extracellular regions of ENaC define the structure of the channel and can influence its function when the channel is expressed at the cell surface. ENaC can be glycosylated (34, 35) and undergo proteolytic cleavage at certain sites in the extracellular domains which in turn can activate ENaC (23). The proteolytic cleavage of ENaC can increase the channel’s open probability (\(P_O\)) and this process does not affect single-channel conductance or its ion selectivity (36, 37). There is however evidence that suggests that the intracellular trafficking of proteolytically processed forms of ENaC is different to its non-processed form. It has been suggested by some that the endosomal sorting complex component of the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) is involved in the selective targeting of cleaved ENaC at the surface pool for degradation (38). Other studies noted that processed ENaC\(\alpha\) selectively associates with GILZ1 to enhance the surface expression of ENaC by reducing the inhibitory effect of the Raf-ERK signalling pathway (39). Additional evidence proposes that ENaC channels with an increased residency time at the plasma membrane are more open to being processed by
proteases (40). ENaC is the key mediator of the apical entry of Na⁺ within the ASDN and its abundance is the paramount determinant of the rate of Na⁺ re-absorption by this segment of the renal tubule (41). ENaC activity is regulated by multiple factors that impact upon its stability in the plasma membrane, subunit abundance through transcription and the processing ENaC subunits following translation that results in its assimilation into the membrane and full activation (42). Functional ENaC is comprised of three subunits, α, β and γ organized into a heterotrimer and is discretely expressed at the apical cell-surface in the ASDN or in sub-apical intracellular pools. ENaCα expression is stimulated by aldosterone in the distal nephron while ENaCβ and ENaCγ are constitutively expressed. In the colon this pattern of expression is reversed such that ENaCα is constitutively expressed and expression of ENaCβ and ENaCγ are induced in the colon (43). We have been focusing our studies on the interaction between aldosterone-induced signalling cascades and ENaC activity.

1.4. Regulation of Na⁺ reabsorption in the distal nephron

The regulation of Na⁺ reabsorption in the distal tubule and collecting duct is via the ENaC at the apical membrane and the Na⁺/K⁺-ATPase at the basolateral membranes of principal cells, under the regulation of the renin angiotensin aldosterone system. ENaC expression, trafficking, stability at the plasma membrane and the open probability of the channel can also be regulated by other hormones such as vasopressin and insulin (1, 44-47). Additionally, the renal sympathetic nervous system can affect Na⁺ reabsorption in the distal tubule by a direct action on adreno-receptors (48). In general, there are several ways in which the activity of transport proteins can be regulated which include changes in the abundance of protein within the whole cell, a redistribution or subcellular trafficking of the transporters and channels and post translational modifications or activation of regulatory proteins.
1.5. Aldosterone

The major hormone regulating Na\(^+\) reabsorption in the distal nephron is aldosterone. Aldosterone target tissues express both mineralocorticoid receptor (MR) and the enzyme 11\(\beta\) - hydroxysteroid dehydrogenase 2 (11\(\beta\)HSD2) (49) which is responsible for the conversion of cortisol into inactive cortisone. Aldosterone (or mineralocorticoid analogues) are known to increase the reabsorption of Na\(^+\) as well as upregulating the expression of the Na\(^+\)/K\(^+\)-ATPase at the basolateral membrane of these tissues (50). An increase in the abundance of NCC and αENaC at the protein level was seen in aldosterone-infused rats (51). In contrast, ENaC\(\beta\) was neither decreased nor unchanged following aldosterone infusion, and ENaC\(\gamma\) underwent a chemical change most likely due to proteolysis. The role of MR in ENaC regulation was established by additional studies showing that spironolactone (an MR antagonist) overturned the upregulation of NCC, ENaC\(\alpha\) and ENaC\(\gamma\) in response to a low Na\(^+\) diet (52). The subsequent restriction of dietary NaCl increased mRNA expression of ENaC\(\alpha\), but not ENaC\(\beta\) or ENaC\(\gamma\), nor NCC in rat kidney showing ENaC\(\alpha\) to be an important transcriptional target of aldosterone action (53).

1.6. Mineralocorticoid receptor

In order to maintain Na\(^+\) homeostasis and normal blood pressure, circulating levels of aldosterone counterbalance changes in the uptake of dietary salt and state of volaemia. In certain conditions such as primary aldosteronism, the level of plasma aldosterone is elevated and so increases the activity of the mineralocorticoid receptor (MR). Consequently, the activation of MR in turn increases Na\(^+\) reabsorption in the kidney and can result in salt-sensitive hypertension. Aldosterone was first characterised in 1953 (54) based on its mineralocorticoid effects on the urinary ratio of Na\(^+\) and K\(^+\). Several years later, aldosterone was shown to bind with high affinity to an intracellular site in the cytosol preparations from the kidney which was initially referred to as a corticosteroid Type I binding site and then termed the MR (55-57). MR has been described in several epithelia that are involved in the transport of electrolytes such as the kidney, the hippocampus of the brain and in non-epithelial tissues that include the A3V3 region of the brain (58), the heart (59) and the vessel wall (60).
Figure 1.4. The steroid hormone nuclear receptor family. (A) The steroid hormones are a large and important family of cell regulators including sex hormones (oestrogen and testosterone) and adrenal cortical hormones (glucocorticoids and mineralocorticoids). These hormones bind with intracellular receptor proteins and convert them to functioning transcription factors which in turn influence gene expression. (B) Domain structures of the steroid hormone nuclear receptor family.

Nuclear hormone receptors have been described as ligand-activated transcription factors that have the ability to regulate gene expression via interaction with specific DNA sequences upstream of their target genes (61). The nuclear receptor family includes steroid hormone receptors, retinoic acid receptors and nuclear bile acid receptors (figure 1.4.). Steroid hormone receptors use their influence in embryonic development as well as in adult homeostasis as hormone-activated transcriptional regulators (62).
The binding of aldosterone to MR promotes its dissociation from a complex with heat-shock proteins in the cytoplasm and translocation to the nucleus where it interacts with specific sequences in the promoter regions of target genes. The activity of ENaC in the distal nephron is tightly regulated by aldosterone through the up-regulation of MR-dependent ENaCα transcription (63). The activation of MR also suppresses the activity of Nedd4-2 ubiquitin ligase which promotes the stability ofpre-expressed ENaC subunits. Aldosterone-responsive SGK1 can phosphorylate Nedd4-2 thus suppressing its activity (64).

The dysregulation of aldosterone release, the functionality of MR and defects in the effector targets contribute towards many rare genetic disorders that affect blood pressure and electrolyte homeostasis. Examples of such rare disorders include pseudohypoaldosteronism type I in which there is a mutation in MR (3) or pseudohypoaldosteronism type II wherein essential transporter regulators such as with no lysine kinases (WNK) type 1 (WNK1) and type 4 (WNK4) are mutated (65). It is by investigation of these genetic disorders that will provide further insight into aldosterone actions and may also lead to the identification of targets within the RAAS offering potential therapeutic interventions in chronic hypertension.

1.7. Renin-Angiotensin-Aldosterone System

The importance of the renin-angiotensin-aldosterone system (RAAS) in regulating blood pressure (BP), fluid and electrolyte balance has long been recognised (66, 67). The role of RAAS in the pathogenesis of cardiovascular, endocrine and renal diseases is however, less understood. In the adrenal cortex, RAAS, through angiotensin II causes the release of aldosterone. Aldosterone, acting on the ASDN in the kidneys, triggers an increase in Na\(^+\) and water reabsorption from the urine which increases blood volume and, in turn increases BP. K\(^+\) is secreted into the tubules in exchange for Na\(^+\) reabsorption to the blood, where it is taken up into the urine and excreted (68).

The primary systemic RAAS is a crucial mechanism through which the kidney is able to detect low systemic blood pressure. This detection enables the compensation for low BP by counteracting the fluctuations in vascular tone and Na\(^+\) conservation (66).
Compensation for low BP occurs through the activation of angiotensin and the subsequent release of aldosterone by the adrenal gland. Angiotensin I is cleaved by angiotensin converting enzyme (ACE) from the lung and kidney to produce angiotensin II (figure 1.5).

In response to hypotension, BP is elevated through the activation of sympathetic nerve activity and arteriolar vasoconstriction by angiotensin II. Angiotensin II also increases Na\(^+\) and water retention by tubular reabsorption through secretion of aldosterone from the adrenal gland (figure 1.5). Vasopressin secretion by the pituitary gland is also stimulated by angiotensin II leading to an increase in water conservation in the CD of the kidney (66). The systemic effects of angiotensin II fuel sodium and water retention to increase effective circulating volume, this results in an increased perfusion to the juxtaglomerular apparatus thus decreasing renin secretion by negative feedback (66).

The intra-renal formation of angiotensin II in the kidney is independent of the circulating RAAS. Intra-renal angiotensinogen is produced mainly in the proximal tubule of the kidney. Additionally, renin is secreted from juxtaglomerular cells. Generation of ACE is from the proximal tubule to the collecting duct and ACE is highly expressed in endothelial cells (69).
Hypertension

Figure 1.5. Schematic of the Renin-Angiotensin-Aldosterone System. Angiotensin II is produced when angiotensin I is cleaved by ACE from the lung and the kidney. With a resulting increase in blood pressure, this is a key factor in hypertension. Aldosterone can regulate renal Na\(^+\) reabsorption and the retention of fluid. Renin is also highly regulated and can easily be stimulated by minor changes for example, a decrease in renal perfusion pressure (70). The AT1 receptor is widely distributed among tubular elements of the kidney which is consistent with the receptors role in the regulation of renin release and sodium reabsorption (69). During periods of juxtaglomerular renin suppression, up-regulated renin is produced in the distal nephron and this may have the ability to maintain continual intra-renal angiotensin II formation resulting in the amplification or preservation of a hypertensive state (71). Production of angiotensinogen in the proximal tubule is activated by angiotensin II via the AT1 receptor (72). Increased renal angiotensinogen is converted to angiotensin II by intra-renal renin and ACE while the intra-renal angiotensin II increases renal vasculature tone and tubular sodium reabsorption. Elevated levels of reactive oxygen species (ROS) in hypertension has been known to produce an oxidized form of angiotensinogen. This oxidized angiotensinogen has a more potent capability to bind...
renin which leads to a transition of angiotensin release and an increase in blood pressure (72).

Under normal physiological conditions, RAAS functions to maintain normal cardiac output through its effects on fluid conservation and vascular tone to sustain venous return. The RAAS system also shifts the balance of cell growth and proliferation in the heart as an adaptive response to myocardial stretch (68). Consequently, the pathologic activation of RAAS and its action on the heart has been put forward as a contributing factor to the development and maintenance of left ventricular hypertrophy (73). There are many direct intrarenal actions of angiotensin II which include renal vasoconstriction, tubular Na\(^+\) reabsorption as well as modulation of pressure-natriuresis and the promotion of renal tissue growth (74).

The RAAS participates in the pathophysiology of hypertension and has been shown to be activated in patients with ADPKD (75-79). Of the studies conducted some (75, 79) but not all (76), observed higher aldosterone and plasma renin levels in patients with ADPKD that had been administered ACE inhibitors in comparison to essential hypertensives. One important growth factor for renal epithelial cells and interstitial fibroblasts is angiotensin II suggesting that RAAS may also have a role in cyst growth and kidney fibrosis (80). Activation of RAAS can occur with an increasing cyst size thus increasing BP leading to a cycle of enhanced cyst growth and hypertension and ultimately ESRD. This highlights the necessity of randomized controlled trials, for example the HALT study (80), in addressing the impact of RAAS in disease progression (80-88). In particular, this thesis will look at the impact aldosterone has on Na\(^+\) and fluid retention in a disease model of ADPKD.

1.8. Non-genomic effects of aldosterone

Aldosterone tightly regulates ion transport by both genomic and non-genomic processes (figure 1.6). Non-genomic effects mediated by aldosterone can result in rapid changes such as the activation of protein kinase C (PKC) and protein kinase D (PKD)(89). During genomic regulation of ion transport, aldosterone binds to the MR
and stimulates the expression of several genes including ENaC, Na⁺/K⁺-ATPase, the serum and glucocorticoid regulated kinase (SGK) and the renal outer medullary K⁺ (ROMK) channels (90-92)(figure 1.7).

**Figure 1.6. Genomic and rapid actions of steroid hormones.** Early, non-genomic effects of aldosterone produce rapid changes such as the activation of extracellular stimulus regulated kinase (ERK) 1/2, PKC isoforms and PKD (91). It is vital to understand how the stimulation of these signalling cascades augments the activity of the fundamental aldosterone-modulated effects including ENaC, Na⁺/K⁺-ATPase, SGK and the ROMK channel in order to establish the physiological relevance of processes that are instigated in advance of transcriptional control (92-94). As MR is at present the only widely recognised receptor that is specific to aldosterone, considerable effort has been exercised in order to understand how nuclear receptors such as MR can initiate protein kinase signalling cascades in the conduct of membrane associated receptors (95).
Aldosterone has been previously shown to exert rapid non-genomic effects, for example, the activation of several kinase families including PKC, PKD, ERK1/2 and MAPK through the trans-activation of EGFR that occurs via the non-receptor tyrosine kinase c-Src. The most widely documented aspect of the rapid responses to aldosterone is the activation of protein kinase signalling cascades. Several research groups have investigated the role of ERK1/2 activation in aldosterone-sensitive models such as Madin-Darby canine kidney (MDCK) cells (96); M1-CCD cells (97, 98); vascular smooth muscle cells (VSMC) (99); cardiac myocytes (100) and the mesangial cells of the glomerulus (101). The activation of ERK1/2 is mostly linked to the variation of cell growth which can occur through hypertrophy (100) or by promoting proliferation (98, 101, 102). The activation of ERK1/2 is modulated by the simultaneous activation of other signalling cascades. In MDCK cells, the activation of ERK1/2 occurs within 5 minutes and can be sustained over a period of hours (96). However, in M1-CCD cells, ERK1/2 activation is linked to the trans-activation of EGFR and subsequent activation of PKD1 which has been shown to be necessary to maintain the activation of ERK1/2 beyond 2 to 5 minutes (98). Additionally, PKD1’s involvement in stabilizing ERK1/2 activation occurs in response to growth factors and does not require the direct phosphorylation of ERK1/2 by PKD1 (103). Aldosterone can also stimulate the prolonged activation of ERK1/2 in A6 renal cells as this process is coupled to Ki-RasA expression where aldosterone can also stimulate Ki-RasA GTPase activity within 15 minutes of treatment (104). Another signalling target of aldosterone is the p38 MAPK sub-family. The biphasic activation of p38 in VSMCs can occur within 1 minute of aldosterone treatment (105) that is followed by a second activation phase that was measurable after 30 minutes. This p38 response in VSMCs is dependent on the co-activation of MR and c-Src and Callera et al connected p38 to the pro-fibrotic effects of aldosterone on VSMCs via the regulation of NADPH. Furthermore, MR-dependent activation of p38 in glomerular podocytes from rats is also promoted by aldosterone and this p38 activation contributes to the induction of apoptosis (106).

The protein kinase C (PKC) family of kinases have been associated with many cellular processes such as apoptosis, proliferation, the formation of tight junctions and subcellular trafficking. For example, aldosterone can promote the activation of PKCα in renal collecting duct cells within 2 to 5 minutes after treatment. This activation occurs in an MR-independent manner (97, 107). This activation of PKCα is reliant on aldosterone binding directly to the kinase (108) along with a simultaneous rise in
intracellular Ca\(^2^+\) ([Ca\(^{2+}\)]). Additionally, PKCδ and PKCε can also be rapidly activated in response to aldosterone, however, this is not reliant on the direct binding of the hormone to the kinase but is coupled to MR through EGFR (109). PKD1 activation follows a similar pattern (94) and has been implicated in the induction of proliferation in M1-CCD cells following aldosterone treatment (98) as well as in the stimulation of hypertrophy in cardiac myocytes (110).

Aldosterone can also promote the activation of secondary messenger responses such as a rise in [Ca\(^{2+}\)], the biosynthesis of cAMP and the release of nitric oxide (NO). There are many studies documenting the rise in [Ca\(^{2+}\)] in response to aldosterone including in M1-CCD cells (111), VSMCs (112), in isolated colonic crypts (113) and in the brain (114). The mechanism by which Ca\(^{2+}\) influx occurs in both the colon and the renal nephron has not been fully described; however, Ca\(^{2+}\) entry into CCD cells was not sensitive to spironolactone and Ca\(^{2+}\) entry into colonic crypt cells is PKC-dependent (115). Aldosterone enhances a stress response in hippocampal cells by stimulating the entry of Ca\(^{2+}\) through L-type calcium channels (116). While the dorsal and ventral hippocampus express MR, the receptor responsible for this Ca\(^{2+}\) response has not yet been elucidated. The PKC-dependent activation of L-type calcium channels elicits vasoconstriction within 5 minutes of aldosterone treatment in the afferent arterioles of the renal microcirculation. In contrast to this, the stimulation of vasoconstriction in efferent arterioles is facilitated by the activation of T-type calcium channels in response to aldosterone (117). Aldosterone induces an increase in the intracellular levels of cAMP within 1 minute coupled with the phosphorylation of CREB following 5 minute treatment in VSMCs (118). It has also been shown in HEK293 cells that treatment with aldosterone suppressed CREB-dependent transcription by stimulating the activity of calcineurin/protein phosphatase 2B (PP2B) (119). While it remained unclear whether aldosterone exerted a rapid effect on the basal levels of CREB phosphorylation in HEK293 cells, it was determined that the pre-incubation of cells with the hormone for 20 minutes suppressed the induction of CREB by forskolin.

The regulation of intracellular pH (pHi) occurs via the Na\(^+\)/H\(^+\) exchanger family (NHE) which is responsible for the exchange of intracellular H\(^+\) for extracellular Na\(^+\). NHE also has a role in regulating cell volume as well as the induction of proliferation and cell growth (120-122). NHE, specifically the NHE1 isoform, is expressed in the basolateral membrane in polarized epithelial cells where it plays a role in the regulation of cell volume and cytoplasmic pH. Aldosterone can activate NHE isoforms to promote
alkalinisation of the cytoplasm within 20 minutes in the kidney of amphibians (123). In MDCK cells, the aldosterone-dependent increase in pH\textsubscript{i} is linked to the activation of NHE. This rise in pH\textsubscript{i} is also dependent on the activation of ERK1/2 along with the rapid increase in [Ca\textsuperscript{2+}] that occurs within 1 minute of treatment with aldosterone (96, 124). Other studies using M1-CCD cells showed that aldosterone induced a concentration-dependent increase in the recovery of pH\textsubscript{i} from an acid load within 5 minutes of treatment. The authors determined that this effect was reduced when PKC\textalpha was inhibited or by the activation of MAPK (97).

Another target of non-genomic aldosterone signalling is the H\textsuperscript{+} pump (H\textsuperscript{+}-ATPase). Aldosterone can stimulate urinary acidification by stimulating H\textsuperscript{+} eflux via the H\textsuperscript{+}-ATPase pump. This response was first described in both the turtle bladder (125) and in the frog skin (126). It was later shown in whole-cell patch clamp recordings of mitochondria-rich cells of the frog skin that aldosterone treatment resulted in the rapid exocytotic insertion of H\textsuperscript{+}-ATPase in to the luminal membrane within 10 minutes of stimulation (127). This insertion of H\textsuperscript{+} pumps into the membrane was sensitive to the inhibition of PKC and disruption to the cytoskeleton. In the kidney, the reabsorption of bicarbonate coupled with the release of H\textsuperscript{+} into the renal ultrafiltrate in the distal nephron accounts for acid-base regulation. Aldosterone has a crucial role in regulating the renal H\textsuperscript{+}-ATPase which is as a result of rapid signalling responses. For example, stimulation with 10 nM aldosterone for 15 minutes resulted in the MR-dependent increase in the exiting of H\textsuperscript{+} from acidic type A intercalated cells of the outer medullary collecting ducts of mice (128). The responses described by Winter et al were similar to that observed in the frog skin whereby this increase in H\textsuperscript{+} pump activity was dependent on the Ca\textsuperscript{2+} induced activity of PKC. Moreover, mice injected with aldosterone displayed an increase in the expression of H\textsuperscript{+}-ATPase in the apical membrane of type A intercalated cells (128) which strengthened the evidence for aldosterone in regulating the trafficking of H\textsuperscript{+}-ATPase in the maintenance of acid-base homeostasis.

In the renal CD, K\textsuperscript{+} enters the principal cells through the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase in the basolateral membrane and is then secreted into the lumen via K\textsuperscript{+} channels along the apical membrane (129). The main K\textsuperscript{+} secreting channel in the kidney is ROMK which is expressed in the apical membrane of cells in the ASDN (130). The function of ROMK is regulated by aldosterone through SGK1 activity which was found to regulate cell surface expression of the channel (131). K\textsuperscript{+} can also enter the cell through K\textsuperscript{+} channels
located in the basolateral membrane of the CCD (132) which may occur due to the stimulation of Na⁺/K⁺-ATPase by mineralocorticoids (133). ATP-dependent K⁺ channel (K⁺ATP) activity in A6 renal cells was rapidly stimulated by aldosterone (15 minutes) and this activation modulated the P₀ of the channel (134). Aldosterone has been shown to mediate a non-genomic inhibition of Ca²⁺-dependent intermediate conductance channels (IKCa) located in the basolateral membrane of colonic crypt cells and this effect was PKC-dependent (135, 136). Additionally, aldosterone can also activate Na⁺/H⁺ exchange through Ca²⁺- and PKC-dependent signalling pathways that result in an upregulation of K⁺ATP and an inhibition of K⁺Ca (113), the effects of which are independent of MR. Taken together, it is evident that aldosterone can induce rapid signalling effects that can impact upon several membrane targets by either having a direct effect on their activity, or, by indirectly via the modulation of MR-dependent transcription.
Figure 1.7. Aldosterone induced signalling intermediates and their modulation of membrane transporters. Fundamental signalling intermediates such as protein kinase A (PKA), protein kinase C (PKC), protein kinase D (PKD), phosphoinositol 3-kinase (PI3K), serum and glucocorticoid activated kinase (SGK), Rho activated kinase (ROK), the with no lysine family kinases (WNKs) and the extracellular stimulus regulated kinase (ERK) are activated by treatment with aldosterone. Once activated, these signalling intermediates modulate the activity of ENaC, ROMK, ATP-sensitive K⁺ channels (K⁺ATP), Na⁺/hydrogen exchanger 1 (NHE1) and the Na⁺/K⁺ pump (Na⁺/K⁺-ATPase) in the principal cells of the collecting duct. Aldosterone induces rapid stimulation of H⁺-ATPase in the intercalated cells via PKC-dependent trafficking of the proton pump into the apical membrane. Large-conductance K⁺ (BK) channel activity is modulated by aldosterone and is involved in the shift of kidney anion exchanger (kAE) activity from the basolateral membrane to the apical membrane. (Modified from (1)).
1.9 Renal transport and hypertension

The earliest transcriptional change initiated by aldosterone is the up-regulation of SGK1. SGK1 is involved in the regulation of several ion channels (137) with ENaC being the first known channel up-regulated by SGK1 activity (138). Responsiveness of ENaC to SGK1 activation has been demonstrated in the CCD of the distal nephron and respiratory epithelial cells (137). The activation of renal SGK1 leads to increased Na\(^+\) reabsorption and blood pressure elevation (137) together with increased ROMK activity (137). ROMK is a K\(^+\) channel in the apical membrane of principal cells and is located in the ASDN (137). In addition to increasing the abundance of ROMK protein in the cellular membrane, SGK1 drives the pH sensitivity of ROMK towards acidic values which subsequently increases the current at normal cytosolic pH (137). The renal excretion of K\(^+\) is regulated by SGK1-dependent ROMK and this ability of the kidney to eliminate K\(^+\) load is impaired in vivo in gene-targeted mice that lack functional SGK1 (137).

The activity of the ubiquitin ligase Nedd4-2 is regulated by SGK1 and is considered to be a vital component in modulating the effects aldosterone has on ENaC. As per consensus models (figure 1.8), SGK1 expression is increased in response to aldosterone which leads to the phosphorylation of Nedd4-2 on crucial serine residues thus promoting its interaction with 14-3-3 proteins. Members of the 14-3-3 protein family have a molecular mass of ~30kDa and are a family of acidic, dimeric proteins that are distributed widely among eukaryotic cells (139-142). 14-3-3 has been linked to several biological activities including the activation of enzymes that are implicated in monoamine synthesis, for example tyrosine and tryptophan hydroxylases (143, 144). It is also implicated in regulating protein products of proto-oncogenes and oncogenes such as Raf-1 and Bcr-Abl protein kinases (145-149), and the retention of ligands in pro-apoptotic pathways (such as those involving Bad and FKHRL1) in the cytoplasm (150, 151). Proteomic studies (152-156) suggest that there are several hundred proteins whose physiological function is regulated by 14-3-3 proteins. It is often observed that binding between 14-3-3 and its target proteins is triggered by phosphorylation of the targets, particularly at specific Ser/Thr residues (157). Thus, the 14-3-3 family is believed to be a key regulator of a wide variety of cell signalling pathways mediated by protein phosphorylation.
This interaction of Nedd4-2 with 14-3-3 reduces the ubiquitin ligase activity of Nedd4-2 with regard to ENaC. This model proposed that ENaC, not highly ubiquitinated, will have prolonged endurance in the plasma membrane (158).

Figure 1.8. Model of ENaC regulation by Nedd4-2. The interaction of aldosterone (aldo) and MR induces the activity of SGK1 which in turn has a suppressing effect on the activity of the ubiquitin ligase Nedd4-2. As seen in step (i), Nedd4-2 normally functions as a catalyst for the addition of ubiquitin moieties (Ub) to ENaC. Ubiquitinated ENaC is subsequently removed from the plasma membrane (step ii) and in turn undergoes degradation (step iii) (Modified from (159)).

There are many studies which indicate that the current model for ENaC regulation by Nedd4-2 mediated-ubiquitination is incomplete (160, 161). These studies demonstrated as expected that Nedd4-2 deletion resulted in a substantial increase in the abundance of the β and γ ENaC subunits. Unexpectedly, their findings also showed that this increase was predominantly intracellular rather than at the plasma
membrane. Taken together, the work from Ronzaud et al (2013) and Traykova-Brauch et al (2008) support the hypothesis that the main effect of Nedd4-2 on ENaC is the modulation of intracellular degradation (Figure 1.8, step iii) instead of the removal of ENaC from the plasma membrane (Figure 1.8, step ii) and that the abundance of ENaCα is regulated by aldosterone. There are also reports that Nedd4-2 and SGK1 cooperate to regulate the activity of ENaC, while others suggest SGK1 regulates ENaC independently of Nedd4-2 (162). This author intends to investigate these hypotheses further in vitro in M1-CCD cell cultures using techniques such as immunoprecipitation and Western blot analysis.

Salt appetite and resulting NaCl intake is enhanced by SGK1 (138, 163, 164) resulting in increased activity which predisposes an individual to extracellular volume expansion and hypertension (165-170). In SGK knock-out mice, normal blood pressure is maintained under a normal diet (163) but when compared to the wild-type, these knock-out mice do not develop hypertension when hyperinsulinism is induced with a high fat or high fructose diet (163, 171-173). Consequently, hyperinsulinism can often lead to hypertension and it has been presumed to be through SGK1-dependent stimulation of renal tubular salt reabsorption (174, 175). Furthermore, SGK1-sensitive transport has been demonstrated to be a contributing factor in glucocorticoid-induced hypertension (175).

1.10. Aldosterone and signalling cascades

A number of strands of evidence point to MR as being the receptor responsible for initiating the aldosterone-induced rapid signalling cascades. The activation of protein kinases such as PKD and ERK1/2 by aldosterone for example can be inhibited with the use of MR-specific antagonists such as spironolactone and eplerenone (94, 102, 110). The ability to exhibit the rapid actions of aldosterone in MR-null cells can be conferred through exogenous expression of the receptor in Chinese hamster ovarian (CHO) and human embryonic kidney (HEK) cells (176). MR can be considered to be a multifunctional receptor. If recombinant MR, which lacks its DNA-binding and coactivator-binding domains is expressed, signalling events can be instigated by a rapid response to aldosterone independent of transcriptional activity (177). In terms of the intermediate phases that couple the aldosterone-MR interaction with the rapid
activation of protein kinases, there are still some questions to be addressed.

One signalling pathway that is known to have involvement in epidermal growth factor receptor (EGFR) trans-activation is the cytosolic tyrosine kinase c-Src pathway (178-180). When c-Src is activated, it phosphorylates EGFR and increases its activity. In a study by Krug et al (2002), it was found that when c-Src was inhibited, the effect of aldosterone was completely abolished. This indicates that aldosterone acts via c-Src (181). Further evidence in support of this was observed when aldosterone induced c-Src phosphorylation and it consequently also induced its activation. Moreover, Krug et al (2002) derived a working model whereby aldosterone stimulates c-Src in turn co-stimulating EGFR when EGF is present and consequently, the raf-MEK-ERK cascade is activated. Phosphorylated ERK1/2 can provoke several cellular responses that range from the activation of Na+/H+ exchange (NHE) to proliferation (95). It is possible for PKC to exercise two-fold action within the signalling network i.e. the inhibition of EGFR and the activation of ERK1/2 phosphorylation (181).
Rapid and Genomic Actions of Aldosterone in CCD

Figure 1.9. Rapid versus genomic effects of aldosterone. Aldosterone can diffuse across the basolateral membrane of the cell and bind to MR which induces dimerization and the translocation to the nucleus. This hormone-receptor complex can bind to GRE response elements and subsequently recruits other transcription factors (TFs). Here the complex acts as a ligand-dependent transcription factor that can induce the expression of genes for example, ENaC and SGK1. The binding of aldosterone to MR can also stimulate rapid protein kinase signalling pathways such as the activation of ERK1/2, PKC and PKD. This rapid activation occurs through the transactivation of EGFR via c-Src. Schematic obtained from (89).

It is well established that the trans-activation of EGFR is a fundamental step in linking this initiation signal to the aldosterone-responsive downstream signalling intermediates (95). It has yet to be determined by which molecular mechanism EGFR and its activation is coupled to MR but it is thought that it is EGFR ligand independent. EGFR is phosphorylated by a small tyrosine kinase, c-Src, within 5 minutes of treatment with aldosterone and this c-Src phosphorylation could be a significant transducing signal (182). Cytoplasmic MR is recruited into a complex of several...
proteins including heat shock protein 90 (Hsp90), this complex dissociates on MR activation (figure 1.9). The aldosterone-induced phosphorylation of EGFR by c-Src can be blocked by antagonising Hsp90 interactions with other proteins using the geldanamycin analogue 17-AAG. This also suppresses EGFR-dependent downstream signalling events initiated by aldosterone that include the activation of PKD (94) and the ERK1/2 mitogen activate protein (MAP) kinase (95).

1.11. Aldosterone signaling via Protein Kinase D

PKD isoforms are diacylglycerol (DAG) and PKC effectors that facilitate the actions of growth factors, hormones and other stimuli that can activate phospholipase C (PLC) (183). PKDs have a pair of C1 domains that bind to DAG and phorbol esters. Membrane-associated DAG can bind to and subsequently activate PKD and in turn recruits PKD via its C1 domains (184). PKC phosphorylates serine (Ser) 744 and Ser748 in the activation loop of PKD. DAG-stimulated PKCs (δ,ε,θ and η) have been shown to be PKD dominant activators (183). However, Ca⁺ and DAG-activated PKCs α, βi and βii have also been demonstrated to activate PKD (185).

PKC isoforms have diverse roles and regulate critical cellular processes such as proliferation and trafficking. In renal CCD cells, PKCα is activated independently of MR within 2 to 5 minutes following treatment with aldosterone (97, 107). During this process, the activation of PKCα is reliant on the kinase binding to aldosterone directly. We have previously shown that two other PKC isoforms, PKCδ and PKCε can also exhibit rapid activation in response to aldosterone. However, this activation is not reliant on the kinase binding directly to aldosterone but is coupled to MR via the trans-activation of EGFR (109). PKD isoforms are downstream targets for active PKCs and can be activated by agonists of G-protein coupled receptors (GPCRs) (186). Aldosterone induces both rapid phosphorylation and activation of PKD1 (94). There are three known members of the PKD family, PKD1/PKCμ (186), PKD2 (187) and PKD3/PKCν. PKD can activate substrates such as cJun N-terminal kinase (JNK) (188) and also has the ability to activate the NHE1 Na⁺/H⁺ antiporter (189). PKD can be phosphorylated and activated by novel PKC isoforms for example PKCε and PKCη (183). We have demonstrated that the activation of PKD1 in M1-CCD cells is PKCε-
dependent (109).

PKD has been implicated in the many facets of the regulation of sub-cellular trafficking by either maintenance of the structure in the Golgi or by the regulation of fission at the trans-Golgi network (TGN)(figure 1.10). PKD can also regulate Golgi to membrane vesicle trafficking by activating phosphatidylinositol (PtdIns) 4-kinase (PI4KIIIβ) (186) and phosphatidylinositol 4-phosphate 5-kinase (PI4P5K), (190).
Figure 1.10. Regulation of cell trafficking by PKD1. Activated PKD1 phosphorylates and activates phosphatidylinositol 4-kinaseIIb (PI4KIIib) at the cis- and trans-Golgi promoting the synthesis of phosphatidylinositol-4-phosphate (PI4P) in the Golgi membrane. Ceramide transport protein (CERT) is released from the endoplasmic reticulum (ER) in complex with ceramide (CER) and binds to PI4P so transporting lipid from the ER to the Golgi. Ceramide is processed at the Golgi to produce sphingomyelin (SM) and diacylglycerol (DAG). DAG recruits PKD1 and novel PKC isoforms to the Golgi, CERT is a substrate by PKD1 and CERT phosphorylation results in its release and re-cycling back to the ER. Multiple proteins are recruited to sites of PI4P biogenesis in the Golgi. These include the arfaptin family proteins. These proteins contain a BAR (Bin/Amphiphysin/Rvs) domain with a concave anionic surface that interacts with negatively charged lipid membranes to facilitate vesicle fission. Other known substrates for PKD1 include actin cytoskeleton regulatory proteins such as coflin, LIM kinase (LIMK) and rhotekin that contribute to actin-independent intra-cellular vesicle trafficking. (21).
Multiple studies have demonstrated how PKD family members undergo rapid redistribution in response to cell stimulation. Both PKD1 and PKD2 translocate to DAG-containing microenvironments present in the plasma membrane from the cytosol (191) while PKC-dependent reverse translocation from the plasma membrane to the cytosol ensued with subsequent nuclear accumulation (192). In comparison, the PKD3 isoform has been shown to shuttle continuously between the nucleus and the cytoplasm (193). The PKD isoforms can collectively localise at the Golgi complex (194, 195) and mitochondria (196). Additionally, PKD1 and PKD2 contain PDZ-binding motifs in the COOH termini and recently, the PDZ-domain containing protein Na⁺/H⁺ exchanger regulatory factor 1 (NHERF-1) was observed interacting with these motifs indicating that PKD can form complexes with NHERF-1 (197). Consequently, PKD has the ability to regulate targets in diverse subcellular locations and therefore control multiple cellular activities. In view of this, PKD has been implicated in regulation of several fundamental biological processes including cell proliferation, migration, differentiation and membrane trafficking (183).

Our rationale for studying PKD is that the abnormal stimulation of cell proliferation in the distal nephron is that it may be a contributing factor in the development of polycystic kidney disease (198). We hypothesize that PKD isoforms have a role in the trafficking and membrane targeting of ENaC subunits and Na/K pumps leading to dysregulation of Na⁺ reabsorption and the manifestation of renal cysts and hypertension which are symptomatic of these patients.

1.12 Cardiovascular and renal effects of aldosterone-MR-PKD signalling

PKD isoforms have been implicated in several pathologies associated with both the cardiovascular and renal systems (figure 1.11). For example, PKD1 has been implicated in cardiac hypertrophy while PKD1 and PKD2 have been observed in the proliferation of endothelial cells and angiogenesis. Aldosterone-MR signalling has been well documented in the kidney and work from our group has demonstrated its effects on the activation of PKD1. Aldosterone-induced PKD1 activation leads to the regulation of Na⁺ reabsorption through its effect on the trafficking and activation of transporters such as ENaC and Na⁺/K⁺-ATPase.
Figure 1.11. Cardiovascular and renal effects of aldosterone-MR-PKD signalling.
Aldosterone can activate PKD isoforms via its interaction with MR. The activation of PKD isoforms have been implicated in cardiac hypertrophy, angiogenesis and endothelial proliferation along with the reabsorption of Na$^+$ in the kidney.

Aldosterone has been shown to promote electrolyte balance, myocardial and vascular fibrosis, damage to the vasculature, impaired arterial compliance and dysfunctionality of the baroreceptor (199-202). Up until the early 1990’s, aldosterone-receptor blockers were not used frequently in patients that exhibited heart failure. In response to these observations, the Randomized Aldactone Evaluation Study (RALES) was initiated in order to determine if a daily treatment with the MR agonist, spironolactone, would have a significant reduction in the risk of death in patients that had severe heart failure as a result of systolic left ventricular dysfunction and who were receiving standard therapy (for example ACE inhibitors) (203). Upon entry to the study, patients were patients were randomly assigned in a double-blind fashion to receive either 25 mg of spironolactone (Aldactone, Searle, Skokie, Ill.) once daily or a matching placebo. After eight weeks of treatment, the dose could be increased to 50 mg once daily if the
patient showed signs or symptoms of progression of heart failure without evidence of hyperkalemia. This study found that spironolactone reduced the risk of death or hospitalisation due to cardiac causes. Kaplan–Meier analysis of the RALES study showed MR antagonism with spironolactone improved the patient survival in hypertension related comorbidity (figure 1.12). The results obtained from this study suggested that not only did spironolactone effect Na\(^+\) retention, but it appears that the antagonist could also be cardio-protective.

Figure 1.12. MR antagonism with Spironolactone improves patient survival in hypertension related comorbidity. Kaplan–Meier Analysis of the Probability of Survival among Patients in the Placebo Group and Patients in the Spironolactone Group. The risk of death was 30 percent lower among patients in the spironolactone group than among patients in the placebo group (P<0.001). (Figure obtained from (203)).
1.13. Other hormonal regulators of renal Na\(^+\) reabsorption

1.13.1 Insulin

It has been shown that changes in the physiological concentration of insulin are adept at affecting electrolyte transport in the kidney. In diabetic patients, it had been documented that natriuresis occurred following the withdrawal of insulin infusion (204) while further studies in both humans and dogs determined that insulin increased plasma Na\(^+\) and reduced Na\(^+\) excretion by the kidney, and that this was independent of blood glucose levels, glomerular filtration rate and plasma levels of aldosterone (205, 206). Furthermore, studies utilising perfusion and micropuncture of distal tubules exposed an increase in Na\(^+\) reabsorption in response to insulin (207). It has also been reported that insulin can activate Na\(^+\)/K\(^+\)-ATPase and ENaC (207, 208) either by inducing an increase in channel abundance at the membrane (209) or by modulating the open probability and the amplitude of the single channel current (46, 210). Knockout studies showed that a loss of the insulin receptor in the collecting duct produced reduced ENaC activity and a reduction in blood pressure. These observations point to a role for insulin in the modulation of blood pressure and renal electrolyte homeostasis.

1.13.2 Vasopressin

Vasopressin greatly increases the reabsorption of Na\(^+\) by the direct activation of ENaC in renal CCD M1 cells and A6 renal epithelial cells as well as perfused tubules from rat and rabbit and rabbit CCD suspensions (211). Vasopressin exerts a pro-absorptive effect that is mediated by the vasopressin V2 receptor (a G-protein coupled receptor) where V2 localisation has been observed in CNT and CD. Chronic treatment of rats with vasopressin either by the infusion of Brattleboro rats (that lack endogenous vasopressin) with a V2 receptor selective agonist (dDAVP) or by the restriction of water in Sprague Dawley rats (which raises endogenous vasopressin) resulted in an increase in the abundance of β- and γENaC, with no consistent effect on αENaC (212) at the protein level. By continually exposing RCCD1 renal cells to vasopressin, mRNA expression of β- and γENaC (but not αENaC) were increased, which coincided with a surge in the uptake of Na\(^+\) by these cells (213).
1.13.3 Angiotensin II

Angiotensin II is upstream of aldosterone in the RAAS signal cascade and has been known to regulate distal tubule Na\(^+\) transporters independently of aldosterone (214). This occurs through binding to G protein coupled angiotensin II receptors (AT1 and AT2) that are expressed in the DCT and CD cells. Na\(^+\) reabsorption is mediated by AT1 receptors while AT2 receptors facilitate natriuresis. It was suggested that the adaptation to low intake of dietary Na\(^+\) coupled with an increase in \(\alpha\)ENaC and NCC abundance was due to an increase in angiotensin II. In AT1a receptor knockout mice, there was a decrease in the upregulation of NCC and \(\alpha\)ENaC in response to a low NaCl diet despite a significant increase in circulating aldosterone levels, compared to wild type mice.

1.14 Sodium transport in renal health and disease

Na\(^+\)/K\(^+\)-ATPase is responsible for the exchange of Na\(^+\) and K\(^+\) along with providing a ubiquitous electrogenic Na\(^+\) pump to actively transport of Na\(^+\) ions out of the cell. Typically, Na\(^+\)/K\(^+\)-ATPase is highly expressed in the basolateral plasma membrane in renal epithelial cells. The distribution of Na\(^+\)/K\(^+\)-ATPase is linked to Na\(^+\) in an apical to basolateral membrane direction. Another crucial role of Na\(^+\)/K\(^+\)-ATPase distribution is the production of Na\(^+\) ion gradients that are in turn regulators of other Na\(^+\) coupled ion measures such as osmotic fluid reabsorption (215-217). One of the significant abnormalities in the pathophysiology of cystic epithelia in ADPKD is the mispolarisation of Na\(^+\)/K\(^+\)-ATPase to the apical membrane. This mislocalisation results in the basolateral-to-apical membrane transport of Na\(^+\) ions which results in the accumulation of fluid at the lumen (215-217). Additional membrane and membrane-associated proteins that exhibit aberrant polarization in cystic epithelia in ADPKD are EGFR, ankyrin and E-cadherin (218). Modifications, such as the reversed polarization and mistargeting of Na\(^+\)/K\(^+\)-ATPase will have detrimental physiological consequences in terms of normal renal growth and function. These consequences include, but are not limited to, the reversal of ion and fluid transport from an absorptive mode to that of a secretory mode. It may also lead to the formation of an “autocrine-loop” for proliferation driven by EGF (219, 220).
We recently demonstrated that aldosterone stimulated the activation of PKD1 in the M1-CCD cell line through an EGFR-coupled signalling cascade that was initiated by the interaction of aldosterone with MR (94). We have demonstrated how PKD1 activation by aldosterone in M1-CCD cells played a role in early, sub-cellular trafficking events that resulted in the redistribution of eCFP-tagged ENaC sub-units within a few minutes of aldosterone treatment (109). The action of aldosterone in regulating transporter activity and trans-activating growth factor signalling cascades raises the question of how aldosterone affects ENaC signalling. Patients with hypertension present with elevated levels of aldosterone are often likely to develop renal fibrosis, therefore it is important to understand aldosterone’s fine tuning of renal Na⁺ handling. As hypertension can occur due to a dysregulation of aldosterone signalling, using inhibitors of MR (such as eplerenone and spironolactone) may provide a novel therapeutic approach to treating pathologies such as kidney disease.

1.15 Polycystic kidney disease

Polycystic kidney disease is one of the most common genetic disorders which presents with the development of large fluid-filled cysts that displace normal renal tubules and disrupt kidney function. There are two types of polycystic kidney disease, autosomal dominant (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD). Not only can polycystic diseases be inherited, they may also be acquired in adult life. Cysts can develop in the kidneys as a consequence of aging, dialysis or hormonal influences (216, 221). However the loss of kidney function is much greater when cyst formation is the result of genetic proliferative syndromes, such as ADPKD (216).

ADPKD disease is largely characterised by the enlargement of cysts in the renal tubules as a consequence of an increase in the proliferation of epithelial cells, abnormal fluid secretion and the mispolarization of many membrane proteins (111-113). Previous studies have shown that similarities between the expression and the distribution of several proteins in ADPKD and foetal epithelia suggest a role for polycystin 1 in the regulation of epithelial polarization (114).

ADPKD is one of the most prevalent inherited genetic diseases with the ultimate outcome being end stage renal disease (ESRD) or even renal failure. The association between hypertension and ADPKD progression is established, but the cause and effect relationship is less clear and may provide novel approaches to treating
progressive kidney disease. In this thesis, two *in vitro* models will be utilized to further understand the role of PKD in the regulation of ENaC.

1.15.1 Autosomal dominant polycystic kidney disease

The most common form of polycystic kidney disease is ADPKD which has an occurrence of 1 in 800 live births. Approximately 6 million people worldwide are affected and accounts for 7-10% of patients undergoing hemodialysis (222). There are two types of ADPKD: type I resulting from mutations in the *PKD1* gene and type II which is caused by mutations in the *PKD2* gene. Type I ADPKD accounts for 85-90% of cases (223) while type II only accounts for the remaining 10-15% of cases (224). Both types of ADPKD display similar physiological and pathological features however, type II presents with a later onset of symptoms coupled with a slower rate of progression to renal failure which increases their life expectancy (69.1 years) when compared to those with type I ADPKD (53 years)(225). There are some cases of patients with ADPKD that do not have a mutation in either *PKD1* or *PKD2* which has suggested that there may be a rare form of the disease (226) determined by a mutation in a proposed *PKD3* gene although this has not yet been identified. Patients with transheterozygote mutations in both *PKD1* and *PKD2* genes have a more severe clinical course when compared to those with only a single mutation (227).

1.15.2 Proliferation in polycystic kidney disease

One of the major characteristics of ADPKD is the vast enlargement of cysts in the kidney. Patients with ADPKD usually present with hypertension, hematuria, polyuria, and are prone to urinary tract infections and often renal stones. Clinically significant cysts are also common in the liver (particularly in women), intestine and pancreas in addition to hundreds to thousands of renal cysts. These patients carry an increased risk of having an aortic aneurysm and sometimes heart-valve defects (228) while some patients are five times more likely than the general population to die suddenly from a ruptured intra-cerebral aneurysm (229).
In ADPKD, cysts of various sizes throughout the medulla and cortex are derived from every segment of the nephron. A single layer of epithelial cells line the tubule wall, expand and rapidly close off from the original tubule (Figure 1.13B)(216). This is in contrast to ARPKD in which smaller and elongated cysts stem from ectatic expansions of collecting ducts. These ARPKD cysts also maintain contact with the nephron the originated from (Figure 1.13C).

**Figure 1.13. Mechanisms of Cyst Formation in Polycystic Kidney Disease.** (A) Cysts originate as expansions from the renal tubule. (B) These cysts arise in every segment of the tubule and rapidly close off the originating nephron in ADPKD. In contrast, the cysts in ARPKD are derived from collecting tubules and these remain connected to the originating nephron (C). (Modified from (222)).

A strictly controlled equilibrium between cell proliferation and apoptosis is required for normal growth and differentiation of the kidney, and also maintenance of the normal postnatal renal structure. However, in polycystic kidney disease, this fundamental process is disrupted. In both ADPKD and ARPKD, apoptosis is abnormally obstinate (230) resulting in the destruction of normal renal parenchyma and subsequent tolerance of cystic epithelial proliferation. Prior to birth, normal renal tubular epithelial cells will cease to proliferate, in contrast to cystic epithelial cells that can proliferate abnormally throughout life in patients with ADPKD (231). Furthermore, epithelial cells from these patients that have been cultured in vitro display an increased intrinsic capability for proliferation and survival, suggesting a disruption of normal apoptotic signalling. Studies that have genetically manipulated the proliferation capacity of tubular epithelial cells in mice have also resulted in the development of renal cystic diseases (232-235).
The movement of fluid which drives cyst formation is stimulated by cAMP and requires the apical membrane expression of the cystic fibrosis transmembrane conductance regulator (CFTR) CFTR and NKCC1 which is a basolateral Na⁺/K⁺/Cl⁻ co-transporter (236-238). The expression, localization and activity of Cl⁻ channels may be affected by polycystin 1 (PC1) and if full length PC1 is expressed with the CFTR channel in vitro results in a decrease in CFTR surface localization and cAMP-stimulated activity of the channel which implicates misregulation of PC1 in ADPKD leading to increased CFTR activity (239). Cl⁻ transport appears to be enhanced when just the C-terminal of PC1 is expressed which prolongs ATP-stimulated Cl⁻ conductance in transfected cortical collecting duct cells and an up-regulated Cl⁻ transport in *Xenopus* oocytes (240, 241). cAMP levels may be regulated by polycystin proteins as there is an association of cystic disease with the misregulation of phosphodiesterases that break down cAMP (242).

EGF plays an important role in renal cyst expansion and epithelial cells from the cysts of patients with ADPKD and ARPKD are typically susceptible to the proliferative stimulus of EGF. Overexpression and uncharacteristic location of EGF receptors on the luminal surface (i.e. the apical side) of cyst-lining epithelial initiates a sustained cycle of autocrine-paracrine prompt of proliferation in cysts (218). Additional cytokines and growth factors in addition to cyclic adenosine monophosphate (cAMP) and adenosine triphosphate (ATP) in the fluid from cysts have shown proliferative effects on epithelial cells in vitro (243-245). EGF-dependent growth of renal cysts may also be stimulated by these factors (246).
1.15.3 Secretion in polycystic kidney disease

Na⁺ ion gradients established by the Na⁺/K⁺-ATPase in the basolateral membrane of tubular cells in conjunction with other ion and fluid transporters located in the basolateral and apical membranes are responsible for the reabsorption of fluid in normal kidneys. In patients with polycystic kidney disease, Na⁺/K⁺-ATPase is located in the apical membrane of tubular epithelial cells (217) while the Na⁺,K⁺,2Cl⁻ symporter is misplaced to the basolateral membrane of the epithelia (247). Studies of the α and β subunits of the Na⁺/K⁺-ATPase complex revealed the normal adult kidneys comprise of a α1β1 complex located at the basolateral area of the tubule. In contrast, patients with polycystic kidney disease contain α1β2 complexes in the apical membrane (248). Na⁺/K⁺-ATPase pumps comprised of a α1β2 complex also occur in the apical membrane of renal tubules in a normal developing fetus (249). It appears that in ADPKD, there is a failure to down-regulate the β2 isoform transcription after birth, which in turn contributes to a miss-targeting of Na⁺/K⁺-ATPase expression into the apical membrane.

Other features related to transport processes in renal cysts include the insertion of water channels aquaporin 1 or aquaporin 2 in the epithelia from patients with ADPKD while patients with ARPKD express aquaporin 2 alone in their cysts (250). The apical membrane releases higher levels of ATP in patients with ADPKD which may further aggravate secretion (251), while levels of intracellular cAMP are also important in regulating secretion in cysts along with the cystic fibrosis transmembrane conductance regulator chloride channels (CFTR) in the apical membranes of cystic epithelial (252).

1.15.4 Mechanism of cyst formation in polycystic kidney disease

Genetically dominant at the organism level, ADPKD is recessive at a cellular level. Individuals with ADPKD inherit one mutated copy of polycystin-1 (PC1) or polycystin-2 (PC2) and the kidneys of these patients develop and function normally. However, over a period of time, cysts begin to form in the kidneys and many studies indicate that the cells lining these cysts lose both functional copies of a polycystin gene (253, 254). This strongly suggests that an additional somatic mutation may result in the formation
of cysts. This model of cyst formation indicates that individual cysts arise as consequence of a distinct somatic mutation event which could explain the slow progression of ADPKD. This coupled with the level of polycystic kidney disease 1 protein expression and the stage of kidney development affected by such a mutation may impact on disease progression (255-259).

In rodent *in vivo* models, temporally controlled inactivation of PC1 or PC2 expression has uncovered that loss of these proteins in a developing kidney results in more severe cystic disease that the loss of these proteins in a mature kidney (260-262). What this data proposes is that a loss of polycystin function during stages of rapid cell growth in the course of postnatal renal development establishes a predisposition to cystogenesis. It is also plausible that when individuals age, the kidneys may experience ischemic injury to the epithelial cells of the tubules.
1.15.5 Cyst expansion in polycystic kidney diseases

One major outcome of ADPKD progression is the formation of fluid-filled cysts which is in strong contrast to normally compact tubules in a healthy kidney (Figure 1.14).

**Figure 1.14. Cyst formation at the level of the cell, nephron and kidney.** Flaws in the encoding of PC1 or PC2 often results in aberrant gene transcription, cell proliferation and ion secretion leading to the formation of fluid-filled cysts. One effect of the cyst growth from individual nephrons is the displacement of normal renal parenchyma with subsequent formation of a cyst-filled kidney with a reduction in its functional capacity. Schema from (263).

As apical CFTR Cl⁻ channel stimulated by cAMP mediates fluid secretion into the lumen of cysts, these factors may present as promising molecular targets for treatment of polycystic kidney disease. An inhibiting compound, CFTRinh172, seems to slow cyst expansion substantially (264). Activity of a basolateral K⁺ channel is required to maintain the electrochemical potential that directs chloride and fluid secretion has also been proposed as a potential target to inhibit cyst expansion (265).
1.16 Thesis hypotheses and aims

Our research group has published a series of papers describing the molecular mechanisms of PKD1 modulation of Na\(^+\) channel trafficking and function by aldosterone (1, 5, 6, 89, 94, 98, 109, 266, 267). PKD is a key modulator of intracellular trafficking through regulation of vesicle fission from the TGN to promote the transport of proteins to the cell membrane. PKD1 directly phosphorylates the TGN-associated PI4KII\(\beta\) that is a key regulator of the lipid chemistry involved in membrane fission (184). PKD1 (and PKD2) can also associate with scaffold protein NHERF1 at the cell membrane, where the activity of the kinase may be directed towards membrane targets (197).

We have shown that PKD1 is involved in the trafficking of the ENaC channel subunits to the membrane in a process that is initiated by aldosterone. We have found that the PKD2 isoform is also regulated by aldosterone and affects ENaC trafficking. We hypothesise that PKD2 has a role in the stabilisation of the channel and that aldosterone disrupts this stability leading to a loss in ENaC activity by preventing PKD2 from trafficking ENaC back into the cytosol. This thesis aims to extend our investigations to include another PKD isoforms (PKD2 and PKD3), which may be an important regulator of sub-cellular trafficking. In particular, this project looks to identify the roles of PKD2 in the regulation of renal Na\(^+\) transport in the cortical collecting duct through the modulation of ENaC activity.

The first objective was to gain new insights into the role of PKD2 in the regulation of Na\(^+\) homeostasis. The work was advanced in an M1 CCD \textit{in vitro} model to establish the contribution of aldosterone-induced activation of PKD2 to Na\(^+\) reabsorption. We investigated if knocking down PKD2 leads to hyper-absorption of Na\(^+\) in the CCD cells thus potentially elucidating a role for PKD2 in hypertension. Pharmacological interventions with antagonists of receptors such as MR (eplerenone) and EGFR (tyrphostin) were conducted in order to predict if a dysregulation in the aldosterone-induced PKD2 signalling pathway could lead to modulation of normal ENaC activity.

A commercially available \textit{in vitro} ADPKD model was used to explore the possible implications aldosterone has on renal fibrosis and cyst formation. WT 9-12 cyst-derived cells were used a model for ADPKD and initial findings (Dooley & Yusef, unpublished) suggest that knocking down PKD2 could lead to altered sodium
trafficking. Taken together, these models were used to assess the impact of a dysregulation of aldosterone signalling in chronic pathologies of the kidney.
Chapter 2

Materials and Methods
2.1 Materials

All reagents and chemicals used in this thesis were molecular-grade.

2.2 Cell Culture

2.2.1 Culturing of M1-CCD cells \textit{in vitro}

The M1 (ATCC, Catalogue Number CRL-2038) murine cortical collecting duct (M1-CCD) cell line was used as an experimental model of the aldosterone-sensitive distal nephron (ASDN). The M1-CCD cell line is derived from renal CCD micro-dissected from a mouse transgenic for the early region of SV40 virus (strain Tg(SV40E) Bri7) (268). M1 cells readily develop into confluent epithelial monolayers with a high electrical resistance when grown \textit{in vitro} (268-272) while retaining features of the CCD including morphology (268, 271), hormone responsiveness (97, 111, 273), specific antigen expression (268, 273) along with the capability for electrolyte transport (268-274). These traits allow M1 cells to characterise an effective reductionist model for the study of ion transport in the CCD. M1 cells were cultured in 25 cm$^2$ or 75 cm$^2$ culture flasks and were maintained in 1:1 Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (DMEM:F-12) without phenol red, supplemented with foetal bovine serum (5%), L-glutamine (2 mM), penicillin (100 units ml$^{-1}$), streptomycin (100μgml$^{-1}$) and dexamethasone (1 μM). Cultures were maintained in an atmosphere of 70% humidity, 5% CO$_2$ at 37°C. While in culture, the medium was replaced every 2-3 days. The cells were passaged when the confluency reached 80 – 90%.

In order to extract protein from cell lysates, cells were cultured on 6 cm$^2$ diameter culture plates until they reached 90% confluency. Cells were washed with serum-free media and maintained in serum-free DMEM:F-12 for 24 hours prior to treatment with either the steroid or antagonists. M1 cells used for immunocytochemistry purposes were cultured on glass coverslips that had been sterilised using 100% ethanol and UV light.
For Ussing chamber experiments M1 cells were seeded on permeable supports with a pore size of 0.4 µm. (Corning, VWR Catalogue Number 29444-282). For experimental purposes, these cells were cultured until they reached a TER > 0.8 kΩ*cm².

2.2.2 Subculture of M1 CCD cells

The media was removed and the cells were washed briefly with 2 ml of trypsin (0.025%). This trypsin was then aspirated and a fresh 2.5 ml was added and the cells were incubated for 5-10 minutes at 37°C until all the cells had detached from the flask. The detached cells were then re-suspended in 10 ml of fresh media and used to seed a new flask or culture plates.

2.2.3 Culturing of WT 9-12 cells in vitro

The WT-9-12 cell line (ATCC-CRL-2833) is an immortalised epithelial cell line derived from over 30 individual renal cysts obtained from 11 patients with ADPKD. The cells were immortalised with wild-type (WT) recombinant adenosimian SV40 virus. WT 9-12 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) without phenol red, supplemented with foetal bovine serum (10%), L-glutamine (2 mM), penicillin (100 units.ml⁻¹) and streptomycin (100 µg.ml⁻¹). Cultures were maintained in an atmosphere of 70% humidity, 5% CO₂ at 37°C. While in culture, cells were fed every 2-3 days. The cells were passaged every 7 days.

2.2.4 Subculture of WT 9-12 cells

The media was removed and the cells were washed briefly with 2 ml of trypsin (0.025%). This trypsin was then aspirated and a fresh 2.5 ml was added and the cells were incubated for 5-10 minutes at 37°C until all the cells had detached from the flask. The detached cells were then re-suspended in 10 ml of fresh media and used to seed a new flask or culture plates.
2.3 Treatment of cells

Cells were serum starved for a period of 24 hours prior to treatment. Dilutions of agonists and antagonists were prepared in serum free media. Aldosterone was prepared as a 50 mM stock solution in ethanol and further diluted in serum-free medium, to a final concentration of 10 nM. Vehicle controls are the equivalent concentration of ethanol diluted in culture medium.

2.4 Immunofluorescence using confocal microscopy

Fluorophores are commonly used in fluorescence microscopy in order to label proteins and structures of interest. In order to distinguish between cell types in cryosections from the rat kidney, the water channel aquaporin-2 (AQP2) was used as a marker for the apical membrane of principle cells. In the case of M1-CCD epithelial monolayers, wheat germ agglutinin conjugated to Alexa 633 was used to stain the apical membrane of the cells. TRITC-phalloidin was used to stain the actin filaments in WT 9-12 cells.

Cells were cultured on glass coverslips until confluent and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Following fixation, samples were permeabilised for 1 hour with 0.3% Triton X-100 prior to blocking with 3% BSA/PBS. Primary antibodies (table 2.1.) were diluted accordingly with blocking buffer and 50 μl was sufficient for each coverslip. Incubation with the primary antibody was overnight at 4°C in a humidified chamber. The secondary antibody was diluted in blocking buffer and the coverslips incubated as before for 30 minutes at room temperature. Coverslips were sealed onto microscopy slides using hard-set Vectashield containing DAPI. Samples were imaged with a Zeiss LSM710 upright laser point scanning confocal microscope. Images were acquired using a Zeiss Plan-APOCHROMAT 63X/1.4 Oil DIC objective.
Table 2.1. List of antibodies used for immunocytochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Diluent</th>
<th>Manufacturer</th>
<th>(Catalogue #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquaporin 2</td>
<td>Mouse</td>
<td>1:100</td>
<td>3% BSA/PBS</td>
<td>Santa Cruz Biotech Ltd</td>
<td>(sc-47710)</td>
</tr>
<tr>
<td>Alexa 488</td>
<td>Goat</td>
<td>1:500</td>
<td>3% BSA/PBS</td>
<td>Invitrogen</td>
<td>(A-11036)</td>
</tr>
<tr>
<td>Alexa 568</td>
<td>Goat</td>
<td>1:500</td>
<td>3% BSA/PBS</td>
<td>Invitrogen</td>
<td>(A-10667)</td>
</tr>
<tr>
<td>ENaCα</td>
<td>Rabbit</td>
<td>1:100</td>
<td>3% BSA/PBS</td>
<td>StressMarq</td>
<td>(SPC-403D)</td>
</tr>
<tr>
<td>ENaCβ</td>
<td>Rabbit</td>
<td>1:100</td>
<td>3% BSA/PBS</td>
<td>StressMarq</td>
<td>(SPC-404D)</td>
</tr>
<tr>
<td>ENaCy</td>
<td>Rabbit</td>
<td>1:25</td>
<td>3% BSA/PBS</td>
<td>Abcam</td>
<td>(ab3468)</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase β1</td>
<td>Mouse</td>
<td>1:200</td>
<td>3% BSA/PBS</td>
<td>Abcam</td>
<td>(ab2873)</td>
</tr>
<tr>
<td>Nedd4-2</td>
<td>Rabbit</td>
<td>1:100</td>
<td>3% BSA/PBS</td>
<td>Abcam</td>
<td>(ab168349)</td>
</tr>
<tr>
<td>Nedd4-2 (pS448)</td>
<td>Rabbit</td>
<td>1:100</td>
<td>3% BSA/PBS</td>
<td>Abcam</td>
<td>(ab46521)</td>
</tr>
<tr>
<td>PKD1</td>
<td>Rabbit</td>
<td>1:25</td>
<td>3% BSA/PBS</td>
<td>Cell Signaling</td>
<td>(90039)</td>
</tr>
<tr>
<td>PKD2</td>
<td>Rabbit</td>
<td>1:25</td>
<td>3% BSA/PBS</td>
<td>Abcam</td>
<td>(ab7281)</td>
</tr>
<tr>
<td>PKD3</td>
<td>Rabbit</td>
<td>1:50</td>
<td>3% BSA/PBS</td>
<td>Cell Signaling</td>
<td>(5655)</td>
</tr>
<tr>
<td>WGA</td>
<td>N/A</td>
<td>1:200</td>
<td>Ice cold PBS</td>
<td>Thermo Fisher Scientific</td>
<td>(W21404)</td>
</tr>
<tr>
<td>TRITC-phalloidin</td>
<td>N/A</td>
<td>1:1000</td>
<td>3% BSA/PBS</td>
<td>Thermo Fisher Scientific</td>
<td>(R415)</td>
</tr>
</tbody>
</table>
2.5 PKD isoform expression in micro-dissected mouse renal tubules

Glass microscopy slides were cleaned with ethanol and lint free optical tissue paper. The slides were prepared by drawing a hydrophobic ring onto the slide and covering the enclosed area with 20 μl of tissue adhesive. The tissue adhesive solution was prepared by mixing 170μl of 0.1 N sodium bicarbonate (NaHCO₃) with 30 μl of Cell Tak.

L-15 Leibowitz medium in powder form was supplemented with L-glutamine and L-amino acids. 20 mg of type II collagenase was dissolved in 15 ml of L-15 medium and the pH adjusted to 7.5 using 1 M NaOH. Anaesthetic was made up adding 400 μl of Ketavet and 150 μl of Rompum to 5 ml of distilled water. 9-week-old female C57/BL6 mice were anesthetised intraperitoneally with 0.5 ml of this anaesthetic solution and allowed for it to take full effect. The collagenase solution was perfused through the mouse arterially and the kidneys extracted. The kidneys were de-capsulated and sliced into <1 mm slices. The tubules were microdissected out using a stereo-microscope and collected before being transferred to the glass microscopy slides and gently held in place with the tissue adhesive. All animal work was carried out according to the principles of German legislation. This study was approved by the state veterinary health inspectorate (license no. 621.2531.32-21.05). Housing and care of the animals was under the governance of the responsible regulatory authority at the Friedrich-Alexander Universität Erlangen-Nürnberg (FAU), Erlangen, Germany.

Dissected renal tubules were fixed for 30 minutes with 4% paraformaldehyde/PBS solution on ice prior to being permeabilized with 0.1% Triton X-100 at room temperature. Blocking was for 1 hour with 1X Roti-Immunoblock also at room temperature. Primary antibodies (table 2.2) were diluted 1:50 with 5% BSA and the samples were incubated overnight at 4°C. The secondary antibody (tagged with Cy3, Dianova, Cat. No. 711165152) was diluted 1:400 in 5% BSA with an incubation period of 2 hours in the dark. The tubules were mounted with Fluoshield containing DAPI (Sigma) and imaged using a Zeiss Apotome. The ZEISS ApoTome used in this experiment uses evenly spaced grids in the aperture plane that serve as a mask through which the sample of interest is illuminated. This grid is inserted into the light path of the microscope and then uses an epi-illuminator lens system that projects a
shadow of the grid lines into sharp focus that are then superimposed on the specimen when observed in the focal plane of the objective. The system acquires three separate images of the sample and gathers them in sequence by adjusting the grid by one-third between each image that is captured. The resulting set of raw images is then processed by the application software resulting in a single, sharp image. The combination of the three raw images acquired by the ApoTome is comparable to a normal widefield image of a thinner sample. An advantage of using the ApoTome over a widefield fluorescent microscope is that the composite image acquired can be observed immediately whereas a widefield image requires deconvolution image processing that requires up to 20 minutes for the computation to be finalised. An additional advantage of using an ApoTome is that the resolution is equivalent to that achieved by confocal microscopy.

Table 2.2. Antibodies and the dilutions used in immunocytochemistry of micro-dissected mouse renal tubules.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Dilution Buffer</th>
<th>Manufacturer</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit PKD2</td>
<td>1:50</td>
<td>5% BSA</td>
<td>Abcam</td>
<td>(ab7281)</td>
</tr>
<tr>
<td>Goat PKD3</td>
<td>1:50</td>
<td>5% BSA</td>
<td>Santa Cruz</td>
<td>(sc-376024)</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG-Cy3</td>
<td>1:400</td>
<td>5% BSA</td>
<td>Dianova</td>
<td>(711-165-152)</td>
</tr>
<tr>
<td>Donkey anti-goat IgG-Cy3</td>
<td>1:400</td>
<td>5% BSA</td>
<td>Dianova</td>
<td>(205-165-108)</td>
</tr>
</tbody>
</table>
2.6 SDS-PAGE and Western Blotting

The electrophoretic transfer of proteins from sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) to nitrocellulose membranes was used to detect proteins in this work as was first described by Towbin et al (1979) (275). Cell lysates were prepared using M2 lysis buffer (table 2.3.) and cleared by centrifugation (14,000 rpm for 15 min) prior to being dissolved in 2X Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH approx. 6.8)(1/1, v/v). Proteins were separated by SDS-PAGE either on 8% or 10% polyacrylamide gels (table 2.4 and 2.5. respectively) unless otherwise indicated. All buffers used are outlined below. Gels were run at a constant voltage (125 V) for approximately 90 minutes.

Table 2.3. M2 lysis buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock Concentration</th>
<th>To make 200ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris pH7.6</td>
<td>1M</td>
<td>4 ml</td>
</tr>
<tr>
<td>0.5% NP40</td>
<td>50 mM</td>
<td>1 ml</td>
</tr>
<tr>
<td>250 mM NaCl</td>
<td>3M</td>
<td>16.6 ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5 mM</td>
<td>0.22 g</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.5 mM</td>
<td>0.23 g</td>
</tr>
<tr>
<td>Na⁺ orthovanadate</td>
<td>0.2M</td>
<td>5 μl</td>
</tr>
<tr>
<td>Boehringer proteinase</td>
<td>1 per 10ml (added</td>
<td></td>
</tr>
<tr>
<td>inhibitor cocktail</td>
<td>freshly)</td>
<td>tablet</td>
</tr>
</tbody>
</table>
Table 2.4. Constituents of the resolving phase for 8% polyacrylamide gels

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume required</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{H}_2\text{O}$</td>
<td>9.3 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH8.8</td>
<td>5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>5.3 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>12 μl</td>
</tr>
</tbody>
</table>

Table 2.5. Constituents of the stacking phase for 10% polyacrylamide gels

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume required</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{H}_2\text{O}$</td>
<td>7.9 ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl pH8.8</td>
<td>5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>6.7 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>8 μl</td>
</tr>
</tbody>
</table>
Table 2.6. Constituents for the stacking phase of polyacrylamide gels

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume required</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>5.5 ml</td>
</tr>
<tr>
<td>1 M Tris-HCl pH6.8</td>
<td>1 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.08 ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.08 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>8 µl</td>
</tr>
</tbody>
</table>

Table 2.7. Running buffer used in SDS-PAGE

<table>
<thead>
<tr>
<th>10X Running Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 mM Tris base</td>
</tr>
<tr>
<td>1.92 M glycine</td>
</tr>
<tr>
<td>35 mM SDS</td>
</tr>
<tr>
<td>dd H$_2$O</td>
</tr>
</tbody>
</table>
Table 2.8. Transfer buffer used in Western blotting

<table>
<thead>
<tr>
<th>10X Transfer Buffer</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>250 mM Tris base</td>
<td>58 g</td>
</tr>
<tr>
<td>1.92 M glycine</td>
<td>29 g</td>
</tr>
<tr>
<td>35 mM SDS</td>
<td>3.7 g</td>
</tr>
<tr>
<td>dd H₂O</td>
<td>Fill to 1000ml</td>
</tr>
</tbody>
</table>

Dilute 1:10 with dd H₂O and 20% methanol

Store at 4°C

Table 2.9. Tris buffered saline for use in Western blotting

<table>
<thead>
<tr>
<th>10X Tris buffered saline</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mM Tris base</td>
<td>24.2 g</td>
</tr>
<tr>
<td>1.5 M NaCl</td>
<td>80 g</td>
</tr>
<tr>
<td>dd H₂O</td>
<td>500 ml</td>
</tr>
</tbody>
</table>

Adjust to pH = 7.6 with HCl

dd H₂O                   | Fill to 1L |
The proteins were transferred to nitrocellulose membranes (constant 15 V for 75 minutes) and blocked with 5% bovine serum albumin (BSA) or 5% milk as specified diluted in 1X Tris buffered saline (table 2.9) for at least 1 hour. Membranes were then probed overnight at 4°C with the specified primary antibodies diluted in blocking buffer supplemented with 0.1% tween-20 (Table 2.9) according to the supplier’s instructions. Membranes were washed X3 for 10 minutes with 1X TBS 0.1% tween-20 and X1 for 5 minutes with 1X TBS. The membranes were blocked again using 5% blocking buffer as before. Bound antibodies were detected using anti-mouse or anti-rabbit horseradish peroxidase (HRP) secondary conjugates that the membranes were incubated with for 1 hour at room temperature. Following secondary antibody incubation, the membranes underwent a series of washes with 1X TBS 0.1% tween-20 and X1 for 5 minutes with 1X TBS. Labelled bands were visualised by enhanced chemi-luminescence with ECL Prime and developed using Kodak Carestream X-OMAT film.
Table 2.10. List of antibodies used for Western blotting

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Predicted molecular weight (kDa)</th>
<th>Dilution</th>
<th>Dilution Buffer</th>
<th>Manufacturer (Catalogue #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse β-actin</td>
<td>43</td>
<td>1:3000</td>
<td>Milk TBST</td>
<td>Sigma (A2228)</td>
</tr>
<tr>
<td>Mouse E-cadherin</td>
<td>80/120/135</td>
<td>1:1000</td>
<td>Milk TBST</td>
<td>Santa Cruz (sc-71009)</td>
</tr>
<tr>
<td>Rabbit ENaCγ</td>
<td>95</td>
<td>1:1000</td>
<td>BSA TBST</td>
<td>Abcam (ab3468)</td>
</tr>
<tr>
<td>Rabbit GAPDH</td>
<td>37</td>
<td>1:1000</td>
<td>Milk TBST</td>
<td>Santa Cruz (sc-47724)</td>
</tr>
<tr>
<td>Rabbit GR</td>
<td>97/135/145</td>
<td>1:1000</td>
<td>BSA TBST</td>
<td>Thermo Fisher (PA1-511A)</td>
</tr>
<tr>
<td>Rabbit MR</td>
<td>102</td>
<td>1:1000</td>
<td>BSA TBST</td>
<td>Santa Cruz (sc-71554)</td>
</tr>
<tr>
<td>Mouse N-cadherin</td>
<td>130</td>
<td>1:1000</td>
<td>Milk TBST</td>
<td>Santa Cruz (sc-8424)</td>
</tr>
<tr>
<td>Mouse β1 Na+/K+ ATPase</td>
<td>35</td>
<td>1:4000</td>
<td>Milk TBST</td>
<td>Abcam (ab2873)</td>
</tr>
<tr>
<td>Rabbit Nedd4-2</td>
<td>120</td>
<td>1:2000</td>
<td>Milk TBST</td>
<td>Abcam (ab46521)</td>
</tr>
<tr>
<td>Rabbit phospho Nedd4-2 (Ser448)</td>
<td>112</td>
<td>1:2000</td>
<td>Milk TBST</td>
<td>Abcam (ab168349)</td>
</tr>
<tr>
<td>Rabbit PKD2</td>
<td>105</td>
<td>1:2000</td>
<td>BSA TBST</td>
<td>Abcam (ab7281)</td>
</tr>
</tbody>
</table>
2.7 Electrophysiological measurements

The Ussing chamber technique is used to measure electrolyte, nutrient and the transport of drugs across the epithelia of tissues (276). The field of epithelial polarity began in 1958 with the Koefoed-Johnsen and Ussing classic paper (277) first describing the use of the “Ussing chamber” and short-circuit current technique to measure transepithelial Na⁺ transport. The apparatus is comprised of a chamber split into two halves that are separated by the supported epithelial monolayer. This arrangement allows the apical and basolateral sides of the epithelium to be electrically and chemically isolated (278). For the purpose of this study, an epithelial monolayer of confluent M1 cells was mounted in the Ussing chamber in order to conduct short-circuit current experiments. One set of electrodes was used to clamp the spontaneous trans-epithelial electrical potential difference (Vt) to zero mV by passing a current across the epithelium. This current is a direct measurement of active ion transport across the epithelium and is referred to as the short-circuit current (Isc) (278, 279). Changes in Isc were measured by connecting the chamber to a voltmeter via Ag-AgCl electrodes (figure 2.1)
Figure 2.1. Schematic representation of the set-up for an Ussing chamber voltage clamp experiment to measure $I_{SC}$. The short circuit current ($I_{SC}$) is defined as the electrical current required to clamp the transepithelial voltage ($V_t$) to zero. The $I_{SC}$ is a measure of transepithelial current. When a blocker of ENaC is applied to the apical bath solution, the changes in $I_{SC}$ reflect changes in ENaC mediated transepithelial Na+ current. The epithelial monolayer was cultured on inserts mounted into the Ussing chamber and bathed in a physiological Krebs solution that was continuously gassed with 95% $O_2$/5% $CO_2$ and maintained at 37°C via a tubing system connected to a water bath.

M1 cells were cultured on Snapwell inserts with an area of 106 cm² until they reached a trans-epithelial resistance (TER) of at least 0.8 kΩ.cm². These inserts were mounted in Ussing chambers (EM-C SYS-6 Ussing Chamber Systems, Physiologic Instruments) and an EVC 4000 Voltage/Current clamp apparatus (World Precision Instruments) was used to clamp the $V_t$ to zero. The $I_{SC}$ was recorded using Ag-AgCl electrodes in salt bridges containing 4% agar in 3 M KCl. The apical and basolateral halves of the chambers were bathed in Kreb’s solution (table 2.11) that was maintained at 37°C and continually gassed with a mixture of 95% $O_2$/ 5% $CO_2$. All preparations were equilibrated until a stable baseline had been achieved prior to experiments being conducted.
The $I_{SC}$ was defined as a positive for the movement of cations ($Na^+$) from the apical to the basolateral side and for the flow of anions in the opposite direction. The results are represented as $\Delta I_{SC}$ where the values were calculated using the following:

$$\Delta I_{SC} = I_{sc(before)} - I_{SC(after)}$$

The $I_{sc(before)}$ is the $I_{sc}$ under basal conditions and before the addition of any agents while the $I_{SC(after)}$ is the measurement of $I_{sc}$ following the addition of any agonists/antagonists.

### 2.7.1 Measurement of amiloride-sensitive current

The $I_{sc}$ can be used as a measure of the electrogenic transport of a specific ion by recording $I_{sc}$ changes following inhibition with a specific ion channel inhibitor or activator. The electrogenic transport of $Na^+$ can be measured in this way by recording the changes in $I_{sc}$ in response to addition of the specific $Na^+$ channel blocker amiloride to the apical side of the epithelium. In order to investigate the effect of aldosterone on the amplitude of amiloride-sensitive $Na^+$ currents, M1 CCD wild-type and M1 CCD PKD2 knock-down cells were cultured on semi-permeable supports and treated for 6 h with aldosterone (10 nM). The inserts were then mounted in Ussing chambers and bathed in Kreb’s solution (table 2.11).
**Table 2.1. Modified Kreb’s solution used for Ussing chamber experiments**

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Stock Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>1.2 mM</td>
<td>1 M</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.2 mM</td>
<td>1 M</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.4 mM</td>
<td>48 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.4 mM</td>
<td>8 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>115 mM</td>
<td>2.3 M</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25 mM</td>
<td>0.5 M</td>
</tr>
<tr>
<td>D - glucose</td>
<td>10 mM</td>
<td>1 M</td>
</tr>
</tbody>
</table>

Following the stimulation of cells with aldosterone, the amplitude of the amiloride-sensitive I_sc uncovered by the addition of amiloride (10 µM) to the apical membrane (figure 2.1).
Figure 2.2. Schematic depicting the protocol for measuring amiloride-sensitive $I_{SC}$. Aldosterone treated cells cultured on semi-permeable inserts were mounted in Ussing chambers and both sides were bathed in Kreb solution. Amiloride (10 µM) was applied to the apical membrane of the epithelium and the aldosterone response on the amiloride-sensitive $I_{SC}$ was recorded.

2.7.2 Measurement of $\text{Na}^+$/K$^+$-ATPase activity

M1 CCD wild-type and M1 CCD PKD2 knock-down cells were cultured on semi-permeable supports and treated for 6 hours and 24 hours with aldosterone (10 nM). The inserts were then mounted in Ussing chambers and bathed in modified Ringer’s solution (table 2.11) until a steady $I_{sc}$ baseline was achieved. The osmolarity of the Kreb’s solution was measured and adjusted if required.
Table 2.12. Modified Kreb’s solution used in the measurement of Na⁺/K⁺-ATPase activity

<table>
<thead>
<tr>
<th></th>
<th>Concentration</th>
<th>Stock Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>1.2 mM</td>
<td>1 M</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.2 mM</td>
<td>1 M</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.4 mM</td>
<td>48 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.4 mM</td>
<td>8 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>15 mM</td>
<td>2.3 M</td>
</tr>
<tr>
<td>NMDG - Cl</td>
<td>100 mM</td>
<td>1.15 M</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25 mM</td>
<td>0.5 M</td>
</tr>
<tr>
<td>D - glucose</td>
<td>10 mM</td>
<td>1 M</td>
</tr>
</tbody>
</table>

This normal Kreb’s solution was removed from both sides of the Ussing chamber and substituted with a modified low Na⁺ Kreb’s solution where NaCl was replaced with equimolar N-Methyl-D-glucamine chloride (NMDG-Cl) to give a final Na⁺ concentration of 40 mM on both sides of the epithelium (table 2.13). Once a steady state Isc had been recovered, the apical membrane was perforated with amphotericin B (10 – 20 µM as indicated) (figure 2.3).

Amphotericin B is an ionophore that forms pores in the cell membrane that are permeable to monovalent cations. This permeabilisation of the apical membrane allows for Na⁺ ions to move freely through the apical membrane which, under the conditions set out in this experiment, produces an equilibration of the apical extracellular and intracellular concentration of Na⁺. The equilibration of Na⁺ on both sides of the apical membrane and the removal of the apical membrane electrical resistance barrier to the movement of cations using Amphotericin B allows the current flow across the basolateral membrane to dominate the generation of the Isc. Ouabain (100 µM) was used as an inhibitor of the Na⁺/K⁺-ATPase pump current and this was
added to the basolateral membrane (figure 2.3). The Na⁺/K⁺ - ATPase pump current was measured as the ouabain-sensitive changes in the I_SC.

![Schematic diagram](image)

**Figure 2.3. Schematic diagram illustrating the protocol used in the measurement of Na⁺/K⁺ - ATPase pump activity.** Inserts containing control and aldosterone treated M1 cell monolayers cells were mounted in Ussing chambers and both sides were bathed in Kreb’s solution. This solution was replaced with a modified Kreb’s solution containing 40 mM Na⁺ and was used to bath both the apical and basolateral side of the chamber. The apical membrane was perforated using 10 – 20 µM amphotericin B. Ouabain (100 µM) was added to the basolateral membrane in order to block Na⁺/K⁺ - ATPase activity.
2.8 Generation of a stable PKD3 knock out in M1 CCD cells using CRISPR

Bacteria have evolved RNA mediated adaptive defence systems that are known as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) that function to protect organisms from the invasion of plasmids and viruses (280-282). These defensive mechanisms rely on small RNAs for the sequence-specific detection and silencing of foreign nucleic acids. The CRISPR/Cas system is comprised of cas genes that are organised in operons and CRISPR arrays that consist of genome-targeting sequences interspersed with identical repeats (280-282).

There are three steps in the process of CRISPR/Cas mediated immunity. The first being the adaptive phase, which occurs when bacteria harbouring one or more CRISPR loci responds to a viral or plasma encounter by integrating short fragments of protospacers (i.e. a foreign sequence) into the host chromosome at the proximal end of the CRISPR array (280-282). The remaining two are the expression and interference phases. It is here that transcription of the repeat spacer element into a precursor CRISPR RNA (pre-crRNA) molecule which then leads to enzymatic cleavage resulting in the yield of short crRNAs that link with complementary protospacer sequences of the invading virus or plasmid target (283-290). The recognition of the target by crRNAs then leads to the silencing of the foreign sequences by Cas proteins that function in a complex with the crRNAs (283, 291-298).

Cas9 is the nuclease that guides the crRNA and the trans-activating crRNA (tracrRNA) in order to cleave specific DNA sequences (285). A guide RNA (gRNA) was designed to include a hairpin that could mimic the tracrRNA-crRNA complex (299). The binding specificity is founded on a gRNA and a three nucleotide NGG sequence that is also referred to as the protospacer adjacent motif (PAM) sequence (300). In this thesis, we utilized this CRISPR/Cas9 system to create a stable knock out of PKD3 in M1 CCD cells. This is the first knock-down cell line produced for PKD3 in the kidney.
Figure 2.4. Vector map of the all-in-one lenti CRISPR vector. The pLV-U6g-EPCG lenti CRISPR vector was used in this study. The target region in this thesis looked to knock out PKD3 in M1 CCD cells by targeting exons 3 and 10. Antibiotic selection was enabled as this vector contained a puromycin resistance gene in addition to expressing GFP.

M1 CCD cells (2 X 10^4/ml) were seeded into a 24-well culture plate and maintained for 24 hours in normal growth media (as described in 2.2.1 above). The media was then removed and replaced with 200 μl of fresh media containing 8 μg/ml of hexadimethrine bromide (Polybrene). In addition to a non-targeting control, two different lentiviral transduction particles were used to target exon 3 and exon 10 of the murine PKD3 sequence (table 2.14).
Table 2.13. Sequences targeted and p24 antigen titre for the LentiCRISPR transduction particles used in this thesis. Two separate sequences that targeted exon 3 and 10 of murine PKD3 were used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Vector</th>
<th>Sequence Verified</th>
<th>p24 Antigen ELISA Titre ≥</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKD3 Exon 3</td>
<td>pLV-U6g-EPCG</td>
<td>GAGAGCGTGAGGGCGGATCTGG</td>
<td>5.4 x 10^7 TU/ml</td>
</tr>
<tr>
<td>PKD3 Exon 10</td>
<td>pLV-U6g-EPCG</td>
<td>GAAATCATCACCAGTACCGTG</td>
<td>5.4 x 10^7 TU/ml</td>
</tr>
</tbody>
</table>

A multiplicity of infection (MOI) of 10 was used in this study therefore, the aim was to get 10 transduction units (TU) to be taken up per cell. The p24 antigen ELISA titre for the transduction particles targeting both regions was 5.4 X 10^7 TU/ml (table 2.14). 3.7 μl of each transduction particle was placed into separate wells and allowed to incubate at 70% humidity, 5% CO₂ at 37°C for 48 hours. Following incubation with the transduction particles the virus containing media was removed from the cells and replaced with normal culture media for 24 hours. Cells were then maintained in media containing 5 μg/ml of puromycin in order to select the cells that had successfully been transduced.

2.9 Generation of a stable PKD2 knock down in M1-CCD cells

Much of the work in this thesis is carried out using a cell line stably knocked down in the expression of PKD2 was established using the pSilencer™2.1-U6 Neo kit (Ambion) (work on the generation of this model was carried out by Dr. Ruth Dooley, postdoc in the Dept. Molecular Medicine, RCSI). Four different target sites of 21 nucleotides were selected along the gene sequence for PKD2, each beginning with an AA dinucleotide sequence. None of the target sites were homologous to other coding sequences when using a BLAST search. Primers were designed to these sequences and annealed to form a hairpin structure as outlined in the pSilencer™2.1-U6 Neo kit (Ambion). These hairpin oligonucleotides were ligated into the pSilencer™2.1-U6 Neo expression vector and amplified. M1 CCD cells were grown in
24-well plastic dishes and transfected with 0.8μg plasmid DNA per well at ~90% confluency using Lipofectamine™ 2000 (Invitrogen) in Opti-MEM® reduced-serum media (Invitrogen). The following day cells were split 1:10 to T25 flasks and transferred to normal growth media as above, containing 5% FBS. 24 hours later, 200 μg/ml G-418 (Invitrogen) was added to each flask. 14 days later all untransfected cells were dead and G-418 was removed from all flasks to allow the surviving transfected cells to repopulate the flasks. The expression of PKD2 was analysed following cell lysis in NP-40 lysis buffer and western blotting. The percentage knockdown was calculated using densitometry and comparing values to untransfected control cells.

2.9 Data analysis and Statistics

All data are reported as mean ± S.E.M for a series of n experiments. Statistical analysis of the data was performed with Prism (GraphPad Software), using paired Student \( t \)-tests for analysis of two matched groups and unpaired \( t \)-tests for analysis of two unmatched groups. One-way ANOVA was used to compare 3 or more matched groups with either the Dunnett’s or the Tukey’s post hoc test which compares all pairs of groups. P values ≤ 0.05 were considered to be statistically significant. Image analysis was carried out using the Zeiss Zen 2009 software. Co-expression of proteins were measured using Mander’s overlapping coefficient and the expression of proteins were analysed using mean intensity fluorescence as calculated by the Zen 2009 software. All image analysis was graphed as the mean of at least three independent experiments ± S.E.M with one representative image.
Chapter 3

Non-genomic activation by aldosterone and regulation of ENaC activity
3. Introduction

3.1. Non-genomic Responses to Aldosterone

Rapid activation of signal transduction cascades are amplified via aldosterone stimulated non-transcriptional responses. Current available evidence indicates that these non-genomic effects are dependent on the interaction of aldosterone with cytosolic MR and not via a non-classical membrane-bound aldosterone receptor (figure 3.1). The signalling cascades that are rapidly activated through the interaction of MR and aldosterone can be inhibited with the use of MR specific antagonists, for example, spironolactone and eplerenone (1).

Figure 3.1. Rapid response of aldosterone on ENaC and Na⁺/K⁺-ATPase via PKD2. This schematic represents the questions being asked in this chapter. We will investigate the potential role of receptors such as MR and EGFR in the activation of PKD2. Subsequently, we will investigate the effect of PKD2 on both ENaC and Na⁺/K⁺-ATPase activity.

The non-genomic responses to aldosterone have long been described in various experimental models. In 1957, Cole described the effect of aldosterone on the renal excretion of intravenously administered saline in adult male albino rats (301). Cole showed that administering aldosterone for 30 minutes reduced the loss of Na⁺ and increased its reabsorption by the renal tubules. It was also documented that the ratio
of Na⁺ to K⁺ was decreased in the urine but there was no significant effect on the glomerular filtration rate (GFR) (301).

In the frog skin, aldosterone can rapidly activate ATP-sensitive K⁺ (K<sub>ATP</sub>) channels. In this study, our group described how aldosterone (10 nM) led to an increase in the open probability of K<sub>ATP</sub> channels within 15 minutes of stimulation which was measured using cell attached patch clamp (134). Aldosterone also stimulated tolbutamide-sensitive K⁺ current in the basolateral membrane of nystatin-permeabilized frog skin within 30 minutes. Furthermore, the stimulatory effect of aldosterone on K<sub>ATP</sub> channels was abolished by an intracellular acidification produced by inhibition of Na⁺/H⁺ exchanger (134). Aldosterone was shown to activate the Na⁺/H⁺ exchanger by shifting its pH activation set-point to more alakine values. The non-genomic effect of aldosterone on K<sub>ATP</sub> channels and Na⁺/H⁺ exchanger was very rapid in the frog skin epithelium and renal A6 CCD cells (within 10 minutes) which was unlike previous findings demonstrating a delay of up to 1 hour before a stimulatory effect on Na⁺ transport in response to aldosterone in the toad urinary bladder (302). Wehling et al investigated the effect of aldosterone on systemic vascular resistance (SVR) in a placebo- controlled, double blinded, randomized trial in 17 patients with coronary heart disease (303). They deduced that effect of aldosterone on SVR was rapid and limited whereby SVR returned to basal levels following 10 minute stimulation with the hormone which was also coupled to a fall in cardiac output in response to the bolus injection. It has also been documented that aldosterone infusion into aldosterone-suppressed rats (by adrenalectomy or infusion with Na⁺ bicarbonate) resulted in the rapid increase in urinary Na⁺ excretion (304). This was followed by a study demonstrating how aldosterone induced the rapid increase in urinary Na⁺ excretion within 15 minutes in the intact rat (305).

### 3.1.2. PKD and trafficking of Na⁺ transporters

PKD has been implicated in the many facets of the regulation of sub-cellular trafficking by either maintenance of the structure in the Golgi or by the regulation of fission at the trans-Golgi network (TGN) (Figure 3.2). PKD can also regulate Golgi to membrane vesicle trafficking by activating phosphatidylinositol (PtdIns) 4-kinase (PI4KIIIβ) (186) and phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) (190). PKD1 phosphorylates
PI4KIIIβ at the Golgi which in turn promotes vesicle fission and subsequently the rate of protein transport to the plasma membrane (186). RhoA-mediated translocation of ENaC to the cell membrane is also associated with PI4P5K activation indicating a critical role for lipid modification in the trafficking of ENaC bearing vesicles (306). ENaC subunits can interact directly with phosphatidylinositide signalling intermediates (307) and a PKD1 activation can be coupled to RhoA-dependent actin rearrangement.

The different PKD isoforms exhibit distinct differences in terms of their sub-cellular localisation, which is also influenced by cell-type (308). We have established that PKD1 is expressed throughout the cytosol of M1-CCD cells but there was an accumulation proximal to the nucleus (266). This structure was identified as the TGN with the use of a specific marker, TGN38. PI4KIIIβ is phosphorylated by PKD1 at the TGN with subsequent up-regulation in vesicle fission, and we found that PI4KIIIβ was also localised to the TGN in M1-CCD cells (266). The TGN association of PI4KIIIβ was not affected by suppression of PKD1 expression. Treatment with aldosterone did not affect the distribution of PKD1 or PI4KIIIβ at the TGN but did promote the formation of an immuno-precipitatable complex between these two kinases within 5 min. This complex remained stable for at least 30 minutes (266), consistent with the sustained auto phosphorylation of PKD1 detectable following treatment with aldosterone (94). The interaction between these two kinases was also observed following the long term stimulation (ranging between 1 and 24 hours) with aldosterone (10nM). Aldosterone-bound MR controls the transcription of ENaCα in renal cells while the remaining subunits, ENaCβ and ENaCγ are expressed constitutively. We have previously shown that the long-term treatment with aldosterone increases the localisation of ENaCα and ENaCβ at the apical membrane in M1-CCD cells which is dependent on PKD1 expression and activation (109). Further studies showed that aldosterone induces the rapid translocation of ENaCβ and ENaCγ to the plasma membrane within 30 minutes of treatment and that this translocation, in common with PKD1 activation was MR-dependent (266). A recent study found that the copper ATPase ATP7B was phosphorylated by PKD1 in the Golgi prior to trafficking to the cell membrane raising the possibility that PKD1 also regulates aldosterone-responses by direct phosphorylation.
3.1.3. PKD2 and trafficking

The question remained if the second PKD isoform, PKD2 has a role in regulating the sub-cellular expression and trafficking of ENaC channel subunits. PKD2 has been shown to localise predominantly in the cytoplasm of unstimulated cells but this localisation can be rapidly altered in response to the GPCR agonist neurotensin (309). This occurs in a two-step process; firstly, PKD2 is translocated to the plasma membrane from the cytosol where secondly, it can rapidly dissociate from the plasma membrane and be trafficked back to the cytosol in a PKC-dependent process. PKD2 is unlike the other PKD isoforms because its activation did not induce a significant accumulation of the kinase in the nucleus (309). Rey et al., provided support for a novel mechanism and their findings suggested that the activation of related signalling molecules by agonists of GPCR could cause diverse physiological responses that are dependent on the differential intracellular distribution of PKD isoforms.

Rey et al., determined that PKD2 is present in the cytoplasm and not in the nucleus of Panc-1, MDCK, COS-7, and NIH-3T3 cells. They showed that the neurotensin mediated activation of PKD2 resulted in the rapid and reversible translocation of PKD2 to the plasma membrane in Panc-1 cells. Furthermore, inhibiting PKC did not stop the translocation of PKD2 to the plasma membrane in response to neurotensin but rather caused a substantial delay in the rapid reverse translocation from the membrane back into the cytosol. These results indicated that the dissociation of PKD2 from the plasma membrane is coupled to its PKC-mediated activation (309).

PKD2 has also been shown to function in the basolateral transport of proteins and Yeaman et al., investigated the effect of knocking down PKD2 had on the trafficking of E-cadherin and β1-integrin (195). They found that in polarised MDCK cells, the transient expression of a PKD2 kinase dead (KD) mutant resulted in the co-accumulation of E-cadherin, β1-integrin and the mutant at the TGN. Moreover, the endogenous proteins were also observed to localise at the basolateral plasma membrane which the authors attributed to transport to the membrane prior to the accumulation of the transiently expressed PKD2 KD mutant (195). Further studies in non-polarised HeLa cells determined that expression of PKD2 KD inhibited proteins that carried basolateral sorting signals from the TGN. This was shown to be
irrespective of the proteins ability to develop apical and basolateral domains of the plasma membrane (195).

It has also been suggested that PKD2 is unlike the other two isoforms because the activation of PKD2 was not shown to induce its redistribution to the nucleus from the cytoplasm (309). It has also been shown that the activation of NFκB by PKD2 occurs in response to oxidative stress and is not dependent on its catalytic activity (310). This suggests that PKD2 could have a distinctive regulatory property in comparison to PKD1 and PKD3. On the basis of our understanding of the role of aldosterone-induced PKD1 trafficking in renal M1-CCD cells, we investigated the potential role of PKD2 trafficking in the kidney. In this chapter, we will demonstrate the subcellular redistribution of PKD2 in response to aldosterone. Similarly, we will describe how PKD2 is involved in the trafficking of ENaC channel subunits following its activation by aldosterone. To date it is unknown whether PKD2 impacts Na⁺ reabsorption in the kidney and this will be investigated further using electrophysiological approaches. Additionally, we will describe the activation of PKD2 in M1-CCD cells is of a consequence of rapid non-genomic response to aldosterone.
3.2. Hypotheses and Aims

Investigations into the role of PKD isoforms in mediating aldosterone actions on renal Na+ transport are still in their infancy, with our group still one of the few actively pursuing this line of research. We have extensively described the role of the PKD1 isoform in regulating early responses to aldosterone and ENaC activation. In this Chapter, our major aim was to test the hypothesis that the PKD2 isoform may also be an aldosterone-MR responsive kinase to exert synergistic or antagonistic actions on ENaC. We tested this hypothesis by determining:

I. The expression profile of PKD2 in the cortical collecting duct
II. The activation dynamics of PKD2 and ENaC trafficking in response to aldosterone
III. The effects of PKD2 and its knock-down on basal and aldosterone-stimulated ENaC activity
IV. Elucidating intermediate signalling pathways involved in the aldosterone activation of PKD2
3.3. Results

3.3.1. PKD2 expression in tubular segments of the aldosterone-sensitive distal nephron.

Our group has demonstrated the rapid non-genomic activation of PKD1 by aldosterone and the implications this has on renal Na\(^+\) absorption in terms of trafficking and activity of transporters such as ENaC and Na\(^+\)/K\(^+\)-ATPase. To date, less is known about the role of PKD2 on ion transport in the distal nephron and in this chapter we describe the functional properties of PKD2 in regulating basal and aldosterone-stimulated Na\(^+\) absorption. PKD expression has never been described in the kidney before and we began this study by determining the expression of PKD2 in mouse and rat renal tubules ex vivo and in M1-CCD cells in vitro.

There is evidence from transcriptomics studies that the \textit{Pkd2} gene is expressed in the mouse distal convoluted tubule and the collecting duct but not in the connecting tubule (311). We examined the distal tubular expression of PKD2 in isolated murine nephron segments. Micro-dissected renal distal tubules from C57/BL6 mice were isolated and subjected to immunofluorescence to determine the expression profile of PKD2 at the protein level. We observed the PKD2 isoform to be ubiquitously expressed along the length of the distal tubule with high expression in the distal convoluted tubule (DCT2) and the connecting tubule with lower levels of expression along the collecting duct (figure 3.2).
Figure 3.2. Expression of PKD2 in segments of distal tubules. C57/BL6 mice were humanely sacrificed in accordance with local legislation. Distal tubules were micro-dissected and mounted onto glass slides for immunocytochemistry. Images were acquired using a Zeiss Apotome. The nuclei were stained with DAPI (blue) while PKD2 was stained with Cy3 (red). PKD2 is more highly expressed in the distal convoluted tubule (DCT2) and connecting tubule (CNT), however, expression was observed along the cortical collecting duct (CCD). Image is representative of 9 individual experiments.
We next investigated the distribution of PKD2 in rats fed a normal Na⁺ diet and a diet poor in Na⁺ in order to elevate plasma levels of aldosterone so as to determine if high aldosterone states can cause shifts in the cellular distribution of PKD2 in the distal nephron. We compared these responses with the effects of aldosterone *in vitro* on PKD2 distribution in M1-CCD cells.

Cryosections from male Sprague-Dawley rats were subjected to immunocytochemistry and the water channel aquaporin 2 (AQP2) was used as a marker for CCD principle cells. PKD2 was found to be expressed primarily in the principle cells and to a lesser extent in the intercalated cells (figure 3.3A). Under basal conditions, the PKD2 cellular distribution appears mainly at the apical membrane (co-localised with AQP2 – yellow staining in merged images figure 3.3A) and also showed low expression in the cytosol. A similar sub-cellular distribution of PKD2 was observed in confluent M1-CCD monolayers which also exhibited PKD2 expression at the plasma membrane (co-localization with WGA – yellow staining in merged images figure figure3 B) and expression in the cytosol.
Figure 3.3. Protein kinase D2 localization in the kidney. (A) Cryosections from male Sprague-Dawley rats were fixed to microscopy slides and analysed using immunocytochemistry. Principle cells were marked with aquaporin-2 (AQP2) (red). PKD2 (green) is expressed in the principle cells (PC) of the collecting duct with little or no expression in the intercalated cells (IC). PKD2 expression is mainly at the apical membrane co-localised with AQP2 (yellow merged image) and in the cytosol. (B) The expression of PKD2 was examined by confocal microscopy in untreated (EtOH vehicle control) M1-CCD cell monolayers. Wheat germ agglutinin (WGA) (red) was used to stain the apical membrane. PKD2 (green) showed apical co-localization with WGA (yellow merged image) and cytosolic expression in M1-CCD cells. (Quinn et al., in preparation).
3.3.2. Aldosterone induces the redistribution of PKD2 in cells of the cortical collecting duct.

We postulated that if PKD2 is an aldosterone-regulated kinase \textit{in vivo}, its cellular distribution should be affected by changes in salt-loading. Rats that were maintained on a low Na$^+$ diet had elevated levels of circulating aldosterone (Harlan: CA170555: 150 mmol K$^+$/ kg, 143 mmol Na$^+$/ kg for the control diet cohort and TD90228: 200 mmol K$^+$/ kg, <0.87 mmol Na$^+$/ kg for the low Na$^+$ diet). Immunofluorescence detection of PKD2 in cells of the distal tubule and collecting duct isolated from rats fed on low Na$^+$ chow showed a marked redistribution of PKD2 from the plasma membrane to the cytosol compared to animals fed a normal Na$^+$ diet (figure 3.4A). These \textit{ex vivo} experiments indicate that high plasma aldosterone can induce subcellular redistribution of PKD2. In order to verify this conclusion, we exposed M1-CCD cells to acute aldosterone treatment and followed the subcellular distribution of PKD2. Treatment of M1 cells with aldosterone for 10 minutes caused a redistribution of PKD2 out of the apical membrane and into the cytosol (figure 3.4B). The rapidity of this response indicates a non-genomic effect of aldosterone on PKD2 trafficking.
Figure 3.4. Aldosterone induces the subcellular redistribution of protein kinase D2. (A) Male Sprague-Dawley rat’s kidneys were cryosectioned and immunofluorescence carried out to determine the localization of PKD2 ex vivo. Aquaporin 2 (AQP2) (red) was used as a marker for the principle cells of the CCD while PKD2 was detected using an antibody conjugated to Alexa 488 (green). In both of the control samples, PKD2 expression was primarily localised at the apical membrane. In contrast, in the cohort of rats maintained on a low Na$^+$ diet and showing an increase in plasma levels of aldosterone, PKD2 localisation was redistributed to the cytosol throughout the distal nephron in particular the collecting duct. (B) The subcellular distribution of PKD2 (green) was examined by confocal microscopy in cells treated with vehicle (ethanol) and aldosterone (10 nM) for 10 minutes. Z-stack analysis was carried out with the apical membrane marked with WGA (red). Co-localization of PKD2 and WGA shown in merged images with yellow staining. Scale 10 μm (Quinn et al. in preparation).
3.3.3. Role of PKD2 in controlling ENaC trafficking

Since we already know that activation of the PKD1 isoform by aldosterone can regulate ENaC subunit trafficking by non-genomic mechanisms, and here we observe a rapid aldosterone-induced re-distribution of PKD2, we postulated that PKD2 may also affect ENaC localisation. We tested this hypothesis using wild-type M1 cells and a PKD2 stable knock-down M1 cell line which we generated for these studies. The subcellular distribution of the ENaCγ subunit was tracked using immunofluorescence confocal microscopy. Under basal conditions M1-CCD cells exhibited cytosolic expression of ENaCγ and treatment with aldosterone for 24 hours induced the redistribution of ENaCγ to the apical membrane (figure 3.5A). Interestingly, we observed that in PKD2 knock-down cells, the basal expression of ENaCγ was already highly abundant at the apical membrane while aldosterone treatment produced a re-distribution of ENaCγ out of the apical membrane into the cytosol (figure 3.5A).

Quantitative analysis of z-stack images was carried out using the overlap coefficient to measure ENaCγ and WGA co-expression at the apical membrane. Aldosterone treatment increased the abundance of ENaCγ at the apical membrane of M1-CCD wild-type cells (figure 3.5B). In contrast, in PKD2 knock-down cells, the co-localization of ENaCγ with WGA was elevated in the apical membrane compared to wild-type cells. Paradoxically, aldosterone increased ENaCγ abundance in the cytosol of M1-CCD cells deficient in PKD2, without significantly increasing expression of the channel subunit at the apical membrane.

In addition to the immunofluorescence studies, we also carried out Western blotting to quantify the effects of PKD2 knock-down on basal and aldosterone-induced expression of ENaC protein in the apical membrane. In wild type M1-CCD cells we found that aldosterone rapidly increased the abundance of ENaCγ in the apical membrane within 5 minutes and 10 minutes (figure 3.5D). However, the response was quite different in PKD2 knock-down M1-CCD cells in which the basal expression of ENaCγ was hugely increased in the apical membrane fraction. Moreover, aldosterone treatment for 5 and 30 minutes produced a rapid loss of ENaCγ protein from the apical membrane fraction (figure 3.5D).
Figure 3.5. Suppression of PKD2 increases the abundance of ENaCγ in M1-CCD cells. (A) The subcellular distribution of ENaCγ (green) was examined by confocal microscopy in cells treated with aldosterone (10 nM) for 24 hours. Z-stack analysis was carried out with the apical membrane marked with WGA (red). The overlap coefficient expresses the amount of ENaCγ and WGA co-localization (yellow staining) and quantified in wild-type M1 CCD cells (B) and in PKD2 knock-down cells (C). Treatment with aldosterone increased the expression of ENaCγ at the apical membrane in wild-type M1 CCD cells (p < 0.05). The effects of PKD2 knock-down on the protein expression of the ENaCγ channel subunit in M1-CCD cells were measured using biotinylation (D). Cells were treated for 5 and 30 minutes with aldosterone (10 nM). Knocking down PKD2 in M1-CCD resulted in a marked increase in the abundance of ENaCγ expressed in the apical membrane in untreated samples. The treatment with aldosterone resulted in a decrease in ENaCγ in M1-CCD PKD2 deficient cells in comparison to its untreated control. Statistics were carried out using a one-way ANOVA followed by Dunnett’s post hoc test. Scale 10 μm (Quinn et al. in preparation).
Taking these *ex vivo* and *in vitro* experiments together, we conclude that PKD2 is an aldosterone responsive kinase in CCD cells which can regulate the sub-cellular distribution of ENaC. Moreover, it would appear that PKD2 is a negative regulator of ENaC since its silencing produced a higher abundance of the channel in the apical membrane. It is interesting to speculate that when PKD2 is present in the apical membrane it suppresses ENaC activity. Moreover, when PKD2 is removed from the membrane under aldosterone control, more ENaC channel subunits are stabilised at the membrane. Thus, we may have revealed a novel regulatory mechanism of ENaC function where an inhibitory kinase, PKD2, suppresses ENaC activity under basal conditions and this brake on ENaC activity is removed when PKD2 is activated by aldosterone and removed into the cytosol to allow increased expression and stabilisation of ENaC channels in the apical membrane. If this conclusion holds true, then PKD2 should act as a suppressor of transepithelial Na\textsuperscript{+} transport under basal conditions. Furthermore, suppression of PKD2 expression in PKD2 knock-down cells should result in enhanced transepithelial Na\textsuperscript{+} transport rates. We can also predict that aldosterone treatment of PKD2 knock-down cells would produce a paradoxical decrease in transepithelial Na\textsuperscript{+} transport since we observed less ENaC in the apical membranes and increased cytosolic abundance of the channel after treatment of PKD2 knock-down cells with aldosterone. This hypothesis was tested in wild-type and PKD2 knock-down M1 CCD monolayers in Ussing chambers to record amiloride-sensitive short-circuit current responses under basal and aldosterone-stimulated conditions.

### 3.3.4. The role of PKD2 in regulating Transepithelial Na\textsuperscript{+} absorption

The basal expression of PKD2 at the apical membrane and its redistribution into the cytosol following aldosterone treatment must be important for regulating transepithelial Na\textsuperscript{+} transport because of the observed effects on ENaC trafficking. We tested this hypothesis by investigating the effects of knocking-down PKD2 on the amiloride-sensitive short-circuit current (I\textsubscript{SC}) in confluent M1-CCD monolayers mounted in Ussing chambers. M1-CCD wild-type and M1-CCD PKD2 knock-down cells were cultured on semi-permeable supports until reaching a TEER of at least 0.8 k\textOmega/cm\textsuperscript{2}. PKD2 suppressed cells exhibited an 8-fold increase in the basal spontaneous short-
circuit current ($I_{SC}$) when compared to the wild-type M1-CCD cells (figure 3.6). The increased $I_{SC}$ could be accounted for mainly by an increase in the specific amiloride-sensitive Na$^+$ current $I_{ENaC}$. Aldosterone (10 nM) treatment for 24 hours increased both the $I_{SC}$ and $I_{ENaC}$ in wild-type and M1-CCD PKD2 knock-down cells with the latter cells showing the largest response. However, when we compared the aldosterone response in PKD2 knock-down cells with the aldosterone response in wild type M1 epithelia, we found that aldosterone actually caused a reduction in both $I_{SC}$ and $I_{ENaC}$ in the PKD2-deficient cells. Long-term treatment (24 hours) of wild-type M1 cells with aldosterone increased the $I_{SC}$ from $1.6 \pm 0.3 \mu A/cm^2$ to $7.2 \pm 1.1 \mu A/cm^2$ ($n=3$, $p=0.01$). The ENaC current showed an increase from $0.9 \pm 0.1 \mu A/cm^2$ in wild-type M1 cells to $4.5 \pm 2.4 \mu A/cm^2$ when treated for 24 hours with aldosterone ($n=3$). This was not an entirely unexpected response since we predicted that aldosterone may actually inhibit Na$^+$ absorption when PKD2 was silenced because of the redistribution of ENaC from the apical membrane into the cytosol. This paradoxical inhibitory effect of aldosterone on Na$^+$ absorption is a completely novel observation in the field of renal research, in which we have revealed inhibitory effects of the hormone on ENaC and transpethelial Na$^+$ transport.
Figure 3.6. ENaC activity in response to long-term aldosterone treatment (24 hours). M1-CCD and M1-CCD PKD2 knock-down (KD) cells were cultured on semi-permeable inserts until they reached a trans-epithelial resistance of at least 0.8kΩcm$^2$. The inserts were mounted in Ussing chambers and the voltage clamped at zero. (A) Trace recordings of cells mounted in Ussing chambers over time. Statistical analysis was carried out using ANOVA and Dunnett’s post hoc test. (B) PKD2 KD using shRNA in M1 cells resulted in an elevated basal short-circuit current ($I_{SC}$) from 1.9 ± 0.2 μA/cm$^2$ in wild-type cells to 9.3 ± 1.4 μA/cm$^2$ in PKD2 KD M1 cells (n=10, p=0.002). PKD2 KD also increased the amiloride-sensitive ENaC current ($I_{ENaC}$) from 1.3 ± 0.3 μA/cm$^2$ in wild-type cells to 6.0 ± 1.0 μA/cm$^2$ in PKD2 knock-down M1 cells (n=11, p=0.0001). Long-term treatment (24 hours) of wild-type M1 cells with aldosterone increased the $I_{SC}$ from 1.9 ± 0.2 μA/cm$^2$ to 4.6 ± 0.7 μA/cm$^2$ (n=7, p=0.008). (C) The ENaC current showed an increase from 1.3 ± 0.3 μA/cm$^2$ in wild-type M1 cells to 3.3 ± 0.5 μA/cm$^2$ when treated for 24 hours with aldosterone (n=8, p=0.001). The effect of aldosterone on both the baseline $I_{SC}$ and $I_{ENaC}$ were abolished in the PKD2 knock-down cells. (Quinn et al., in preparation).
Normally, the genomic responses of aldosterone on short-circuit current in M1-CCD cells appear after 6 hours post-hormone treatment and we wished to determine if PKD2 was important in this latent stage of aldosterone action on Na\(^+\) absorption. Treatment of wild type M1 cells with aldosterone (10 nM) for 6 hours produced a 5-fold increase in \(I_{SC}\). Knocking down PKD2 in M1-CCD cells led to a 7-fold increase in ENaC activity (\(I_{ENaC}\)) under basal conditions when compared to wild-type cells. Furthermore, stimulating the PKD2 knock-down cells for 6 hours with aldosterone led to a 5-fold increase in \(I_{ENaC}\) which was still below the maximum spontaneous \(I_{ENaC}\) recorded under basal conditions in PKD2 knock-down cells (figure 3.7). By knocking down PKD2 in M1 cells, the basal \(I_{SC}\) was increased from 1.9 ± 0.5 \(\mu\)A/cm\(^2\) in wild-type cells to 8 ± 1.4 \(\mu\)A/cm\(^2\) in PKD2 knock-down M1 cells (n=3, p=0.008). PKD2 KD also increased the amiloride-sensitive ENaC current (\(I_{ENaC}\)) from 1.2 ± 0.1 \(\mu\)A/cm\(^2\) in wild-type cells to 7.4 ± 1.6 \(\mu\)A/cm\(^2\) in PKD2 knock-down M1 cells (n=3). Thus, it would appear from these data that the early non-genomic effects of aldosterone on PKD2 (and ENaC trafficking) have repercussions during the latent genomic phase of stimulating transepithelial Na\(^+\) transport.
Figure 3.7. ENaC activity in response to short-term aldosterone treatment (6h).
M1 CCD and M1 CCD PKD2 knock-down (KD) cells were cultured on semi-permeable inserts until they reached a trans-epithelial resistance of at least 0.8kΩcm². The inserts were mounted in Ussing chambers and the voltage clamped at zero. (A) Trace recordings of current from cells mounted in Ussing chambers. (B) PKD2 knock-down using shRNA in M1 cells resulted in an elevated basal short-circuit current (I_{SC}) from 1.9 ± 0.5 μA/cm² in wild-type cells to 8 ± 1.4 μA/cm² in PKD2 knock-down M1 cells (n=3, p=0.008). (C) PKD2 knock-down also increased the amiloride-sensitive ENaC current (I_{ENaC}) from 1.2 ± 0.1 μA/cm² in wild-type cells to 7.4 ± 1.6 μA/cm² in PKD2 knock-down M1 cells (n=3). Statistical analysis was carried out using one-way ANOVA followed by Dunnett's post hoc test. (Quinn et al., in preparation).
In addition to the $I_{ENaC}$ experiments, we began a pilot study to look at the effect of knocking down PKD2 on the Na$^+$/K$^+$ pump activity (figure 3.8). Previous work from our group showed that knocking down PKD1 in M1 CCD cells diminished the Na$^+$/K$^+$ pump current (266) when compared to the wild-type. Here we investigated the effect of PKD2 on the pump activity by performing Ussing chamber experiments on an Amphotericin B perforated epithelium. In this case, we were unable to demonstrate that the ouabain-sensitive $I_{SC}$ in PKD2-deficient cells is effected in comparison to M1 cells.
Figure 3.8. Pilot investigation into the effect of aldosterone on Na⁺/K⁺ pump activity in PKD2 deficient cells. M1 CCD and M1 CCD PKD2 knock-down (KD) cells were cultured on semi-permeable inserts until they reached a trans-epithelial resistance of at least 0.8kΩcm². Cells were treated with 10 nM aldosterone (or equivalent vehicle control) for 6 h. The inserts were then mounted in Ussing chambers and the voltage clamped at zero. Once a steady baseline has been achieved, the epithelium was perforated using Amphotericin B. Ouabain was added to the chambers and the pump recordings extracted. (A) Trace recording representative of the experiment (n = 1). (B) I_{sc} comparison between control and aldosterone treated M1 and PKD2 KD cells. (C) I_{pump} comparison between wild-type and PKD2 deficient M1 cells.
3.3.5. Mechanisms of action of Aldosterone activation of PKD2

The rapid effects of aldosterone on PKD2 subcellular re-distribution point to a non-genomic activation of PKD2. Classically, protein kinase activation requires phosphorylation and we hypothesised that aldosterone would produce a very rapid phosphorylation of PKD2 which would be receptor-mediated. We have previously shown that aldosterone treatment produces a very rapid (within 2 minutes) phosphorylation activation of PKD1 in M1-CCD cells via MR transactivation of the EGF receptor (94). Thus, it is reasonable to propose a similar mechanism of activation for PKD2 which we tested in M1-CCD cells. We investigated the potential non-genomic activation of PKD2 over a 30 minute period of aldosterone (10 nM) treatment using Western blotting to determine the phosphorylation state of PKD2 at Ser£876. Aldosterone produced a rapid phosphorylation of PKD2 which started to become evident within 5 minutes and was maximal at 10 minutes of hormone treatment (figure 3.9A). The aldosterone-induced phosphorylation of PKD2 presented a cyclical activation profile, with troughs and peaks of activation occurring at a frequent interval of approximately every 10 minutes. The phosphorylation of PKD2 was sustained and enhanced over periods of observation from 2 to 24 hours (figure 3.9B).
Figure 3.9. Phosphorylation of PKD2 in response to aldosterone. (A) M1-CCD cells were treated with aldosterone (10 nM) or vehicle control over a time course of up to 30 minutes and the phosphorylation of PKD2 was determined by Western blotting and quantified by densitometry (n = 5, * = p < 0.05). Aldosterone induced the rapid and cyclical increase in PKD2 phosphorylation peaking initially 10 minutes post-treatment with the hormone. (B) M1-CCD cells treated with aldosterone (10 nM) for 2 hours and 24 hours displayed sustained phosphorylation levels of PKD2 (n = 5, * = p < 0.05 and ** = p < 0.01). Statistical analysis was carried out using a one-way ANOVA followed by Dunnett’s post hoc test. Error bars are representative of SEM.
3.4. Discussion

Hormones such as aldosterone can mediate rapid signalling effects, for example, the activation of kinase cascades which allow for the regulation of transcriptional events either by phosphorylation of receptors like MR, of coactivators or via direct phosphorylation of the protein itself. Rapid responses to aldosterone can occur through the classical nuclear MR or via an alternative membrane bound receptor. In renal CCD cells, PKD1 acts as a key regulator of ENaC and Na⁺/K⁺-ATPase trafficking and activity. The activation of PKD1 at the TGN by aldosterone is an important regulatory mechanism of ENaC trafficking. Aldosterone can induce the interaction between PKD1 and PI4KIIIB which can regulate the signalling of protein kinases and the lipid modification that is essential for vesicle fission. The rapid activation of PKD1 signalling by aldosterone primes cells for subsequent transcriptional events and increases the expression of proteins. Vital roles of PKD isoforms including PKD2 are emerging with the identification of novel substrates for this kinase family that often include other kinases and transcription factors responsible for modulating gene expression and intracellular trafficking.

The level of ENaC activity at the apical membrane in principle cells of the CCD is the rate-limiting factor for Na⁺ reabsorption in the distal nephron. The role of early rapid signalling responses to aldosterone stimulation in Na⁺ transport has become clearer. Previous work from our group have shown that PKD1 is activated in M1-CCD cells within 5 minutes in response to aldosterone (94). In our studies presented here, aldosterone stimulated the auto-phosphorylation of PKD2 within 10 minutes. We have shown that under high aldosterone states, a shift in the cellular distribution of PKD2 can occur in the distal nephron. We observed a translocation of PKD2 from the cytosol to the cell membrane in response to hormone stimulation. This redistribution of PKD2 from the cytosol could have an effect on the localization of transporter proteins such as ENaC.

We observed that the activation of PKD2 in response to aldosterone was a potential consequence of stimulating multiple convergent signalling processes or it could be due to the result of a cycling of the kinase activation in response to a sustained stimulation (seen in the increase in PKD2 phosphorylation at 30 minutes, 2 and 24 hours stimulation with aldosterone). However, subsequent experiments failed to repeat this observation which cannot for sure state that the cyclical activation of PKD2 could
provide a mechanism for rapidly modulating the response to changes in the external environment for example through \( \text{Na}^+ \) loading (by regulating ENaC and \( \text{Na}^+ \) pump localization) or by epigenetic alterations that could affect gene expression. Here we demonstrate for the first time that PKD2 is a potentially novel aldosterone targeted kinase that can regulate ENaC trafficking and activation.

We have observed the subcellular redistribution of PKD2 from the apical membrane to the cytosol after treatment with aldosterone. Here we have also shown a novel role for PKD2 in the trafficking of ENaC channel subunits. Under basal conditions, ENaC\( \gamma \) is located in the cytosol. Upon stimulation with aldosterone (10 nM), ENaC\( \gamma \) is trafficked to the apical membrane. Interestingly, when we knocked down PKD2 in M1-CCD cells we found that the basal expression of ENaC\( \gamma \) was already highly abundant at the apical membrane. Aldosterone treatment produced a re-distribution of ENaC\( \gamma \) out of the apical membrane into the cytosol in M1-CCD cells deficient in PKD2. We have previously reported that the formation of membrane-bound structures that were found to be rich in ENaC subunits was observed following aldosterone stimulation for 5 min (109). It has been proposed that an ER-Golgi intermediate compartment could be the initial site for the post-ER sorting of proteins (312). This is consistent with the subcellular redistribution of ENaC channel subunits observed within 2 min of aldosterone stimulation (109). The data presented in this chapter further demonstrates the promotion of rapid transport of pre-expressed ENaC subunits to membrane bound structures following aldosterone stimulation. Therefore, the translocation of ENaC is dependent on PKD isoform coupled signalling cascades that may potentiate or synergize with a delayed effect of aldosterone on ENaC subunit trafficking.

Previous studies on ENaC-related acid-sensing ion channel (ASIC) suggested that a functional heterodimeric ENaC assembles in the ER (15) prior to it undergoing post-translational modifications as it passes through the Golgi. ENaC is found in vesicles throughout the cytoplasm of cells under high \( \text{Na}^+ \) where its depletion or exposure to aldosterone results in the subsequent translocation of ENaC to the apical membrane without undergoing transcriptional changes (313). The rapid surface translocation of ENaC and its increased activity has been reported in response to agonists; for example, a two-fold increase in the amiloride-sensitive \( I_{\text{sc}} \) was observed after 25 min of treatment with forskolin in CCD cells (314). This increase in \( I_{\text{sc}} \) coincided with an increase in the surface exposure of ENaC. Work from our group reported an increase in ENaC activity
in M1-CCD cells 2 to 4 hour post treatment with aldosterone. Maximal stimulation of ENaC activity was observed after 16 to 24 hour treatment with the hormone (5). Suppressing PKD2 in M1-CCD cells resulted in a measurable increase in Isc under basal conditions (seen at both 6 and 24 hours). This increase in ENaC activity corresponded with an increase in basal expression of ENaCγ in M1-CCD PKD2 knock-down when compared to wild-type cells. Aldosterone treatment stimulated an increase in the insertion of ENaCγ into the apical membrane in PKD2 deficient cells. Insertion of ENaC into the apical membrane is a prerequisite for its ubiquitination and proteasomal degradation. A failure of PKD2-dependent trafficking of ENaC channel subunits into the cytosol results in an increase in both the total ENaC abundance in the membrane and the increase in channel activity seen here. Another possibility for the increase in Isc in the PKD2 knock down cells could stem from an increase in the PKD1-dependent trafficking of ENaC channel subunits. There is also a possibility that PKD1 activity could increase in order to compensate for the absence of PKD2 in our model by upregulating for example, PI4KIIIβ and therefore affecting ENaC activity. Some of the results here present a paradox in terms of the effect of aldosterone in decreasing the Isc in PKD2-deficient M1 cells. We would presume that aldosterone would continue to increase ENaC activity in the absence of PKD2 in this model due to an upregulation of PKD1-dependent trafficking. However, from these findings, we propose that that PKD1 and PKD2 exert opposite effects of ENaC membrane abundance and that aldosterone, by activating PKD2 and removing it from the membrane, releases a tonic inhibition of ENaC stability exerted by unphosphorylated PKD2 (figure 3.10) These results present a paradoxical inhibitory effect of aldosterone on ENaC current in PKD2 KD. This is most likely due to the loss of PKD2 which uncovers an inhibitory effect of aldosterone on ENaC stability in the membrane. One possible explanation is that Ca\textsuperscript{2+} mobilization occurs or EGFR is activated. The both of these cases are known to inhibit ENaC activity but these effects appear to be overridden when PKD2 is normally expressed in wild type CCD. Our pilot investigation into the effect of PKD2 on Na\textsuperscript{+}/K\textsuperscript{+} pump activity did not yield any findings to date. The negative recordings observed could be due to the cells losing their TER when they were mounted in the Ussing chambers. Measures such as ensuring that the temperature and CO\textsubscript{2}/O\textsubscript{2} control are in working order can be taken. As this was a pilot study, it will require further optimisation of the protocol prior to repeating the experiment.
Figure 3.10. PKD isoforms exert opposite effects on the abundance of ENaC in the apical membrane. The PKD1 isoform is involved in the insertion of ENaC channel subunits into the apical membrane of CCD cells. Aldosterone has been shown to activate the PKD1-dependent trafficking of ENaC subunits to the membrane. In contrast to this, PKD2 has a role in the trafficking of ENaC into the cytosol under basal conditions. Stimulating CCD cells with aldosterone results in the inhibition of ENaC retrieval back into the cytosol which results in an increased abundance of ENaC at the apical membrane along with an increase in ENaC activity.

The rapid phosphorylation and activation of PKD2 in response to aldosterone seen in this chapter is indicative of a non-genomic response. As this observation excludes the translocation of the ligand bound receptor to the nucleus, the binding of chromatin or transcription, we investigated whether a non-classical receptor signal transduction mechanism resulted in the rapid phosphorylation of PKD2 in M1-CCD cells. The non-genomic activation of PKD1 by aldosterone required the rapid transactivation of MR/EGFR (94). The trans-activation of EGFR is typically an intermediate step in the transduction of signalling responses stimulated by other steroid hormones such as estrogen (315) as well as G-protein coupled receptor agonists (316, 317).

The ErbB family of receptor tyrosine kinases (including its member EGFR) can be
activated independently of ligand binding via phosphorylation of specific residues that are distinct from auto-phosphorylation sites. For example, EGFR can be phosphorylated at Tyr\(^{845}\) by Src tyrosine kinases which result in the activation of EGFR without requiring binding of the receptor to EGF (318-320). Additionally, other groups have shown that there is an increase in the overall phosphorylation of EGFR following the treatment of aldosterone and this is coupled to ERK1/2 activation and an increase in intracellular Ca\(^{2+}\) (321, 322).

The activation of PKD has been considered to be a consequence of stimulation at the membrane that could be initiated via GPCRs or tyrosine kinases. The as yet “unknown” aldosterone receptor may be an undiscovered novel receptor or it could be a well characterised signalling molecule. Aldosterone can bind directly to the C2 domain of PKC\(\alpha\) and with a binding affinity of between 0.5 and 1 nM, it results in the auto-phosphorylation of PKC\(\alpha\) (108). There have also been numerous reports proposing GPR30 (a G protein coupled receptor) as a novel estrogen receptor. GPR30 can bind to estrogen and result in the intracellular mobilization of Ca\(^{2+}\) and nuclear synthesis PIP\(_3\) (325). Furthermore, the rapid responses to aldosterone in smooth muscle has been linked to the GPR30 coupled signalling pathway in which the expression of GPR30 is required for the MR-independent rapid effects of aldosterone (326). However, the capacity of GPR30 to bind to several steroid ligands remains controversial.

The transient activation of cAMP signalling by aldosterone has been shown in CCD cells and the phosphorylation of CREB following aldosterone treatment was found to be PKA-dependent (327). Some research groups have reported aldosterone inducing the activation of PKA however, they also describe an inhibitory effect between the physiological response stimulated by forskolin and that stimulated by aldosterone. There is also evidence suggesting that suppressing CREB-dependent transcription is via the upregulation of protein phosphatase 2\(\beta\) (PP2\(\beta\)) activation by aldosterone (119). This could be due in part to the activation of isoforms of adenylate cyclase and PKA by forskolin and aldosterone. This activation could result in the compartmentalised signalling in cells. Conversely, this could also be due to a negative feedback loop that
is intrinsic to aldosterone signalling thus making cells more refractive to further PKA stimulation after the initial aldosterone-induced response. It is possible that PKA could be involved in crosstalk with PKD2. Forskolin induces a 2-fold increase in the amiloride-sensitive $I_{SC}$ following 25 minutes of treatment which coincided with an increase in the exposure of ENaC subunits at the surface of the cell (314). While questions surrounding the mechanism of PKD2 activation by aldosterone in M1-CCD cells still remain unanswered, we can conclude the aldosterone induced the subcellular redistribution of the kinase.
Chapter 4

Evidence for independent PKD2 and SGK1 signalling in the regulation of ENaC
4.1. Introduction

4.1.1 ENaC Ubiquitination by the E3 ligase Nedd4-2

The insertion of ENaC into the apical membrane, its retrieval and its recycling have been recognised as important factors in regulating the function of the channel (328-331). The process of ubiquitination is carried out by E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzymes and the E3 ubiquitin-protein ligases. In terms of cascade specificity, it is the E3 ubiquitin ligases that take part in the recognition of proteins (332). The neural precursor cell expressed developmentally downregulated protein (Nedd4) like family fits into the HECT class of E3 ligases and include Nedd4, Nedd4-2/Nedd4L (333). These proteins each contain an NH₂ terminal (which is responsible for the Ca²⁺ dependent binding of lipids), a C2 domain for binding to the membrane (334), a region that contains between two and four WW domains (for interaction with the targeted protein) (335) and a COOH terminal HECT domain (333, 336).

Since determining that WW domains of Nedd4/Nedd4-like E3 ligases can bind to proline-rich PY motifs in ENaC, a role of ubiquitination has been observed in regulating the expression of transporters and ion channels at the cell surface as well as in endocytosis (337). In a study conducted by Staub et al (1996), Nedd4-2 was found to interact with ENaC when the PY-containing region of ENaCβ was used to conduct a two-hybrid screen to identify proteins that interact with the channel. It has also been determined that each ENaC channel subunit contains a PY motif in the COOH terminus (337-339). It has since been shown that the increase in the expression of ENaC at the cell surface that is a characteristic of Liddle’s syndrome could be a result of the ubiquitination and degradation of the channel (340). It has been shown that in Liddle’s syndrome, mutations that enhance Na⁺ transport do so by impacting on the surface expression and open probability of ENaC (40). Additionally, ENaC is multi-monoubiquitinated at the cell surface (341) while the mutated ENaC PY motifs in Liddle’s syndrome regulate internalisation, sorting and recycling of the channel (342). Furthermore, Nedd4-2 is now known to be a catalyst for the ubiquitination and degradation of ENaC at the plasma membrane (343).
4.1.2 SGK interaction with Nedd4-2

Serum glucocorticoid kinases (SGK) are present in all eukaryotes and there are multiple isoforms for example SGK1, 2 and 3. All isoforms have been shown to promote ENaC activity with SGK1 and SGK3 being the most potent stimulators of this activity when they have been co-expressed in *Xenopus* oocytes (344, 345). Aldosterone and other glucocorticoid hormones induce the transcription of SGK1 by acting via nuclear receptors that bind to the response elements in the SGK1 gene. The subsequent rapid increase in SGK1 at both the protein and mRNA level stimulated ENaC mediated Na\(^+\) currents in the epithelium of several tissues such as the kidney, lung, colon and ocular epithelial cells (346-349).

SGK1 can interact with and phosphorylate key consensus sites in the WW domain of Nedd4-2 which decreases the binding affinity with ENaC proteins that most likely induce conformational changes. SGK1 can also promote binding of Nedd4-2 to 14-3-3. The 14-3-3 protein consists of a family of acidic dimeric proteins of approximately 30 kDa in molecular mass that are distributed extensively among eukaryotic cells (139-142). This family of protein has been associated with several biological activities for example, the activation of enzymes such as tyrosine and tryptophan hydorxylases (143, 144) that are involved in monoamine synthesis, the regulation of protein products of oncogenes like Raf-1 and Bcr-Abl protein kinase (145-149) as well as the retention of ligands in pro-apoptotic pathways in the cytoplasm (150, 151). In many cases, the binding of 14-3-3 to target proteins is initiated by the target phosphorylation, principally at specific Ser/Thr residues (157). Therefore, it is believed that the 14-3-3 family could be a key regulator of cell signalling pathways that are mediated via protein phosphorylation.

Nedd4-2 is sequestered by 14-3-3 which inhibits its binding to ENaC. This includes all three Nedd4-2 residues, however, the phosphorylation of S444 and T363 of *Xenopus* Nedd4-2 is crucial in promoting the binding of 14-3-3 and the promotion of ENaC surface expression and activity (350-353). SGK primarily phosphorylated T246 in human embryonic kidney cells (HEK-293) and the mutation of this residue strongly inhibits the binding of Nedd4-2 to 14-3-3 (350, 352).
SGK also contains a PY motif and although some studies in vitro did not present an interaction (353-355), surface plasmon resonance has recently demonstrated that the WW domains of Nedd4-2 interact with an SGK peptide containing the PY motif (356). The effect of activated SGK on stimulating ENaC function was more than the non-active form of the kinase or SGK that lacked a PY motif. This suggests that the interaction of Nedd4-2 and SGK is functionally significant in the control of ENaC activity (351, 353). SGK and Nedd4-2 may regulate the abundance of each other in a reciprocal manner. This was observed when the Ser^{327} residue of SGK was phosphorylated, it promoted the stability of Nedd4-2 when expressed in HEK293-T cells thus increase its half-life independent of binding to 14-3-3 (350). Additionally, Nedd4-2 phosphorylation in COS-7 cells induced the ubiquitination of SGK and its degradation in the proteasome and therefore, through phosphorylating Nedd4-2, SGK stimulated its own degradation (357).

4.1.3 PKD2 and the regulation of ENaC trafficking by ubiquitination

It is widely accepted that the aldosterone-induced increase in the expression of ENaC at the cell surface and the channel open probability (P_O) is regulated in two stages through 1) the promotion of trafficking and stabilization of pre-expressed ENaC subunits at the apical membrane and 2) via the MR-dependent regulation of each subunit’s transcription. A rapid increase in ENaC activity by elevation in the channel density at the membrane has been linked to the rapid vesicle trafficking that is coupled to the activation of the RhoA small GTPase (306).

As described in the previous chapter, we have observed rapid activation of PKD2 by aldosterone and have shown how the effects of aldosterone on both the basal short-circuit current (I_{sc}) and ENaC activity (I_{ENaC}) are abolished with the knock-down of PKD2 (Quinn et al.). By revealing this novel pleiotropic affect on sodium reabsorption, this data suggests complex interactions between various PKD isoforms in regulating ENaC function. For example, as seen in this thesis, treatment of the PKD2 knock-down M1 cells with aldosterone prevented the ENaC channel subunits from being trafficked back into the cytosol, leaving them vulnerable to ubiquitination and degradation. Thus it is important to understand the interactions between protein kinase D isoforms and Nedd4-2 and SGK.
4.2 Aims

The primary aim of this chapter was to investigate the effect of knocking down PKD2 on the phosphorylation and activation of Nedd4-2. Specifically, we examined:

I. The effect of PKD2 knock-down on the abundance of Nedd4-2
II. The rapid phosphorylation of Nedd4-2 in response to aldosterone in the M1 CCD PKD2 knock-down model.
III. The effect of PKD knock-down on aldosterone-induced SGK1 phosphorylation
4.3 Results

4.3.1 Nedd4-2 is increased in the absence of PKD2 in M1 CCD cells

We have shown in Chapter 3 that the stimulation of M1 CCD PKD2 knock down cells with aldosterone resulted in a decrease in both the baseline $I_{SC}$ and $I_{ENaC}$. We hypothesised that this paradoxical inhibitory effect of aldosterone on ENaC could be due to enhanced ubiquitination and degradation of surplus ENaC in the membrane. The work described in this Chapter sought to demonstrate a possible role for the PKD2 isoform in the regulation of aldosterone-induced ENaC activity in M1-CCD cells. This work builds upon our previous studies that indicate suppressing PKD2 in vitro inhibits the retrieval of ENaC channel subunits out of the apical membrane which coincides with a decrease in ENaC activity following stimulation of PKD2 knock-down M1-CCD cells with aldosterone. The studies to investigate potential changes in the abundance of Nedd4-2 were carried out simultaneously in order to look for changes between the wild-type and PKD2 deficient M1 cells.

Firstly, in order to determine the localization of Nedd4-2 under basal conditions, M1-CCD and M1-CCD PKD2 knock down cells were cultured on sterilised coverslips. Wheat germ agglutinin (WGA) was used in these experiments as a marker for the apical membrane. Following fixation, immunocytochemistry was performed using confocal microscopy to establish whether knocking down of PKD2 altered the localisation of Nedd4-2 (figures 4.1 and 4.2).
Figure 4.1. The sub-cellular distribution of the E3 ubiquitin ligase Nedd4-2 in M1 CCD cells under basal conditions. M1 monolayers cultured on sterilized glass coverslips were labelled with a Nedd4-2 antibody (green, Alexa Fluor 488 nm) and wheat germ agglutinin (WGA) as a marker of the plasma membrane (red, Alexa Fluor 633 nm) which was added apically prior to cell permeabilisation. Z - stack images are representative of 3 independent experiments (scale = 10 μm).
Figure 4.2. The sub-cellular distribution of the E3 ubiquitin ligase Nedd4-2 in M1 CCD PKD2 knock down cells under basal conditions. M1 CCD PKD2 knockdown monolayers cultured on sterilized glass coverslips were labelled with a Nedd4-2 antibody (green, Alexa Fluor 488 nm). Wheat germ agglutinin (WGA) was used as a marker of the plasma membrane (red, Alexa Fluor 633 nm) which was added apically prior to cell permeabilisation. Z - stack images are representative of 5 independent experiments (scale = 10 μm).
As can be seen in figure 4.1, Nedd4-2 is expressed in the cytoplasm of M1 CCD cells under basal conditions (n = 3). Knocking down of PKD2 resulted in an increase in the abundance of Nedd4-2 in the cytosol (n = 5). In addition, the immunocytochemistry further demonstrated the high expression of Nedd4-2 in the cytosol of M1 CCD PKD2 knock down cells (figure 4.2).

The potential change in the abundance of Nedd4-2 was also investigated using the mean fluorescence intensity values for the confocal images (figure 4.3) and by evaluating the abundance of Nedd4-2 at the protein level by Western blotting (figure 4.4). PKD2 knock-down had no significant effect on the absolute expression of Need4-2 protein in the cell. It would appear that PKD2 may alter the distribution of Need4-2 without changing expression levels of the protein.
Figure 4.3. Knocking down PKD2 does not increase the abundance of Nedd4-2 in M1 CCD cells. The mean fluorescence intensity was calculated for confocal images acquired from M1 CCD and M1 CCD PKD2 knock down cells that were immunodetected for Nedd4-2. A student's t-test was carried out and revealed that knocking down PKD2 in M1 CCD cells does not increase the abundance of Nedd4-2.
Figure 4.4. Densitometry analysis for the comparison of the abundance of Nedd4-2 in M1 CCD and M1 CCD PKD2 knock down cells. (A) Protein lysates from M1 CCD and M1 CCD PKD2 known down cells were electrophoresed using SDS-PAGE and subjected to immunoblotting where the membranes were probed with a primary antibody specific to Nedd4-2. Unstimulated cells (represented in the red box) were used. Knocking down PKD2 in M1 cells did not result in an increase in the abundance of Nedd4-2 at the protein level. (B) Densitometric analysis was carried out using a two-tailed student’s T test (n = 3).
4.3.3 Aldosterone increases Nedd4-2 phosphorylation in M1 CCD PKD2 deficient cells

It was observed that knock-down of PKD2 increased both the basal $I_{\text{SC}}$ and the amiloride-sensitive $I_{\text{ENaC}}$ current. This novel finding indicates that PKD2 somehow acts as a ‘brake’ on ENaC function or stability in the membrane. We postulated that the increase in both $I_{\text{SC}}$ and $I_{\text{ENaC}}$ could be due to changes in the abundance of Nedd4-2 in both basal and aldosterone-stimulated M1-CCD PKD2 knock-down cells. Cell lysates underwent SDS-PAGE and Western blotting to determine whether the PKD2 deficient cells had an increase in the abundance of Nedd4-2. Densitometric analysis was carried out and showed no significant increase in Nedd4-2 abundance due to the loss of PKD2 (figure 4.4). In the case of the PKD2 knock-down alone, stimulation of these cells for 24 hours with aldosterone led to a 4-fold decrease in the baseline $I_{\text{SC}}$ and a decrease in ENaC specific current. We hypothesise that perhaps the loss of PKD2 in M1-CCD cells resulted in the retention of ENaC at the apical membrane where it could be subjected to ubiquitination and degradation. We have shown that both PKD1 and PKD2 are rapidly phosphorylated and activated when stimulated by aldosterone, so we explored the possibility that aldosterone could have a rapid effect on the abundance of Nedd4-2 in M1-CCD PKD2 knock-down cells. These cells were treated for 0, 10, 30, 60 and 120 minutes with aldosterone (10 nM) prior to undergoing immunoblotting. Densitometry was carried out and it was observed that aldosterone did not significantly affect the abundance of Nedd4-2 in either M1-CCD or M1-CCD PKD2 knock-down cells (figure 4.5).
Figure 4.5. Densitometry analysis for the comparison of the abundance of Nedd4-2 in M1 CCD and M1 CCD PKD2 knock down cells in response to aldosterone. M1 CCD and M1 CCD PKD2 knock down cells were stimulated with aldosterone (10 nM) for 0 to 120 minutes. Protein lysates from these cells were subjected to SDS-PAGE and immunoblotting where the membranes were probed with a primary antibody specific to Nedd4-2. Aldosterone did not increase the abundance of Nedd4-2 in M1 CCD cells. Additionally, knocking down PKD2 in M1 cells did not result in an increase in the abundance of Nedd4-2 at the protein level. A one-way ANOVA was carried out with Tukey’s post-hoc test (n = 3).
Wild-type M1-CCD and M1-CCD PKD2 KD cells were treated for up to 120 minutes with aldosterone (10 nM) and we investigated if Nedd4-2 was phosphorylated in response to aldosterone (figures 4.6 and 4.7). We have previously shown that PKD2 is rapidly phosphorylated in response to aldosterone (<10 minutes and here we explored whether Nedd4-2 phosphorylation was increased in M1-CCD PKD2-deficient cells. As can be seen in Fig. 4.6, acute treatment with aldosterone did not induce an increase in the rapid phosphorylation and activation of Nedd4-2 in M1-CCD cells (n = 3). In contrast to this, aldosterone (10 nM) increased the phosphorylation of Nedd4-2 in M1-CCD PKD2 knock down cells at 30 minutes when compared to the untreated control (n = 6, p = < 0.05), (figure 4.6).
Figure 4.6. Effect of acute aldosterone treatment on the phosphorylation of Nedd4-2 in M1 CCD cells. M1 CCD cells grown on culture plates were treated with aldosterone (10 nM) for 0, 10, 30, 60 and 120 minutes. The phosphorylation of Nedd4-2 in response to aldosterone was assessed using Western blotting. Densitometric analysis showed that aldosterone did not have an effect on the rapid phosphorylation of Nedd4-2 in these cells. A one-way ANOVA was carried out alongside Dunnett’s post-hoc test (n = 3, p = < 0.05).
Figure 4.7. Effect of acute aldosterone treatment on the phosphorylation of Nedd4-2 in M1 CCD PKD2 knock down cells. M1 CCD PKD2 knock down cells grown on culture plates were treated with aldosterone (10 nM) for 0, 10, 30, 60 and 120 minutes. The phosphorylation of Nedd4-2 in response to aldosterone was assessed using Western blotting. Densitometric analysis shows that aldosterone increases the phosphorylation of Nedd4-2 in these cells following 30 minutes stimulation with the hormone. A one-way ANOVA was carried out alongside Dunnett’s post-hoc test (n = 6, p = < 0.05).
4.3.4 Effect of aldosterone on SGK1 phosphorylation in M1 CCD PKD2 knock down cells

It has been proposed that Nedd4-2 can facilitate some of the effects of SGK1 on the abundance of ENaC at the cell surface. SGK1-dependent phosphorylation of Nedd4-2 has been shown to have an inhibitory effect on Nedd4-2’s interaction with the PY-motif in the C terminus of the ENaC channel subunits. It is therefore likely that SGK1-induced phosphorylation prevents Nedd4-2 induced ubiquitination of ENaC and inhibits the endocytotic retrieval and degradation of ENaC (97, 107, 109). Here we investigate the phosphorylation of SGK1 in response to aldosterone (figures 4.7 and 4.8).
Figure 4.8. Effect of acute aldosterone treatment on the phosphorylation of SGK1 in M1 CCD cells. (A) M1 CCD cells grown on culture plates were treated with aldosterone (10 nM) for 0, 10, 30, 60 and 120 minutes. The phosphorylation of SGK1 at Ser^{422} in response to aldosterone was assessed using Western blotting. (B) Densitometric analysis showed that aldosterone did not have an effect on the rapid phosphorylation of SGK1 in these cells. A one-way ANOVA was carried out alongside Tukey’s post-hoc test (n = 3, p < 0.05).
Figure 4.9. Effect of acute aldosterone treatment on the phosphorylation of SGK1 in M1 CCD PKD2 knock down cells. M1 CCD PKD2 KD cells grown on culture plates were treated with aldosterone (10 nM) for 0, 10, 30, 60 and 120 minutes. The phosphorylation of SGK1 at Ser\textsuperscript{422} in response to aldosterone was assessed using Western blotting. Densitometric analysis showed that aldosterone did not have an effect on the rapid phosphorylation of SGK1 in these cells. A one-way ANOVA was carried out alongside Tukey’s post-hoc test (n = 3).
We have previously shown that PKD2 is rapidly phosphorylated in response to aldosterone (<10 minutes and here we explored whether SGK1 phosphorylation was increased in M1-CCD PKD2-deficient cells. As can be seen in Fig. 4.8, acute treatment with aldosterone did not induce an increase in the rapid phosphorylation and activation of SGK1 in M1-CCD cells (n = 3). Similarly, aldosterone (10 nM) did not increase the phosphorylation of SGK1 in M1-CCD PKD2 knock when compared to the untreated control (n = 3), (figure 4.9).
4.4 Discussion

SGK has dominated the field of aldosterone-induced proteins regulating ENaC function for the past 25 years. Our studies have described a new player in both the rapid (non-genomic) and chronic (genomic) regulation of ENaC trafficking and function via aldosterone-sensitive protein kinase D isoforms. It is still uncertain whether SGK can directly phosphorylate ENaC in order to modify its function. Previous studies using patch clamp recordings from *Xenopus* oocytes that express ENaC showed that SGK1-dependent phosphorylation did not increase the $P_0$ or the kinetics of ENaC (358). Outside-out patch clamp recording of *Xenopus* oocytes that contained recombinant constitutively active SGK1 showed stimulation of ENaC activity that was independent of the effects of SGK1 on Nedd4-2. It has also been determined in the rat kidney that an SGK consensus motif $^{616}$RSRYWS$^{621}$ in the carboxyl terminal region of ENaCα is essential for mediating the effects observed (162, 359). Conversely, replacing the serine at the similar consensus site of human ENaCα (S622A) did not inhibit the overall increase in ENaC activity by SGK (360). It is interesting to note that the activity of the channel that contain the mutations observed in the β and γ subunits in Liddle’s syndrome was stimulated by the co-expression of SGK1 while the infusion of aldosterone into the renal tubules of Liddle’s syndrome mice also promoted ENaC activity. The effects observed could implicate the silencing of Nedd4-2 binding to PY motifs in ENaCα or could support the notion that ENaCα can be phosphorylated by SGK (338, 358, 361).

There have been reports using renal collecting duct cells and *Xenopus* oocytes that show that the activation of SGK did not decrease the rate of ENaC endocytosis that would be expected by Nedd4-2 activation. However, these reports also state that an increase in the abundance of ENaC was also observed suggesting that SGK1 promotes insertion of ENaC channel subunits into the membrane (358, 362). AS160, which is an aldosterone-induced rab protein regulator, has been shown to be vital for the stabilisation of the intracellular pool of ENaC and its subsequent translocation to the cell membrane (363). Butterworth et al., showed that the phosphorylation of AS160 at SGK1 sites Ser$^{751}$ and Tyr$^{568}$ stimulated the binding of AS160 to 14-3-3 β and ε subunits which allowed for the translocation of ENaC into the plasma membrane. Therefore, they propose that SGK1 may promote the trafficking of ENaC to the
membrane via AS160 phosphorylation (363). From the observations outlined in this thesis, knocking down PKD2 in M1-CCD cells did not result in an increase in the abundance of Nedd4-2 without changing its overall protein expression levels.

This group found that PKD1 is a crucial regulator of the apical membrane directed trafficking of ENaC. Furthermore, it was shown that PKD1 regulates the membrane localisation of the Na⁺/K⁺-ATPase pump and knocking down PKD1 resulted in miss-localisation of the pump β subunit to the apical membrane and bulk accumulation of the pump protein in the cytosol rather than in the basolateral membranes. (266). Similarly, work from this group has shown that aldosterone stimulation of PKD1 knock-down M1-CCD cells failed to increase the basolateral membrane abundance of the Na⁺/K⁺ pump normally seen in wild-type cells.
Figure 4.10. ENaC insertion, its stability and removal from the membrane could be balanced by the interactions between PKD1, PKD2 and SGK1. Upon phosphorylation by aldosterone, PKD1 stimulates the insertion of ENaC into the apical membrane. This PKD1 dependent insertion of ENaC increases endocytosis of pre-formed ENaC subunits into the apical membrane. Additionally, SGK1 is phosphorylated in order to prevent the ubiquitination of ENaC. In the basal unphosphorylated state, PKD2 stimulates the retrieval of ENaC from the apical membrane. When PKD2 is phosphorylated by aldosterone, PKD2 may increase deubiquitination thus stabilizing ENaC in the membrane. When PKD2 is knocked down in M1 cells, it results in the stabilization of ENaC in the apical membrane due to a decrease in the activity of deubiquitinases. Knocking down SGK1 in M1 cells stimulates the retrieval of ENaC from the membrane while suppressing PKD1 results in the inhibition of ENaC insertion in response to aldosterone.
Short term treatment with aldosterone (30 minutes) resulted in an increase in the phosphorylation of Nedd4-2 in M1 CCD PKD2 deficient cells. However, this increase was not observed in the M1 cells. We reported in Chapter 3 that the aldosterone used may not have been producing an effect on PKD2 phosphorylation therefore, further control measures will need to be taken to ensure that the effect of aldosterone on the phosphorylation of Nedd4-2 is a true reflection of what is happening. We have shown that knocking down PKD2 does not have an effect on the abundance of Nedd4-2. Our results suggest that hormonal treatment does not alter the abundance of Nedd4-2 in either the wild type of the PKD2 deficient M1 cells. Again, in order to confirm that aldosterone does not have an effect on Nedd4-2 abundance, more stringent controls should be applied and the experiments repeated.

There may also be parallel pathways impacting SGK1 that are independent of PKD2 that could be exposed in our PKD2 KD M1 cell model. Recently, the serine-threonine with no lysine (K) kinases (WNKs) has been linked to the control of the ionic permeability of epithelial tissues. Specifically, two members of this family, WNK1 and WNK4, are associated with pseudohypoaldosteronism type 2 (PHA2), a disease that is characterized by hypertension, hyperkalemia, and metabolic acidosis (65, 364). WNK kinases form a novel signalling pathway that appears to be modulated by aldosterone which may play a role in regulating renal electrolyte homeostasis. WNK1 has been shown to phosphorylate SGK1 and subsequently incite ENaC activity (365, 366) while WNK4 and SGK1 have the ability to phosphorylate each other (367, 368). These kinases have been shown to act on ENaC in the connecting tubule and the cortical collecting duct (366, 369). Recent studies by Yu et al., demonstrate that endogenous ENaC single channel activity and amilorise-sensitive ISC are reduced by WNK4 (370). They showed using biotinylation that WNK4 reduced both the apical and total expression of ENaC.

It has been proposed that WNK4 inhibition of ENaC is dependent on Nedd4-2 mediated ubiquitination of ENaC due to the fact that WNK4 does not inhibit the channel when the C-terminal tails of ENaC β and γ are truncated (368). Additionally, there is no additional inhibitions of WNK4 following the initial Nedd4-2 inhibition of ENaC (370) which could imply that WNK4 mediated inhibition of ENaC is dependent on the function of Nedd4-2. Forskolin promotes the movement of ENaC to the surface of the cell membrane from a sub-apical recycling pool. Nedd4-2 promotes the trafficking of
retrieved ENaC into a degradative pathway rather than returning it to the recycling pool. This reduced the amount of ENaC that is available for insertion into the membrane. Furthermore, WNK4 and Nedd4-2 are not associated with one another in the same complex which suggests that WNK4 is unlikely to directly affect Nedd4-2 ubiquitination of ENaC (370). Yu et al., report that WNK4 reduced the ENaC recycling pool but had no effect on the time course of ENaC recycling between early endosome and apical membrane. Moreover, they demonstrated that WNK4 enhanced the internalization of ENaC and its retrograde trafficking.
Chapter 5

Characterisation of a novel aldosterone responsive kinase: protein kinase D3
5.1. Introduction

5.1.1 Protein kinase D

Protein kinase D (PKD) is a serine/threonine kinase that include three isoforms PKD1 (PKCμ), PKD2 and PKD3 (PKCν) (371). PKD isoforms contain a tandem repeat of zinc finger-like cysteine-rich motifs at the N terminus that exhibit a strong affinity for DAG or phorbol ester as well as a pleckstrin homology domain and a C-terminal catalytic domain that has a similar homology with calmodulin-dependent kinases (371). PKD isoforms have been linked to many cellular processes such as proliferation, trafficking, vesicle fission, gene expression and reorganisation of the actin cytoskeleton (371-373). While the PKD family contain a homologous catalytic domain, each isoform varies with respect to their subcellular localization, expression and regulation (371, 374-376).

Previous studies have shown the PKD isoforms undertake rapid redistributions in response to cellular stimulation. Both PKD1 and PKD2 are known to translocate from the cytosol to DAG-containing microenvironments in the plasma membrane (191) which is followed by PKC-dependent reverse translocation from the membrane to the cytosol where they subsequently accumulate in the nucleus (192). In contrast to the first two isoforms, PKD3 constantly shuttles between the cytoplasm and the nucleus (193). The PKD family members can pool and localise at the Golgi complex (194, 195) and the mitochondria (196). Additionally, PKD1 and PKD2 contain short PDZ-binding motifs in their COOH termini namely VSIL in PKD1 and ISVL in PKD2. Kinkel and colleagues determined that the Na⁺/H⁺ exchanger regulatory factor 1 (NHERF-1) interacts with these motifs of PKD1 and PKD2 which suggests that PKD isoforms can form complexes with NHERF-1 (197). This provides further evidence for the ability of PKD isoforms to regulate targets in multiple subcellular locations, thereby controlling various cellular activities. The PKD family of kinases have been implicated in the regulation of multiple biological processes such as proliferation, polarity, migration, differentiation, membrane trafficking, inflammation and hypertrophy (183) as they are often well positioned to regulate nuclear, cytoplasmic and membrane events.
5.1.2 Protein kinase D signalling

PKD isoforms are known to vary the relative activity of both the ERK and JNK pathways whereby they can attenuate the c-Jun phosphorylation and JNK activation in response to the activation of EGFR (192, 193) while stimulating the ERK and Ras pathways (191, 377-379). The PKD family of kinases can regulate budding of secretory vesicles from the trans-Golgi network (380, 381) and this process is required for locomotion and localization and activity of the Rac1-dependent leading edge in fibroblasts (183). In addition to a major regulatory role in cell trafficking and motility, PKD also stimulates the recruitment of integrin to newly formed focal adhesions (382) as well as the invasion of cancer cells (197, 383). Moreover, PKD has been shown to have a role in the regulation of apoptosis (384), the differentiation of T cells in transgenic models (385), re-introduction of DNA synthesis that can be induced by phorbol esters and regulatory peptides that act through Gq-coupled receptors (377, 386) and cardiac hypertrophy (103). PKD has been implicated as a facilitator of stress and multiple disease states for example, human hypertrophic cardiomyopathy (110), the activation of NFkB which is induced by Bcr-Abl in human myeloid leukaemia (310) and in oxidative stress responses (387-389). PKD isoform involvement in facilitating a wide array of both normal and abnormal biological actions in different subcellular compartments is most likely to be dependent on dynamic alterations in the isoform spatial and temporal localisation in combination with their substrate specificity (183) (figure 5.1).
Figure 5.1. Multiple biological functions of PKD signalling. Active PKD isoforms can phosphorylate multiple cellular targets at specific sites therefore regulating its subcellular localization or activity. Solid lines indicate direct phosphorylation of substrates while broken lines represent processes in which PKD is implicated but the sequence of molecular events has not been elucidated. Several scaffolding proteins and endogenous inhibitors have been connected to the regulation of the intensity and the duration of the ERK pathway. Class II histone deacetylases (HDACs) can regulate chromatin structure through their interaction with transcription factors to repress their activity. PKD phosphorylates specific residues in class II HDACS leading to their association with 14-3-3 proteins in endothelial cells and other cell types. In terms of epithelial cell polarity, PKD1 and PKD2, but not PKD3, regulate the production of TGN carriers that are to be trafficked to the basolateral membrane rather than to the apical membrane, and consequently PKD family members may play an important role in the generation of epithelial polarity. Another key mechanism required to establish cell polarity is facilitated by the evolutionary conserved PAR (partitioning-defective) genes, including Par-1. (Image modified from (186)).
PKD3 is unlike the other two isoforms whereby it is present in the nucleus as well as the cytoplasm in unstimulated cells (390). The rapid (and reversible) translocation of PKD3 to the plasma membrane is stimulated by agonists of GPCR (for example neurotensin) and B cell antigen receptor engagement (390, 391). Similar to the PKD1 isoform, the rate of PKD3 entry into the nucleus is increased by the activation of GPCR (390). It has been proposed that the variations in the intracellular distribution of the PKD isoforms may be responsible for their ability to carry out multiple functions at distinct subcellular locations. It is also possible that the GPCR-induced trans-activation of EGFR is required for the production of Ras-GTP (the partner of Raf) (381). The activation of PKD enables the interaction of Ras/Raf by phosphorylating RNI1 which translocates to the cytosol when formed in a complex with 14-3-3.

A novel PKCε/PKD3 pathway has been identified in prostate cancer cells in which it controlled the nuclear localization of PKD3 while regulating downstream ERK1/2 and Akt activities (392). PKD3 was seen to be up-regulated in malignant tumours and more aggressive cell lines that were relative to PKD3 expression in the secretory epithelial cells of normal prostate glands and benign primary prostate epithelial cells (PrEC). Chen et al., found that correlation between the nuclear retention of PKD3 and higher-grade tumours coincided with the distribution of PKD3 being predominantly nuclear in more metastatic prostate cancer cells (392). Their findings suggest that the nuclear localization of PKD3 may play a role in the advancement of prostate cancer.

5.1.3 PKD3 involvement in cell polarity and tight junction integrity

The establishment and the maintenance of cell polarity are essential for the functions of several cell types including epithelial cells. In polarised epithelial cells, PKD1 and PKD2 regulate the production of TGN carriers that are intended to locate to the basolateral membrane which consequently suggests that PKD isoforms may have a key role in the generation of epithelial polarity (195). Cell polarity is also mediated by partitioning-defective (PAR) genes including Par-1. Treating cells with phorbol-12-myristate-13-acetate (PMA) induced PKD-mediated phosphorylation of Par-1 on a Ser400 residue thus promoting the binding of Par-1 to 14-3-3. This complex promotes its dissociation from the lateral plasma membrane and subsequently inhibits its activity.
The results presented by Watkins et al., (393) suggest that PKD-induced phosphorylation of Par-1 may have a role in the regulation of cell polarity.

Rozengurt outlined in a review the substantial evidence they have gathered that demonstrates how PKD, especially PKD3, impairs the formation of apical intercellular junctions (186). The reassembly of these junctions decreased the trans-epithelial resistance (TER) and increases the paracellular permeability to sodium fluorescein in human bronchial epithelial cells (16 HBE 14o) and primary human SAEC monolayers. They further showed that in epithelial monolayers, PKD preferentially down-regulates claudin-1, but not other tight junction proteins (claudin-3, claudin-4, claudin-5, occludin and ZO-1) and adherent junctional proteins such as E-cadherin and β-catenin. Tight junctions are comprised of transmembrane and cytoskeletal proteins as well as peripheral scaffolding which work together to control paracellular permeability. Tight junctions associate with the underlying actin cytoskeleton in order to stabilize and enhance their barrier integrity (394). Rozengurt et al., found that over-expressing PKD3 resulted in the disorganisation of cell to cell contacts that resulted in the loss of cell tension leading to gaps in airway epithelial cells, thus implicating a possible loss of association with the actin cytoskeleton. This could be as a result of a defunct PKD-mediated down-regulation of claudin-1 in the airway epithelium.

To date, the role of PKD3 in the kidney remains unknown. Preliminary data from our group demonstrated expression of the kinase in non-stimulated M1-CCD cells. It is possible that PKD3 is having a similar effect on trafficking as the remaining two PKD isoforms. By advancing our investigation into M1-CCD cells, we have an opportunity to elucidate a possible role of PKD3 and hopefully determine a link to aldosterone-MR signalling cascades.
5.2. Aims

The primary aim of this chapter was to lay the foundation for studies on the physiological functions of PKD3 in the principle cells of the renal cortical collecting duct. Specifically, we examined:

I. The expression profile and localization of PKD3 in M1 CCD cells
II. The effect of knocking-down of PKD3 expression on ENaC subunit expression
III. To generate transient and stable PKD3 knock-down M1 cell lines using siRNA and CRISPR
5.3. Results

Our group has demonstrated the critical signalling interactions between PKD1 and PKD2 and the implications on the trafficking of ENaC channel subunits (5, 89, 94, 98, 109, 266). We have also found that increased plasma levels of aldosterone in male Sprague-Dawley rats fed a low Na⁺ diet, induced the translocation of PKD2 from the apical membrane to the cytosol (Quinn et al., Kidney Int). Suppressing PKD2 in M1-CCD cells did not prevent the polarization of the epithelial monlayer as the distribution of polymerized actin or Na⁺/K⁺-ATPase was similar to that of the wild-type. The up-regulation of SGK1 is the earliest transcriptional and translational response that is elicited by aldosterone. Knocking-down PKD2 in M1-CCD cells did not significantly affect the earliest phases of aldosterone-induced gene expression nor did it affect the early aldosterone-induced translocation of ENaC (109). Ussing chamber experiments with PKD2-deficient M1 CCD cells demonstrated a significant increase in the amiloride-sensitive $I_{SC}$ at both 2 and 4 hours following the treatment of aldosterone and this increase was maintained at 24 hours (5). Similarly, knocking-down PKD2 in M1-CCD cells resulted in an increase in the abundance of ENaCγ at the apical membrane along with aldosterone increasing the amiloride-sensitive $I_{SC}$ at 6 and 24 hours post-treatment. Given what we know in terms of other PKD isoforms, the work described in this chapter sought to characterise the potential role of PKD3 as an aldosterone-MR target kinase and to determine the role of this kinase in regulating ENaC activity.

5.3.1. PKD3 is expressed in principle cells of the cortical collecting duct

We began this investigation by determining whether M1-CCD cells expressed PKD3 (figure 5.2). NIH3T3 cells were used as a control and to further confirm expression of PKD3 in the CCD, we used mpkCCD cl14 cells. Cells were cultured under normal growth conditions and whole-cell protein lysates were prepared and separated by SDS-PAGE. Immunoblotting was subsequently carried out and we determined that PKD3 is expressed in M1-CCD cells.
Figure 5.2. PKD3 is expressed in principle cells of the cortical collecting duct. Protein lysates from M1 CCD, mpkCCD cl14 and NIH3T3 cells were electrophoresed using SDS-PAGE and subjected to immunoblotting where the membranes were probed with a primary antibody specific to PKD3 (predicted molecular weight 110 kDa).

Additionally, M1=CCD cells were cultured on sterilized glass coverslips so that by using immunofluorescence we could verify the localization of the kinase under basal conditions (figure 5.3A). Z-stack analysis using confocal microscopy exhibited cytosolic and nuclear expression of PKD3 in M1 CCD under basal conditions (figure 5.3B).
Figure 5.3. PKD3 expression and localization in M1 CCD cells. (A) M1 monolayers cultured on sterilised glass coverslips and wheat germ agglutinin (WGA) was used as a marker of the plasma membrane (red, Alexa Fluor 633 nm) which was added apically prior to cell permeabilisation. Cells were probed with an antibody conjugated to Alexa 488 targeting PKD3 (green). (B) The expression pattern of PKD3 in M1 cells was predominantly cytosolic with some nuclear staining. Z stacks and images are representative of 3 independent experiments (scale = 10 μm).
In order to determine the expression of PKD3 \textit{ex vivo}, normotensive male C57BL/6J mice were fed for 2 weeks on mouse chow containing 3.0 mg Na\textsuperscript{+}/g pellet prior to extraction of the kidneys. 5 μm cryosections of the kidneys were obtained and subjected to immunofluorescence and confocal microscopy (figure 5.4). The water channel aquaporin-2 was used as a marker for the apical membrane of cortical collecting duct and these cryosections were also probed with an antibody specific to PKD3. PKD3 was observed in the principle cells of the mouse cortical collecting duct \textit{ex vivo}.
Figure 5.4. PKD3 expression in ex vivo mouse cryosections. Male C57BL/6J mice were fed for 2 weeks on mouse chow containing 3.0 mg Na+/g pellet prior to extraction and the cryoprotection of the kidneys. Following fixation in 4% PFA, 5 µm thick slices of the kidneys were cryosectioned and fixed to microscopy slides. The cryosections were probed with an antibody specific to the aquaporin-2 water channel (red) which was used as a marker for the apical membrane of principle cells of the collecting duct. PKD3 was immune-detected with an antibody conjugated to Alexa 488 (green). The regions surrounded by a box in the representative image are indicating a region of the cortical collecting duct. PKD3 expression in the collecting duct of mice is cytosolic (Scale = 10 µm, n = 3).
5.3.2. Aldosterone effects on PKD3 localization in the CCD

In order to determine whether aldosterone had an effect on the subcellular localisation of PKD3 ex vivo, male Sprague-Dawley rats were maintained on either a normal chow diet or a low Na⁺ diet for 10 consecutive days. Rats maintained on a low Na⁺ diet exhibited a higher concentration in serum plasma aldosterone than their control diet counterparts and this increase in the circulating hormone was verified by ELISA. Cryosections of kidney tissue were obtained and immunofluorescence coupled with confocal microscopy were used to determine the effect of aldosterone on PKD3 localisation ex vivo (figure 5.5). Expression of PKD3 in sections obtained from rats maintained on a normal diet exhibited cytosolic expression of the kinase (figure 5.5A). Increasing the concentration of aldosterone by maintaining animals on a low Na⁺ diet did not induce the intracellular redistribution of PKD3 (figure 5.6).
Figure 5.5. PKD3 expression in rat kidney cryosections. Male Sprague-Dawley rats were humanely sacrificed according to local legislation and their kidneys removed. The kidneys were cryosectioned and immunofluorescence carried out to determine the localisation of PKD3 *ex vivo*. Aquaporin 2 (AQP2) (red) was used as a marker for the principle cells of the CCD while PKD3 was detected using an antibody conjugated to Alexa 488 (green). The regions surrounded by a box in the representative image are indicating a region of the cortical collecting duct. (A) Expression of PKD3 in sections obtained from rat maintained on a normal diet was observed in the cytosol. (B) Cytosolic expression of PKD3 was exhibited in the cohort of rats maintained on a low Na⁺ diet with an increase in levels of aldosterone. Images are representative of 5 animals per group. Scale 10 µm.
Figure 5.6. Aldosterone does not increase the expression of PKD3 in the rat CCD. Male Sprague-Dawley rats maintained on both a normal and a low Na⁺ diet were humanely sacrificed according to local legislation and their kidneys removed. The kidneys were cryosectioned and immunofluorescence carried out to determine the localisation of PKD3 ex vivo. The mean fluorescence intensity was then calculated for each cohort. Increasing the serum plasma concentration of aldosterone via a low Na⁺ diet did not increase the expression of PKD3 in the rat kidney.
Here we investigated whether aldosterone can produce this rapid response from PKD3 in vitro. M1-CCD cells cultured on glass coverslips were treated for 10 minutes with aldosterone (10 nM) or equivalent vehicle control. Cells were fixed and probed with an antibody specific to PKD3. Images were acquired using a confocal microscope and z-stack analysis was used to compare the subcellular localisation of PKD3 following aldosterone treatment (figure 5.7). In M1-CCD control samples, expression of PKD3 was cytosolic. Stimulation of these cells with aldosterone did not provoke the redistribution of PKD3 following 10 minutes of hormone treatment.
Figure 5.7. Aldosterone effects on the subcellular redistribution of PKD3 in M1-CCD cells. The subcellular distribution of PKD3 (green) was examined by confocal microscopy in cells treated with vehicle (ethanol) and aldosterone (10 nM) for 10 min. Z-stack analysis was carried out with the apical membrane marked with WGA (red). Untreated M1-CCD cells exhibited cytosolic expression of PKD3. Images are representative of 3 independent experiments. Scale 10 μm.
5.3.2. PKD3 knock-down in M1-CCD cells

PKD1 and PKD3 were originally considered to be members of the PKC family (395) however, they lack the C2 domain that is responsible for Ca\(^{+}\)-sensitivity exerted by conventional PKCs. Furthermore, the catalytic domain of PKD exhibits a low homology to the conserved kinase domain of PKCs and PKD also displays a distinct inhibitor and substrate specificity (396-398). The NH\(_2\)-terminal part of PKD contains a pleckstrin homology (PH) domain and lacks the typical auto-inhibitory pseudosubstrate motif present in PKCs (399) (figure 5.8).
Figure 5.8. Modular structure of PKD family members. All three PKD isoforms share parallel modular structures that are comprised of an N-terminal regulatory domain and a C-terminal kinase domain. In the case of PKD1 and PKD2, the N-terminus begins with an apolar region that is abundant in alanine and/or proline residues. In contrast, PKD3 does not contain a hydrophobic domain. All three PKD isoforms contain two cysteine-rich Zn fingers that are disconnected via a 14–20 amino acid long Zn-finger linker region. Furthermore, this region contains a serine-rich stretch in PKD2. The Zn fingers in all three isoforms come before a region of negatively charged amino acids and a PH domain. AP, alanine- and proline-rich domain; P, proline-rich domain; S, serine-rich domain; CYS, cysteine-rich Zn finger domain; AC, acidic domain; PH, pleckstrin homology domain; KINASE, kinase catalytic domain. (Image obtained from (308)).
Currently, investigations of PKD3-dependent signalling pathways in the kidney are lacking while studies in other tissues and cell types have been reliant on the use of non-specific pharmacological inhibitors or the use of small interfering RNA (siRNA) (figure 5.9).

Figure 5.9. Knock-down of PKD3 in M1-CCD cells using siRNA mediated silencing. RNA interference (RNAi) is widely used to develop a knock-down in order to carry out functional studies of genes within a cell. During this process, siRNA is synthesized with a complementary sequence for a gene of interest prior to being introduced into the cell. RNAi does not completely block the expression of the gene. siRNA’s can interfere with gene expression by hybridising to its corresponding RNA sequence in the target mRNA. This results in the subsequent activation of the degrading mRNA to prevent its translation into protein. (Image obtained from Abcam).
To date, it is unknown if PKD3 is an important kinase in the regulation of Na\(^+\) absorption in the kidney. Here we utilised two separate siRNA’s targeting murine PKD3 (PKD3 siRNA 1 and 2 respectively) and verified the transient knock down of PKD3 in M1 CCD (figure 5.10). Western blotting using a PKD3-specific antibody revealed the different degrees of effectiveness that each of the target siRNA’s had in suppressing PKD3. PKD3 siRNA 2 was found to be the most effective in suppressing PKD3 expression in M1 CCD cells. The sample lysates were also probed with an antibody particular to PKD1 to ensure that the siRNA’s were target specific.
Figure 5.10. Transient knock-down of PKD3 in M1 CCD cells. (A) Two different target sites (PKD3 siRNA 1 and PKD3 siRNA 2) were selected along the gene sequence for PKD3. The housekeeping gene GAPDH was used as a control. M1-CCD cells were cultured in 6-well plates in media containing 1% charcoal stripped serum and without antibiotics. A final concentration of 100 pmol siRNA was used to transfect the cells for 24 hours. This transfection was repeated for an additional 24 hours (i.e. a double transfection) and cells collected. Cells were then lysed, and their proteins separated by SDS-PAGE. Western blotting was used to confirm the knock down of PKD3 and its efficiency (PKD1 was used to show specificity of the siRNA for PKD3) with β-actin used as a loading control. As there were no changes in the expression of PKD1, we can be assured that the siRNA’s used were target specific (B). Densitometric analysis confirmed that both PKD3 siRNA 1 and 2 knocked down PKD3 (n = 2). The transfections were carried out by Dr. Cormac Jennings (Research assistant, Dept. Molecular Medicine, RCSI).
One of the earliest transcriptional and translational effects elicited by aldosterone is the up-regulation of SGK1. Our group has shown that knocking-down PKD1 in M1 CCD cells did not significantly affect the earliest phases of aldosterone-induced gene expression. Additionally, knocking-down PKD1 did inhibit the early aldosterone-induced translocation of ENaC into the apical membrane (109). Here we investigated the effect of knocking-down PKD3 on the expression of the ENaC channel subunits at the protein level (figures 5.11 – 5.13). We transiently transfected M1-CCD cells with PKD3 siRNA 1 and PKD3 siRNA 2 in order to knock down PKD3 \textit{in vitro}. Protein lysates from the cells collected were subjected to SDS-PAGE and Western blotting to determine if PKD3 could act as a transcription factor thus regulating ENaC. Knocking down PKD3 in M1 CCD cells did not affect the expression of the ENaCα subunit (figure 5.11). Multiple bands were observed on the ENaCα Western blot and these could be splice variants that are being detected by the primary antibody. Suppressing PKD3 in M1-CCD cells did not affect the expression of ENaCβ at the protein level (figure 5.12). This provides additional evidence to suggest that PKD3 is not acting as a transcription factor in these cells. Similarly, we did not observe any alterations in the protein levels of ENaCγ in PKD3-deficient M1-CCD cells (figure 5.13) thus suggesting that PKD3 cannot transcribe DNA to produce ENaC mRNA. As these experiments were preliminary, they will require repetition. Additional experiments to confirm that PKD3 is not a transcription factor should also be carried out at the mRNA level.
Figure 5.11. Knocking down PKD3 in M1 CCD cells does not affect the expression of ENaCα. (A) Two different target sites (PKD3 siRNA 1 and PKD3 siRNA 2) were selected along the gene sequence for PKD3. The housekeeping gene GAPDH was used as a control. M1 CCD cells were cultured in 6-well plates in media containing 1% charcoal stripped serum and without antibiotics. A final concentration of 100 pmol siRNA was used to transfect the cells for 24 hours. This transfection was repeated for an additional 24 hours (i.e. a double transfection) and cells collected. Cells were then lysed and their proteins separated by SDS-PAGE. Western blotting was used to confirm the knock down of PKD3 and its efficiency with β-actin used as a loading control. Additionally, Western blot was used to probe for expression of the ENaCα subunit (B). Densitometric analysis confirmed that both PKD3 siRNA 1 and 2 did not decrease the level of ENaCα expression in M1 CCD cells (n = 2). The transfections were carried out by Dr. Cormac Jennings (Research assistant, Dept. Molecular Medicine, RCSI).
Figure 5.12. Knocking down PKD3 in M1 CCD cells does not affect the expression of ENaCβ. (A) Following a double transfection with two different target sites along the gene sequence for PKD3 (at a final siRNA concentration of 100 pmol), M1 CCD cells were lysed and their proteins separated by SDS-PAGE. The housekeeping gene GAPDH was used as a control. Western blotting was used to confirm the knock down of PKD3 and its efficiency with β-actin used as a loading control. Additionally, Western blot was used to probe for expression of the ENaCβ subunit (B). Densitometric analysis confirmed that both PKD3 siRNA 1 and 2 did not decrease the level of ENaCβ expression in M1 CCD cells (n = 1). The transfections were carried out by Dr. Cormac Jennings (Research assistant, Dept. Molecular Medicine, RCSI).
Figure 5.13. Knocking down PKD3 does not alter the expression of ENaCγ in M1-CCD cells. (A) Two different target sites (PKD3 siRNA 1 and PKD3 siRNA 2) were selected along the gene sequence for PKD3. The housekeeping gene GAPDH was used as a control. M1 CCD cells were cultured in 6-well plates in media containing 1% charcoal stripped serum and in the absence of antibiotics. A final concentration of 100 pmol siRNA was used to double transfect the cells which were then harvested and lysed prior to their proteins being separated by SDS-PAGE. Western blotting was used to confirm the knock down of PKD3 and its efficiency with β-actin used as a loading control. Additionally, Western blot was used to probe for expression of the ENaCγ subunit (B). Densitometric analysis confirmed that both PKD3 siRNA 1 and 2 did not decrease the level of ENaCγ expression in M1 CCD cells (n = 2). The transfections were carried out by Dr. Cormac Jennings (Research assistant, Dept. Molecular Medicine, RCSI).
It is known that the aldosterone-induced increase in the expression of ENaC at the cell surface and the channel $P_o$ is regulated in two stages through the promotion of trafficking and stabilisation of pre-expressed ENaC subunits at the apical membrane and through the MR-dependent regulation of each subunit’s transcription. The phosphorylation and inactivation of Nedd4-2 by SGK1 results in the stabilisation of ENaC channel subunits in the membrane in order to increase the channel density by suppressing channel degradation after treatment with aldosterone. (351, 400). Our investigation into the effect of PKD isoform suppression on Nedd4-2 suggests that knocking down PKD2 in M1 CCD cells down removes the limitation of ENaC activity either by preventing or delaying the endocytotic retrieval of ENaC from the apical membrane back into the cytosol. In this thesis, we propose that PKD2 may act as a prolonged brake on ENaC activity in the basal state by maintaining Nedd4-2 at the membrane. To further understand the role of PKD isoforms in ENaC regulation, we examined the effect of knocking down PKD3 on the expression of Nedd4-2 (figure 5.14). In this case, we found that transiently knocking-down PKD3 in M1 CCD cells did not alter the expression of Nedd4-2 in vitro.
Figure 5.14. Knocking down PKD3 in M1 CCD cells does not affect the expression of Nedd4-2. (A) Following a double transfection with two different target sites along the gene sequence for PKD3 (at a final siRNA concentration of 100 pmol), M1 CCD cells were lysed and their proteins separated by SDS-PAGE. The housekeeping gene GAPDH was used as a control. Western blotting was used to confirm the knock down of PKD3 and its efficiency with β-actin used as a loading control. Additionally, Western blot was used to probe for expression of the E3 ubiquitin ligase Nedd4-2 (B). Densitometric analysis confirmed that both PKD3 siRNA 1 and 2 did not increase the level of Nedd4-2 expression in M1 CCD cells (n = 2). The transfections were carried out by Dr. Cormac Jennings (Research assistant, Dept. Molecular Medicine, RCSI).
The results observed in this chapter suggest that PKD3 may play a role in the regulation of renal Na⁺ reabsorption however it does not seem to do so by affecting the expression of ENaC channel subunits or by altering expression of the E3 ubiquitin ligase Nedd4-2. Transient transfections with siRNA’s can often lead to variations in the efficiency of the knock-down and factors such as the recycling of proteins can add to the consistency of maintaining the suppression of the kinase. In order to overcome this, we sought to generate a stable knock-down for PKD3 in M1 CCD cells be using CRISPR.

5.3.3. Generation of a stable PKD3 knock-down using CRISPR

Using a new gene editing technique such as CRISPR has allowed us to generate a stable known down of PKD3 in M1 CCD cells. In this thesis, we used CRISPR lentiviral transduction particles that were designed for two specific sites at exon 3 and exon 10 of the mouse PKD3 gene. Images were acquired following successful antibiotic selection with puromycin to track and morphological changes the cells might undergo (figure 5.15). M1-CCD PKD3 knock out cells displayed a similar morphology to that of both non-transduced and non-targeted cells. Once maintained in culture, PKD3-deficient cells become fully confluent and begin to form compact tight junctions that is characteristic of an epithelial monolayer. PKD3 knock-down cells were then lysed and Western blotting was used to determine the efficiency in suppressing this kinase (figure 5.16).
Figure 5.15. Morphology of M1-CCD PKD3 knock out cells generated using CRISPR lentiviral transduction particles. M1-CCD cells were transduced with non-targeting, exon 3 and exon 10 targeting lentiviral transduction particles for 48 h. Successfully transduced cells were selected using puromycin (5 µg/ml) for 14 days. Once confluent, cells were seeded into a T25 culture flask (day 0) and their image acquired on day 1. The morphology of the cells is similar to that of M1-CCD wild-type cells. PKD3 knock out cells reached full confluency at approximately day 5 where they showed characteristics of becoming a polarized epithelial monolayer. Images were acquired using a light microscope.
Figure 5.16. Generation of a PKD3 knock-down in M1 CCD cells using CRISPR. M1-CCD cells were transduced using CRISPR lentiviral transduction particles targeting two separate exons along the mouse PKD3 gene (exon 3 and 10 respectively). Non-targeting CRISPR lentiviral transduction particles were used as a control. Following the antibiotic selection of successfully transduced cells with puromycin, M1-CCD PKD3(-) cells were lysed and their protein content separated by SDS-PAGE. Western blotting was used to determine the efficiency of the knock-down. When compared to the non-transduced control, the particles targeting exon 10 was the most successful in knocking-down PKD3 in M1 CCD cells (n = 2).
5.3.4. Role of PKD3 in trafficking of ENaC subunits

As described in previously chapters, PKD isoform have a role in the subcellular trafficking of ENaC channel subunits to and from the plasma membrane. Here we investigated whether PKD3 initiated trafficking of ENaCγ in response to aldosterone. M1-CCD PKD3 knock out cells were cultured on glass coverslips and treated with aldosterone (10 nM) or vehicle control (EtOH) for 10 min. WGA was again used as a marker for the apical membrane prior to fixation with 4% PFA. In M1-CCD wild-type cells, aldosterone initiated a redistribution of ENaCγ to the sub-apical cytosolic space after 10 minutes (figure 5.17A). Knocking out PKD3 in M1-CCD cells does not appear to have an effect on the expression and localisation of ENaCγ (figure 5.17B). Stimulation of these cells for 10 minutes with aldosterone (10 nM) did not increase expression of ENaCγ at the apical membrane. In fact, ENaCγ remained in the cytosol. This data is representative of one experiment and therefore we cannot make any assumptions or conclude that a loss of PKD3 expression leads to altered trafficking of ENaC channel subunits in response to aldosterone.
Figure 5.17. PKD3 does not affect ENaCγ trafficking in response to aldosterone. M1-CCD and M1-CCD PKD3 knock out cells were cultured on glass coverslips prior to being treated with aldosterone (10 nM) or equivalent vehicle control (EtOH) for 10 min. Prior to fixation, the apical membrane of the cells was stained with WGA (red). The coverslips were then probed with an antibody specific to ENaCγ (green) while the nuclei were stained with DAPI (blue). Confocal microscopy was carried out using a Zeiss LSM710 microscope. (A) Z – stack analysis in M1-CCD cells demonstrated cytosolic expression of ENaCγ under basal conditions. Stimulation of M1-CCD cells with aldosterone did not increase the trafficking of ENaCγ to the apical membrane. (B) The expression pattern of ENaCγ in PKD3 knock out cells was predominantly cytosolic. Z – stack analysis did not exhibit the redistribution of ENaCγ in response to aldosterone. Images are representative of 1 independent experiment (scale = 10 μm).
Figure 5.18. Aldosterone stimulation does not affect the abundance of ENaCγ in M1 or M1 CCD PKD3(-) cells. Z-stack analysis on the confocal images acquired looked to compare the effect of aldosterone on ENaCγ expression in M1 and PKD3(-) knock out cells. The mean intensity fluorescence was calculated using ImageJ. This figure is representative of n = 3. A one-way ANOVA was carried out along with Tukey’s post hoc test. Aldosterone did not have an effect on the abundance of ENaCγ in the wild type or PKD3 deficient cells.
5.4. Discussion

There are multiple studies that suggest a similar model could clarify the regulation of the catalytic activity and intracellular distribution of the PKD2 and PKD3 isoforms in response to DAG generation induced by agonists. In these studies, the model proposed that the distribution of inactive PKD2 and PKD3 isoforms before cell stimulation are in the cytosol and nucleus which results from their respective rates of nuclear import and nuclear export (192, 309, 401). This proposed model suggests that the production of DAG in the plasma membrane can trigger alterations in the localization, phosphorylation and catalytic activation of PKD2 and PKD3. The results presented in this chapter demonstrate both cytosolic and nuclear localization of PKD3 in principle cells of the cortical collecting duct. Going forward, further confirmation of PKD3 in specific sub-cellular compartments (such as the nucleus or the cytoplasm) can be carried out with the use of Western blotting by separating out each of the sub-cellular fractions from whole-cell lysates. The use of immunoblotting for house keeping genes known to be expressed in each fraction can be used to ensure that each fraction has not been contaminated by the others. Additionally, stimulation M1 CCD cells with aldosterone can be carried out to determine whether hormone treatment can induce a redistribution of PKD3 from these cellular compartments. Initial observations from immunofluorescent experiments looking at PKD3 expression and localization suggest that perhaps a similar mechanism of PKD activation could in produce diverse physiological responses based on the differential distribution of each PKD isoform. As the localization of PKD3 presented here is subjective, further investigation is required.

The association between PKD3 and transporters such as ENaC remains unknown. Here we report using siRNA to transiently knock down ENaC and Nedd4-2. Knocking down PKD3 with an siRNA did not have an effect on the expression of ENaC channel subunits, nor did it have an effect on the expression of Nedd4-2 at the protein level. As this study only looked at the effect that knocking down PKD3 had on protein expression, we do not know whether the knock down has an affect at the mRNA level. This is something that can be explored and may answer the question as to whether PKD3 is involved in the genomic regulation of ENaC.
A disadvantage of using siRNA to suppress gene expression is that it is often difficult to reproduce and can lead to variations in expression. To overcome this, the more up-to-date technology of CRISPR/Cas9 was used to delete the PKD3 gene from M1 cells.

Rozengurt et al., determined that PKD preferentially down-regulated claudin-1 in epithelial monolayers and that over-expressing PKD3 resulted in the disorganisation of cell to cell contacts that resulted in the loss of cell tension leading to gaps in airway epithelial cells (394). They indicate that this could be due to loss of association with the actin cytoskeleton. An advantage of using technology such as CRISPR/Cas9 to delete the PKD3 gene from M1 cells is that it allowed for better reproducibility when looking at the effect that suppressing the kinase had on the morphology of M1 cells. Knocking-down PKD3 in M1 cells did not appear to affect the tight junction integrity. PKD3 knock out M1 cells produced in this pilot study did not appear to have altered morphological characteristics when compared with both the non-transducing control and wild-type M1 cells. This CRISPR knock out also behaved in a similar manner to the PKD2 knock-down that was generated using shRNA suggesting that suppression of these kinases alter the phenotype of the M1 cells. Further investigation into the ability of PKD3-deficient cells to form a polarized epithelial monolayer by measuring TEER are required. We have however determined that knocking-down PKD3 is not lethal in M1 CCD cells.
Chapter 6

Characterisation of WT 9-12 cells: an *in vitro* model for autosomal dominant polycystic kidney disease
6.1 Introduction

6.1.1 Autosomal Dominant Polycystic Kidney Disease

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder that often results in the formation of fluid-filled cysts in the renal tubules which in turn lead to the gradual destruction of the renal parenchyma and renal failure. ADPKD is caused by the mutations in two genes, \textit{PKD1} and \textit{PKD2} which encode polycystin-1 and polycystin-2 (not to be confused with protein kinase D1). Mutations in \textit{PKD1} account for 85\% of cases of ADPKD. Renal cysts can originate from any segment of the nephron in ADPKD including proximal and distal tubules and/or collecting ducts (402, 403). Over half of the patients with ADPKD present with hypertension often before the onset of renal deficiency (404-406) and it is a key factor in the progression of end-stage renal disease (ESRD). However, the mechanisms that lead to hypertension in ADPKD remain to be elucidated.

6.1.2. Evidence for the role of RAAS in ADPKD

It has previously been suggested that the RAAS is involved in ADPKD though a consistent connection between BP, plasma renin activity or plasma aldosterone concentrations (75, 404, 407, 408). There is however, indirect evidence supporting the involvement of RAAS in the control of BP in patients with ADPKD (75, 404, 408). The converting enzyme inhibitor captopril resulted in a significant increase in the plasma renin activity in hypertensive versus normotensive patients with ADPKD suggesting an increased RAAS activity in ADPKD (404). Conversely, is it difficult to correlate the increase in systemic RAAS activity with hypertension in ADPKD but some studies support the hypothesis that there is over active intrarenal RAAS in ADPKD (78). Torres \textit{et al.}, observed strong renin immunostaining in dilated tubules and cysts in kidneys with ADPKD (78). They also demonstrated that the cyst-derived epithelial cells \textit{in vitro} expressed renin mRNA suggesting local renin synthesis (78). While these studies showed the ability of the cyst epithelium to produce renin, it did not look at where other
RAAS components were also present and whether there was an increase in the production of angiotensin II.

In more recent studies carried out by Loghman-Adham et al., (409) it was determined that in addition to renin, the cysts and dilated tubules in ADPKD also contain angiotensinogen, ACE, the angiotensin II receptor and peptide. Based on their findings, Loghman-Adham et al., proposed that ectopic renin and antiotensinogen production by the cyst epithelial could increase the creation of angiotensin I and subsequent angiotensin II production (409). Higher concentrations of angiotensin II could result in the increase in Na\(^+\) and water reabsorption by the functioning tubules leading to the development of hypertension.

### 6.1.3 Possible mechanisms of the upregulation of intrarenal RAAS in ADPKD

The production of renin in juxtaglomerular cells can be as a result of fluctuations in extracellular NaCl or intracellular Ca\(^{2+}\) (410). Polycystin-2 could generate a non-selective Na\(^{+}\) channel (224, 411) that requires the assembly with polycystin-1 in order to form a functioning ion channel (412). It is therefore possible that polycystins could have a role in the regulation of renin production through the modulation of Na\(^{+}\) or Ca\(^{2+}\) fluxes crossing the cyst epithelium. Additionally, dysfunctional channel activity that results from mutations in polycystin (413, 414) could lead to a reduction in the concentrations of intracellular Na\(^{+}\) and Ca\(^{2+}\) and the stimulation of renin production. It has been shown that polycystins can act as flow sensors in renal tubules that transduce mechanical fluid flow signals into Ca\(^{2+}\) signals (415). Nauli et al., showed that in vitro models that contained an homozygous mutation in polycystin had cilia that failed to sense fluid flow (415). Loghman-Adham et al., speculated that the increase in renin expression by the epithelial cells lining the cyst could be in part related to the failure of mechanosensation that arise from mutations in polycystin (409). Here they proposed a model of autocrine/paracrine intrarenal RAAS that accounted for salt and water retention and the subsequent hypertension that is observed in polycystic kidney disease.
6.1.4. Protein Kinase D mediated phosphorylation of polycystin-2 in cell growth and channel activity

Polycystin 1 and 2 have been shown to function as a heterodimeric complex (412, 416) that activate multiple key signalling pathways which regulate various cellular functions such as proliferation, tubulogenesis and fluid secretion. These two polycystins are prone to functioning together but there is evidence to suggest that they can also function independently (417). Polycystin-2 is a 968-amino acid with six transmembrane spans, a pore-forming region and contains cytoplasmic N- and C-terminal tails. It has also been included as a member of the transient receptor potential (TRP) channel superfamily (TRPP2) that is highly conserved and can function as non-selective cation channels that respond to various stimuli. Polycystin-2 has been demonstrated to function at several cellular locations such as primary cilia (415), the basolateral membrane (418) and the endoplasmic reticulum (ER) (419). Polycystin 1 and 2 have also been shown to reconstitute a receptor-ion channel complex at the plasma membrane and the junction between the ER and plasma membrane (420).

Phosphorylation of proteins is a key post-translational mechanism for the regulation of the function of most proteins including the TRP family of ion channels (421). In most cases, phosphorylation results in an increase in channel activity. The activity of TRPC1 is increased by the phosphorylation by PKCα (422) while TRPM4 is also increased via PKC phosphorylation (423, 424). Furthermore, phosphorylation may also regulate polycystin-2 channel activity (425, 426). Previous studies have identified two mammalian polycystin-2 serine residues that are phosphorylated (425, 427). The phosphorylation of polycystin-2 at Ser812 is crucial for many functions such as Ca$^{2+}$ dependence, the retrograde trafficking via binding to adaptor proteins and the retention of the cell cycle regulatory protein Id2 in the cytoplasm (425, 428, 429). Additionally, GSK3 dependent phosphorylation at Ser76 has also been shown to be crucial for the retention of polycystin-2 at the basolateral membrane in vitro and is vital for the pronephric development in vivo (427).

Streets et al., demonstrated a new phosphorylation site within the polycystin-2 terminus at Ser801 that contained a conserved recognition sequence for Protein Kinase D (PKD) (430). They also showed that in Madin-Darby canine kidney cells (MDCK) with inducible expression of polycystin-2, phosphorylation at this site specifically
regulated cell growth that is mediated through permissive effects on Ca\(^{2+}\) transients from ER-located polycystin-2 channels. A pathogenic mutation at Ser\(^{804}\) within the PKD recognition consensus sequence eliminated the phosphorylation of polycystin-2 at Ser\(^{801}\), indicating that it plays a significant physiological role (430). They also investigated Ser\(^{801}\) phosphorylation under conditions of PKD overexpression or knockdown and found that the phosphorylation of Ser\(^{801}\) was increased in wild-type polycystin-2 when co-transfected with PKD but not in a polycystin-2 Ser\(^{801}\) mutant. In HEK cells, knocking down PKD led to the elimination of polycystin-2 Ser\(^{801}\) phosphorylation (430).

The activation of PKD following growth factor stimulation has been widely described (183, 431). Streets et al., looked at whether the phosphorylation of Ser\(^{801}\) could be altered by stimulation with EGF or serum and found that a significant increase in endogenous polycystin-2 Ser\(^{801}\) phosphorylation was detected after the addition of serum in murine collecting duct epithelial cells (M8) (416). The effect of serum and EGF stimulation was also seen in transiently transfects HEK cells and stably transfected MDCK cells.
6.2. Aims

Loghman-Adham et al. reported on the expression of transporters such as ENaC, cystic fibrosis transmembrane conductance regulator (CFTR), Na⁺/H⁺ exchanger (NHE3), the water channel aquaporin-1 (AQP1) and Na⁺/K⁺-ATPase at the mRNA level (432). They demonstrated the expression of EGFR by both immunocytochemistry and RT-PCR and determined that it was expressed in the cyst-derived cells of both distal and proximal tubule origin. Notably, Na⁺/K⁺-ATPase showed weak expression in cells that originated from a distal cyst (432). Cyst-derived cells that originated from a distal tubule were shown to express renin however, cells originating from proximal tubules express angiotensinogen (433). Unpublished observations from Loghman-Adham et al., describe how both cell types express ACE and AT1 receptors. Their study did not look at the effect of RAAS components on cyst-derived cells. This group has previously shown how aldosterone rapidly phosphorylates and activates PKD2 in M1 CCD cells. Similarly, PKD1 can be trans-activated by EGFR which is coupled to MR. Given the knowledge of this group, this chapter sets out to:

I. Further characterise the WT 9-12 in vitro model of ADPKD
II. Determine whether ADPKD cells are responsive to aldosterone stimulation
III. Establish the effect of aldosterone on PKD isoforms in WT 9-12 cells

The overall aim was to determine if miss-polarisation of ENaC and Na/K pump is a feature of AKPKD cells and if PKD isoforms under the control of Aldo-MR played a role in miss-localisation of these ion transporters to reverse fluid secretion into the lumen and produce fluid-filled cysts.
6.3. Results

In this chapter, we focused on the autosomal dominant form of this disease. Not only can polycystic diseases be inherited, they may also be acquired in adult life. Throughout the acquired forms, cysts can develop in the kidneys as a consequence of aging, dialysis or hormonal influences (216, 221) and these renal cysts are often manifestations resulting from genetic proliferative syndromes (216). ADPKD disease is largely characterised by the enlargement of cysts in the renal tubules as a consequence of an increase in the proliferation of epithelial cells, abnormal fluid secretion and the mispolarisation of many membrane proteins (97, 107, 185). Previous studies have shown that similarities between the expression and the distribution of several proteins in ADPKD and foetal epithelia suggest a role for polycystin 1 in the regulation of epithelial polarisation (109).

6.3.1 Characterisation of an *in vitro* model of ADPKD – WT 9-12 cyst-derived cultures

ADPKD is one of the most prevalent inherited genetic diseases with the ultimate outcome being end stage renal disease (ESRD) or even renal failure. The association between hypertension and ADPKD progression is established, but the cause and effect relationship is less clear and may provide novel approaches to treating progressive kidney disease. In this thesis, two in vitro models were utilised to determine if the polarisation of ENaC and Na⁺/K⁺-ATPase is a feature of ADPKD cells and whether PKD isoforms have a role in the trafficking of these transporters. Our group has previously determined that the activation of PKD1 in the normal collecting duct epithelium is dependent of PKCε (109). We began this study by determining the differentiation status of WT 9-12 cells (figure 6.1). The American Type Culture Collection (ATTC) propose that M1 CCD cells are propagated in the presence of the glucocorticoid hormone dexamethasone and previous studies from this lab corroborated this finding (94). There is also evidence that dexamethasone markedly enhances morphological and functional cell differentiation in epithelial cells of a proximal tubule origin (434). The colonic epithelial cell line HT29 cl.19A were used as
a control as they display the characteristics of a well differentiated cell line while DLD1 cells derived from colorectal adenocarcinoma were used as an example of a less differentiated model (figure 6.1).

**Figure 6.1. Dexamethasone does not induce differentiation in an in vitro model of ADPKD.** WT 9-12 cyst derived cells were cultured in the presence or absence of media supplemented with the glucocorticoid hormone dexamethasone (5 µM). HT29 cl.19A cells were used as a control for a well differentiated epithelial cell line while DLD1 cells were used to represent a less differentiated cell model. Cell lysates were separated electrophoretically by SDS-PAGE and Western blotting performed. (A) HT29 cl.19A and DLD1 cells both express E-cadherin which was used as a characteristic of high differentiation. Dexamethasone (5 µM) did not increase the expression of E-cadherin in WT 9-12 cells. (B) N-cadherin was used as a marker for undifferentiated cells. WT 9-12 cells express N-cadherin. Treatment with dexamethasone (5 µM) did not decrease the expression of N-cadherin in these cells. HT29 cl.19A and DLD1 cells did not show expression of N-cadherin. n = 3.
Cadherins are comprised of a large family of membrane-associated glycoproteins or transmembrane proteins that facilitate specific cell to cell adhesion that is often dependent on Ca\(^+\). Cadherins also function as important molecules in organ morphogenesis (435-437). E-cadherin is a type I cadherin and its adhesive properties in V79 Chinese hamster lung cells have been attributed to a Ca\(^+\) independent agglutination and Ca\(^+\) dependent cell to cell adhesion (438). As seen in figure 6.1A, HT29 cl.19A and DLD1 cells both exhibited expression of E-cadherin confirming their status as well differentiated cell lines. However, WT 9-12 cells did not show expression of E-cadherin under basal conditions (figure 6.1A). In order to determine whether glucocorticoids could initiate differentiation in these cyst-derived cells, WT 9-12 cells were maintained in normal growth media that had been supplemented with dexamethasone (5 µM). The treatment of these cells with dexamethasone did not induce differentiation (figure 6.1A).

N-cadherin is known to promote survival in cancer cells as well as having an inhibitory effect on cell proliferation (439-441). It is also associated with migration and cell invasion while aberrant expression of N-cadherin enables cells to become more motile and invasive as they are undifferentiated during this process. Neither the HT29 cl.19A nor DLD1 cells showed expression of N-cadherin (figure 6.1B). WT 9-12 cells exhibited N-cadherin expression that was not enhanced with the treatment of dexamethasone (5 µM). Based on these observations, it would seem that these cells do not represent a well differentiated model for studying ADPKD. Additionally, treatment with dexamethasone did not induce differentiation in WT 9-12 cells.
6.3.2. Nuclear hormone receptors in WT 9-12 cultures

In order to determine the responsiveness of WT 9-12 cells to aldosterone, we first established whether these cells express MR (figure 6.2) and GR (figure 6.3).

![Image of Western Blot Analysis]

**Figure 6.2. Mineralocorticoid receptor expression in an in vitro model of ADPKD.** NIH 3T3, HEK293T and WT 9-12 cells were lysed and their proteins separated by SDS-PAGE. Subsequent immunoblotting was carried out to determine the expression of MR. Each cell line expressed MR with a molecular weight of 115 kDa and a smaller band at 82 kDa.
MR is known to be expressed by the principal cells and to a lesser extent by intercalated cells of the distal renal CCD (193), however it has been suggested that MR is not expressed in the M1 CCD cell line or that it is inactive as a transcription factor but capable of eliciting some rapid signalling responses (108, 194, 195). Our investigations demonstrated that the WT 9-12 cell model of ADPKD expresses MR under basal conditions. As MR is predominantly expressed in the distal nephron, this suggests that our cell model of ADPKD is derived from the distal tubule. The feature of MR in WT 9-12 cells suggests a characteristic sensitivity for aldosterone which distinct effects of this hormone on the handling of electrolytes in this part of the nephron.

Glucocorticoids can modulate a range of functions such as cardiovascular, behavioural and immune processes. The biological actions of glucocorticoids are facilitated by the activation of the glucocorticoid receptor (GR). This receptor belongs to the same nuclear hormone receptor super-family as MR and can also function as a ligand-dependent transcription factor (61, 442-444). GR is expressed in the kidney and its mRNA has been detected in most cell types while studies of the receptor-ligand interactions in the collecting duct have shown that mineralocorticoids can also bind to GR (445). It has also been shown that GR can interact with aldosterone in both the mouse and rat kidney (446) whereby nuclear translocation is used as an alternative to receptor activation. In the collecting duct, GR can activate the transcription of Sgk1 and GILZ (447, 448). Glucocorticoids can stimulate the electrogenic transport of Na⁺ when present in excess in humans which is the case in Cushing's syndrome. However, it is still unclear whether this contributes to hypertension in metabolic syndrome or in cases of moderate glucocorticoid excess (449).
Figure 6.3. Glucocorticoid receptor expression in an *in vitro* model of ADPKD. NIH 3T3, HEK293T and WT 9-12 cells were lysed and their proteins separated by SDS-PAGE. Subsequent immunoblotting was carried out to determine the expression of GR. NIH3T3 cells did not express GR. In contrast, both the HEK293T and the WT 9-12 AKPKD cells exhibited expression of the receptor.
We investigated the expression of GR in our in vitro model of ADPKD. Unlike the expression of MR, the murine cell lines (M1 CCD and NIH3T3) did not show expression of GR (figure 6.3). Conversely, the cyst derived WT 9-12 cells and the HEK293T cells exhibited expression of GR at the protein level (figure 6.3). This provides further evidence that these cells have been isolated from a distal tubule. The presence of GR in these cells points to sensitivity of this model to glucocorticoids which may play a role in the hypertensive pathology that is displayed by patients with ADPKD.

6.3.3. Transporter expression in WT 9-12 cells

Here we confirmed the expression and localisation of Na⁺/K⁺-ATPase in WT 9-12 cyst-derived cells using the β1 subunit as a marker using Western blot coupled with immunofluorescence (figures 6.4 and 6.5). Interestingly, in the case of ADPKD, the expression of the Na⁺/K⁺-ATPase β1 pump is mislocalised to the cytosol and the apical membrane (248). This abnormal localisation was investigated in WT 9-12 cells by immunofluorescence (figure 6.5). The representative images demonstrate expression of the Na⁺/K⁺-ATPase β1 subunit at the apical membrane in WT 9-12 cyst-derived cells.
Figure 6.4. Expression of the Na\(^+\)/K\(^-\)-ATPase \(\beta1\) subunit in WT 9-12 ADPKD cyst-derived cells. Commerciaally available human brain cerebellum whole cell lysate was used as a positive control for the Na\(^+\)/K\(^-\)-ATPase \(\beta1\) subunit. 10\(\mu\)g of protein from each sample was subjected to SDS-PAGE and western blotting before being probed for expression of the Na\(^+\)/K\(^-\)-ATPase \(\beta1\) subunit (predicted size 45kDa). WT 9-12 cells do express Na\(^+\)/K\(^-\)-ATPase but exhibit a decrease in its expression in comparison to other tissues such as the brain (n=3).
Figure 6.5. Na\(^+\)/K\(^+\)-ATPase expression and localisation in an in vitro model of ADPKD. WT 9-12 cyst-derived cells were cultured on glass coverslips and immunocytochemistry performed. The nuclei were stained with DAPI (blue), the cytoskeleton with TRITC-phalloidin (red) and the primary antibody for the β1 Na\(^+\)/K\(^+\)-ATPase subunit was couple to Alexa 488 (green). (A) This representative image shows that β1 Na\(^+\)/K\(^+\)-ATPase subunit is expressed in these cells. (B) Further z-stack acquisition demonstrates localisation of β1 Na\(^+\)/K\(^+\)-ATPase to be at the apical membrane under basal conditions. Scale 20μm.
The WT 9-12 ADPKD cyst-derived cells not only express both polycystin 1 and polycystin 2 but also express a diverse range of other markers that are typical of renal tubules. In order to confirm the presence and localisation of each of the ENaC channel subunits, immunofluorescence studies were performed (figures 6.6 – 6.8).
Figure 6.6. ENaCα channel subunit expression and localisation in an in vitro model of ADPKD. WT 9-12 cyst-derived cells were cultured on glass coverslips and immunocytochemistry performed. The nuclei were stained with DAPI (blue), the cytoskeleton with TRITC-phalloidin (red) and ENaCα was couple to Alexa 488 (green). (A) ENaCα (green) is expressed in these cells. (B) Z-stack was used to show the localisation of ENaCα within the cell. Scale 10μm.
Figure 6.7. ENaCβ channel subunit expression and localisation in an in vitro model of ADPKD. WT 9-12 cyst-derived cells were cultured on glass coverslips and immunocytochemistry performed. The nuclei were stained with DAPI (blue), the cytoskeleton with TRITC-phalloidin (red) and ENaCβ was couple to Alexa 488 (green). (A) ENaCβ (green) is expressed in these cells. (B) Z-stack acquisition demonstrating ENaCβ localisation within the cell. Scale 10µm.
Figure 6.8. ENaCγ channel subunit expression and localisation in an in vitro model of ADPKD. WT 9-12 cyst-derived cells were cultured on glass coverslips and immunocytochemistry performed. The nuclei were stained with DAPI (blue), the cytoskeleton with TRITC-phalloidin (red) and ENaCγ was coupled to Alexa 488 (green). (A) This representative image confirms the expression of ENaCγ in these cyst-derived cells. (B) Z-stack showing the localisation of ENaCγ in the z-plane of WT 9-12 cells. Scale 10μm.
Fluorescence analysis of the localisation profile of the ENaC channel subunits was conducted using immunocytochemistry. ENaC α, β and γ channel subunits were stained with Alexa Fluor 488 (green) (figures 6.6 – 6.8). Imaging was carried out using a Zeiss LSM710 confocal microscope. TRITC-phalloidin was used to mark the cytoskeleton (red) and DAPI was used to label the nuclei (blue). As seen in the representative images (Figures 6.6 – 6.8), ENaC channel subunits (green) appear to be expressed in the mainly in the cytosol under basal conditions.

6.3.4. Aldosterone-sensitive protein kinase D in ADPKD cells

There have been some studies that point to PKD having a role to play in the phosphorylation of several serine sites along polycystin 2 (183, 430, 431). With the use of SDS-PAGE and Western blotting, the expression profiles of each of the three PKD isoforms were determined. Immunofluorescence was also conducted to confirm the presence or absence of PKD2 in ADPKD WT9-12 cells.

Figure 6.9. Protein kinase D1 expression in WT 9-12 cyst-derived cells. Whole cell lysates from the murine cells lines WT 9-12, NIH3T3 and human HEK293T cells were separated by SDS-PAGE. Subsequent immunoblotting was carried out to determine the expression of PKD1 (predicted molecular weight 115 kDa). HEK293T cells were used a positive control for expression of PKD isoforms in human in vitro models. PKD1 is expressed in WT 9-12 cyst-derived cells.
Figure 6.10. Localisation of protein kinase D1 in an *in vitro* model of ADPKD. WT 9-12 cells were cultured on sterilized glass coverslips and stained with wheat germ agglutinin (WGA, red) prior to fixation. Nuclei were stained with DAPI (blue) while the PKD1 primary antibody was conjugated to Alexa 488 (green).
PKD1 is the isoform that is well characterised and has been implicated in many processes such as proliferation, migration and trafficking. The catalytic function, intracellular localisation and regulation of PKD1 is intricately connected to its structural composition. Western blotting confirmed that PKD1 is expressed in WT 9-12 cyst-derived cells (figure 6.9). Further evidence to support this was provided following immunofluorescence to determine the localisation of PKD1 in WT 9-12 cells (figure 6.10). WT 9-12 cells had their nuclei stained with DAPI (blue) and the apical membrane stained with WGA (red). An antibody specific to PKD1 was immune-detected with a secondary antibody conjugated to Alexa 488 (green). Image acquisition was carried out using confocal microscopy and this confirmed expression of PKD1 in our *in vitro* model of ADPKD.

Similar to determining PKD1 expression in WT 9-12 cells, we also looked at expression of the second isoform (PKD2) *in vitro*. In 2001, Sturany *et al.*, cloned the serine/threonine protein kinase, PKD2 (187). PKD2 has a predicted molecular mass of 97 kDa which they showed to contain an N-terminal hydrophobic area predicted to be a transmembrane region, 2 cysteine-rich motifs that form zinc finger-like repeats, a pleckstrin homology domain, and a putative kinase domain containing the ATP-binding consensus sequence (187). Northern blot analysis detected a constitutively low expression of a 4-kb transcript although higher expression levels were seen in the heart, lung, smooth muscle, brain and pancreas while the kidney and liver exhibited lower levels of expression. SDS-PAGE and Western blot analysis resulted in the detection of PKD2 at the protein level with a molecular mass of 105 kDa (187). Here we have also used Western blotting to confirm expression of PKD2 at the protein level in WT 9-12 cells (figure 6.11).
Figure 6.11. Protein kinase D2 expression in WT 9-12 cyst-derived cells. Whole cell lysates from the murine cells lines WT 9-12, NIH3T3 and human HEK293T cells were separated by SDS-PAGE. Subsequent immunoblotting was carried out to determine the expression of PKD1 (predicted molecular weight 105 kDa). HEK293T cells were used a positive control for expression of PKD isoforms in human in vitro models. PKD2 is expressed in WT 9-12 cyst-derived cells.
A. Figure 6.12. Localisation of protein kinase D2 in an in vitro model of ADPKD. WT 9-12 cells were cultured on sterilized glass coverslips. Nuclei were stained with DAPI (blue), the actin cytoskeleton with TRITC-phalloidin (red), while the PKD2 primary antibody was conjugated to Alexa 488 (green). (A) Immunofluorescence confirmed the expression of PKD2 in vitro. (B) Further z-stack acquisition was used to show the localization of PKD2 in these WT 9-12 cells.
Isolated proteins from WT 9-12 cells were analysed using Western blotting and probed with a specific antibody for PKD2. HEK293T cells were used as a control and WT 9-12 cells demonstrated a lower expression profile for PKD2 (figure 6.11). Immunofluorescence suggests that PKD2 is localized in the cytosol of WT 9-12 cells (figure 6.12).

Using similar techniques, we determined the expression of the remaining isoform (PKD3) along with its localization (figures 6.13 – 6.14). PKD3 is expressed at the protein level in WT 9-12 cyst-derived cells. Western blot analysis resulted in a band of approximately 95 kDa which is close to the predicted mw for PKD3 (figure 6.13). Confocal microscopy confirmed expression of PKD3 in WT 9-12 cells (figure 6.14A). Further z-stack analysis determined the expression of this kinase to be predominantly cytosolic but with some cells showing nuclear staining (figure 6.14B). This is consistent with studies that show PKD3 shuttling from the cytosol to the nucleus.

**Figure 6.13. Protein kinase D3 expression in WT 9-12 cyst-derived cells.** Whole cell lysates from the murine cells lines M1 CCD, NIH3T3 and human HEK293T and WT 9-12 cells were separated by SDS-PAGE. Subsequent immunoblotting was carried out to determine the expression of PKD3 (predicted molecular weight 110 kDa). HEK293T cells were used a positive control for expression of PKD isoforms in human *in vitro* models. PKD3 is expressed in WT 9-12 cyst-derived cells.
A. Figure 6.14. Localisation of protein kinase D3 in an *in vitro* model of ADPKD. WT 9-12 cells were cultured on sterilized glass coverslips. Nuclei were stained with DAPI (blue), the actin cytoskeleton with TRITC-phalloidin (red), while the PKD3 primary antibody was conjugated to Alexa 488 (green). (A) Immunofluorescence confirmed the expression of PKD3 *in vitro*. (B) Further z-stack image acquisition was used to view the localisation of PKD3 in these WT 9-12 cells.
We have shown that PKD isoforms 1 and 2 to be rapidly phosphorylated and activated by aldosterone in M1 cells. We investigated here the role of aldosterone in phosphorylating the different PKD isoforms in the cyst-derived cells. We began by treating WT 9-12 cells with various concentrations of aldosterone (0.1 nM to 1 µM) for 10 minutes. Western blot analysis was used to determine the effect of aldosterone on the phosphorylation of each of the three PKD isoforms (figures 6.15 – 6.17). In the case of PKD1, 10 nM aldosterone prompted the biggest response in the phosphorylation of PKD1 at Ser$^{916}$ when treated for 10 minutes (figure 6.15). Aldosterone (1 nM) induced an increase in the phosphorylation of PKD2 at Ser$^{876}$ (figure 6.16) while it does not appear that aldosterone phosphorylates PKD3 at Ser$^{41}$ in WT 9-12 cells following hormone treatment for 10 minutes (figure 6.17).
Figure 6.15. Phosphorylation of protein kinase D1 by aldosterone. WT 9-12 cells were stimulated with 0.1 nM, 1 nM, 10 nM, 100 nM and 1 µM aldosterone for 10 minutes prior to being lysed and their protein content collected. Proteins were separated by SDS-PAGE and immunoblotting was carried out using an antibody specific to PKD1 that is phosphorylated at Ser$^{916}$. Membranes were probed with an antibody that recognises total PKD1 to determine whether treatment with hormone had any effect on the total PKD1 expression. β-actin was used as a loading control in these experiments (n = 2).
Figure 6.16. Phosphorylation of protein kinase D2 by aldosterone. WT 9-12 cells were stimulated with 0.1 nM, 1 nM, 10 nM, 100 nM and 1 µM aldosterone for 10 minutes prior to being lysed and their protein content collected. Proteins were separated by SDS-PAGE and immunoblotting was carried out using an antibody specific to PKD2 that is phosphorylated at Ser^{876}. Membranes were probed with an antibody that recognises total PKD2 to determine whether treatment with hormone had any effect on the total PKD2 expression. β-actin was used as a loading control in these experiments (n = 2).
Figure 6.17. Phosphorylation of protein kinase D3 by aldosterone. WT 9-12 cells were stimulated with 0.1 nM, 1 nM, 10 nM, 100 nM and 1 µM aldosterone for 10 minutes prior to being lysed and their protein content collected. Proteins were separated by SDS-PAGE and immunoblotting was carried out using an antibody specific to PKD3 that is phosphorylated at Ser^{41}. Membranes were probed with an antibody that recognises total PKD3 to determine whether treatment with hormone had any effect on the total PKD3 expression. β-actin was used as a loading control in these experiments (n = 2).
Previous work from our group has demonstrated that aldosterone can rapidly phosphorylate and activate PKD isoforms by a non-genomic mechanism in M1-CCD cells. Here we set out to investigate the effect of aldosterone (1 nM) on the rapid phosphorylation and activation of PKD1 and PKD2 in ADPKD cells. WT 9-12 cells were stimulated with aldosterone (1 nM) for 2, 5, 10 and 30 minutes prior to cell lysis. Immunoblotting was carried out to determine the phosphorylation of PKD1 and PKD2 isoforms in response to aldosterone (figures 6.18 – 6.19). Aldosterone (1 nM) rapidly increased the phosphorylation of PKD1 (at Ser916) following 10 and 30 minute treatment with the hormone (figure 6.18). A vehicle response was observed at 2 minutes but this could be due to cell swelling induced by the ethanol used as a vehicle. Similarly, aldosterone (1 nM) elicited an increase in the phosphorylation of PKD2 at Ser876 following stimulation with the hormone for 10 and 30 minutes, with the biggest increase in PKD2 phosphorylation being observed at the later time point (figure 6.19). There are also vehicle responses at 2 and 5 minutes. These vehicle control responses may be due to the pH within the environment. Stress induced by fluctuations in pH has been shown to activate PKD’s and as such further investigation into the activation of PKD isoforms by aldosterone is warranted.
Figure 6.18. Aldosterone-induced phosphorylation of protein kinase D1 in WT 9-12 cells. WT 9-12 cells were stimulated with aldosterone (1 nM) or equivalent vehicle control (ethanol) for 2, 5, 10 and 30 minutes. Whole cell lysates from these cells were separated using SDS-PAGE and subjected to Western blotting to determine the phosphorylation of PKD1 at Ser^{916}. Total levels of PKD1 expression were also analysed (n = 2). Densitometric analysis demonstrated that PKD1 was rapidly phosphorylated following 10 and 30 minutes stimulation with aldosterone. The vehicle control also prompted a rapid response following 2 min treatment.
Figure 6.19. Aldosterone-induced phosphorylation of protein kinase D2 in WT 9-12 cells. WT 9-12 cells were stimulated with aldosterone (1 nM) or equivalent vehicle control (ethanol) for 2, 5, 10 and 30 minutes. Whole cell lysates from these cells were separated using SDS-PAGE and subjected to Western blotting to determine the phosphorylation of PKD2 at Ser\textsuperscript{876}. Total levels of PKD1 expression were also analysed (n = 2).
6.3. Discussion

ADPKD is a systemic condition that is characterised by the early onset of hypertension prior to the loss of normal renal function. To date, a causal role for hypertension in the acceleration of kidney growth in ADPKD has not yet been elucidated. There have been several studies (75, 76, 79) that have reported a higher plasma aldosterone level and a distinct decrease in the renal vascular resistance following treatment with an ACE inhibitor in individuals with ADPKD when compared with individuals with essential hypertension. There are also multiple randomised controlled trials that address the impact of RAAS inhibition on the progression of ADPKD (81-88). Investigations, such as the HALT study (80), are essential in order to address the query of using RAAS inhibitors in ADPKD. The HALT polycystic kidney disease study is the first major multi-centre, interventional trial in patients with ADPKD that tests both new kidney structural end-point in early disease (study A) and along with a more typical kidney functional end-point in late disease (study B) while using the same RAAS inhibition as a potential therapeutic intervention (80).

Here we have determined the expression profile of MR both under basal conditions and when supplemented with dexamethasone. MR is known to be expressed by the principal and to a lesser extent by intercalated cells of the distal renal CCD, however it has been suggested that MR is not expressed in the M1 CCD cell line or that it is inactive as a transcription factor but capable of eliciting some rapid signalling responses (111, 182). We have proposed that expression of MR in the M1 CCD cells is glucocorticoid dependent and that differences in culture conditions employed by different groups can account for the discrepancies in the published literature concerning expression in cell lines derived from the CCD (94). Our investigation deduced that the WT 9-12 cell model of ADPKD did not display changes in the expression or abundance of MR in cells cultured in dexamethasone when compared to those without. As MR is predominantly expressed in the distal nephron, this suggests that our model of ADPKD is derived from distal tubule. The feature of MR in WT 9-12 cells suggests a characteristic sensitivity for aldosterone which distinct effects of this hormone on the handling of electrolytes in this part of the nephron. As we cannot be certain that these cells are derived from a cyst originating in the distal tubule, single cloning of these cells should be carried out. The isolation of single cells will allow for
further characterisation to occur. Going forward, the expression of the water channel AQP2 should be investigated at both the protein and mRNA levels in order to single out cells that are derived from the distal nephron. Isolating these cells will aid in determining whether this model is aldosterone sensitive.

The data presented here demonstrates that each of the three PKD isoforms are expressed in WT 9-12 cyst derived cells and further investigation revealed that these cells are sensitive to aldosterone. The data obtained in this chapter points to aldosterone phosphorylating PKD1 in WT 9-12 cells however the mechanism of activation remains elusive. Until the type of tubule of origin for these cells is confirmed, it is presumed that the model is of mixed phenotype. Previous work from our group (Dooley, unpublished) showed that PKD2 is rapidly phosphorylated (<10 minutes) in M1 CCD cells in response to aldosterone. These rapid effects on PKD2 activation in WT 9-12 cells were also observed following treatment with aldosterone. To further understand this role, interactions of PKD2 with ENaC and transporters must be considered. Transporters such as Na+/K+-ATPase are mislocalised to the apical membrane in renal cyst epithelial cells resulting in the accumulation of fluid at the lumen (215-217) Such alterations can impact on normal renal growth as the reversal of ion and fluid transport from an absorptive mode a secretory one. Furthermore, a mutation in PC2 may also affect Ca2+-induced reorganisation of actin thus potentially allowing the Na+/K+-ATPase pump to mislocalise to the wrong membrane. The immunofluorescence experiments reported here investigating the mislocalisation of the pump did not exhibit a strong signal with TRITC. This will require further optimization of the antibody concentrations, exposure time and laser strength using the confocal microscope. We can use biotinylayion experiments coupled with immunofluorescence to confirm this altered trafficking of transporters in our working model. Additionally, it is possible to knock down PKD isoforms in these cells either by shRNA (as carried out with the M1 PKD1 and M1 PKD2 knock down used by this group) or by using newer techniques such as CRISPR/Cas9 (as reported in this thesis in the case of PKD3) to determine the effect these kinases are having on Na+ handling in our model.
Figure 6.20. ENaC and Na/K pump membrane targeting is under the control of PKD1. PKD1 regulates the correct targeting of transporters such as ENaC and Na⁺/K⁺-ATPase to the apical and basolateral membranes, respectively. Knocking down PKD1 in renal cells results in the membrane sorting of ENaC and the pump being inverted. Trafficking and sorting of transporters are targeted to the wrong membrane in ADPKD and this could be due to defective PKD1 function in these cells.

We have demonstrated expression of PKD2 in WT 9-12 cells along with ENaC channel subunits and transporter’s such as Na⁺/K⁺-ATPase. By investigating the impact of aldosterone on normal renal growth, we can determine if a dysregulation of transporters such as Na⁺/K⁺-ATPase initiates the reversal of ion and fluid transport. Preliminary pilot experiments described above suggest that aldosterone could rapidly phosphorylate PKD2 in WT 9-12 cells thus lending support to our theory that the PKD family of kinases play a vital role in the regulation of Na⁺ handling. Our data may point to a role for PKD2 in regulating the trafficking and activity of aldosterone-sensitive apical membrane transporters such as ENaC in the distal nephron, and activation of these protein kinases. Further investigation may reveal a novel facet of aldosterone regulation of ENaC by these kinases that could contribute to the hypertensive effects of renal health and disease.
Chapter 7

General Discussion
7.1 Discussion

This study demonstrates a novel function for PKD isoforms in the regulation of Na\(^+\) reabsorption in response to the steroid hormone aldosterone. It also provides novel insights into the molecular mechanisms of PKD activation in response to hormone stimulation. The effects of suppressing PKD2 produce changes in ENaC function that have not been described before and reveal a completely novel pleiotropic effect of aldosterone on ENaC. The study presents a paradigm shift in our understanding of the renal response to aldosterone and its effect on Na\(^+\) reabsorption. This chapter will discuss the relevance of the findings presented here and their impact on Na\(^+\) handling in the context of physiology and pathophysiology.

There are three known isoforms of the PKD family of kinases, PKD1/PKC\(\mu\) (115), PKD2 (116) and PKD3/PKC\(\nu\). It is also known that PKD can be phosphorylated and activated by novel PKC isoforms such as PKC\(\varepsilon\) (109). Our group has previously established that the activation of PKD1 in M1 CCD cells is PKC\(\varepsilon\)-dependent (114). At the outset of this thesis, we hypothesised that the different PKD isoforms can work in tandem to regulate the trafficking of ENaC channel subunits from the cytosol to the apical membrane. We have shown previously that PKD1 is responsible for the forward trafficking and insertion of ENaC into the membrane and this process is initiated by aldosterone. Conversely, we propose that PKD2 under basal conditions stimulates the retrieval of ENaC subunits from the membrane where they are trafficked back into the cytosol. Additionally, it is possible that PKD2 could also suppress the insertion of ENaC into the apical membrane. We further suggest that this inhibitory effect on ENaC function is impeded following the stimulation by aldosterone. In the context of renal disease, we postulate that these kinases could have a role in regulating cyst fluid volume in pathologies such as ADPKD and could also contribute to the hypertensive status observed in these patients. The basis for this study was to gather novel insights by advancing our work into an M1-CCD in vitro model to establish the contribution of PKD2 to basal and hormone-regulated regulated Na\(^+\) reabsorption. Utilising techniques such as shRNA and pharmacological interventions, we have revealed for the first time the role of PKD2 in regulating the absorption of sodium by the kidney. This novel signalling pathway may be important in understanding the dysregulation in
the aldosterone-induced signalling pathway which leads to hypertension through chronic stimulation of ENaC activity.

Our work also has implications for understanding the physiological role of non-genomic actions of aldosterone and the consequences for genomic responses. The most widely documented facet of rapid responses to aldosterone is the activation of protein kinase signalling cascades while the mechanism behind how these signalling cascades impact the renal tubule cell and kidney physiology are now being exposed. For example, the activation of several members of the MAP kinase family has been documented in numerous aldosterone-responsive tissues. In particular, the activation of ERK1/2 has been extensively studied (97-102).
Figure 7.1. Signalling pathways associated with protein kinase D. The activity of crucial signalling intermediates such as PKD, Rho-activated kinase (ROK), protein kinase A (PKA), phosphoinositide 3-kinase (PI3K), PKC, ERK, SGK and with no lysine family kinases (WNK) can be modulated by aldosterone. Aldosterone activates some or all of these signalling pathways to modulate ENaC channel activity along with other channels such as the ATP-sensitive K⁺ (KATP) channel, the Na⁺/H⁺ exchanger 1 (NHE1), the Na⁺/K⁺ pump (Na⁺/K⁺-ATPase) the renal outer medullary K⁺ (ROMK) channel in principle cells of the CCD. The activation of MR leads to a suppression of Nedd4-2 ubiquitin ligase activity which promotes ENaC stability. Aldosterone can instigate transcriptional changes in promoting SGK-1 thus inactivating Nedd4-2. This inactivation of Nedd4-2 leads to an increase in ENaC abundance.
The rapid signalling effects that are induced by aldosterone affect multiple membrane targets, either by directly affecting their activity, or indirectly through the modulation of MR-dependent transcription (figure 7.1). Both PKCδ and PKCε can be rapidly activated in response to aldosterone, however, this is not reliant on the direct binding of aldosterone to the kinase. The rapid activation is instead coupled to MR via the trans-activation of EGFR (109). The rapid activation of PKD1 in response to aldosterone is now known to be a substrate for novel, Ca\textsuperscript{2+}-independent PKC isoforms (nPKCs) for example PKCδ and PKCε. In M1-CCD cells, aldosterone stimulates PKD1 activation in the same manner as aldosterone-induced activation of nPKC isoforms. This activation of PKD1 is coupled to MR through the trans-activation of EGFR (94, 109) (figure 7.2). This model of rapid actions of aldosterone has been the premise for our investigation into the mechanism of PKD2 activation in M1-CCD cells.
PKCδ and PKCε can be rapidly activated in response to aldosterone. The rapid activation is coupled to MR via the trans-activation of EGFR through c-Src. PKD1 is downstream of PKCδ and PKCε and once activated, it is responsible for the trafficking of ENaC channel subunits from the cytosol to the apical membrane. To date, the mechanism of PKD2 activation in collecting duct cells remains unknown.
A number of strands of evidence point to MR as being the receptor responsible for initiating the aldosterone-induced rapid signalling PKD cascades. The activation of protein kinases such as PKD and ERK1/2 by aldosterone for example can be inhibited with the use of MR-specific antagonists such as spironolactone and eplerenone (94, 102, 110). MR can be considered to be a multifunctional receptor. If recombinant MR which lacks its DNA-binding and coactivator-binding domains is expressed, signalling events can be instigated by a rapid response to aldosterone independent of transcriptional activity.

In terms of the intermediate phases that couple the aldosterone-MR interaction with the rapid activation of protein kinases, there are still some questions to be addressed. It is well established that the trans-activation of the epidermal growth factor receptor (EGFR) is a fundamental step in linking this initiation signal to the aldosterone-responsive downstream signalling intermediates (95). It has yet to be determined by which molecular mechanism EGFR and its activation is coupled to MR but it is thought that it is EGFR ligand independent. EGFR is phosphorylated by a small tyrosine kinase, c-Src, within 5 minutes of treatment with aldosterone and this c-Src phosphorylation could be a significant transducing signal (182). Cytoplasmic MR is recruited into a complex of several proteins including heat shock protein 90 (Hsp90), this complex dissociates on MR activation. The aldosterone-induced phosphorylation of EGFR by c-Src can be blocked by antagonising Hsp90 interactions with other proteins using the geldanamycin analogue 17-AAG. This also suppresses EGFR-dependent downstream signalling events initiated by aldosterone that include the activation of PKD1 (94) and the ERK1/2 mitogen activated protein (MAP) kinase (95).

In order to rule out the trans-activation of EGFR as the receptor involved the activation of PKD2, these studies need to be re-investigated. Identifying the inconsistencies between the pilot study conducted by Dooely and the experiments conducted during the investigation into the mechanism of PKD2 activation could provide a more realistic overview of how this kinase fits into the signalling pathway regulating ENaC activity. Failing that, the experimental approach to this study could also be improved by using alternative concentrations of pharmacological agents or by knocking down the receptors in M1-CCD cells to determine the effect on PKD2 activation in response to aldosterone.
One possible mechanism by which aldosterone-induced signal transduction cascades could influence ENaC activity would be to enhance the rate of ENaC integration into the membrane. Such an action could potentiate the subsequent transcriptional effects of MR (5). Our previous studies have shown that a significant increase in the activity of ENaC in M1-CCD cells is detected between 2 and 4 hours following treatment with aldosterone that peaked between 16 and 24 hours, an effect that correlated with the subcellular redistribution of ENaCβ (5)]. Aldosterone treatment stimulated the apical translocation of ENaCα in wild-type M1-CCD, but in PKD1 knock-down cells, aldosterone treatment failed to increase ENaCα abundance at the apical membrane, but rather remained localized in the cytoplasm. This indicates that the trafficking process of ENaCα to the apical membrane is defective in cells suppressed in PKD1 expression and indicates a critical role of protein kinase in regulating sub-cellular trafficking of ENaC activity by aldosterone.

We found that a rapid redistribution of PKD2 from the membrane into the cytosol occurs in response to aldosterone. Furthermore, suppression of PKD2 in M1-CD cells resulted in an increase in the abundance of ENaCγ at the apical membrane in response to aldosterone. Our study reports the novel finding of increased ENaC activity in PKD2-suppressed M1-CCD cells under both basal and aldosterone treated conditions. This significant finding is important as the very high Na+ currents through ENAC we observed in PKD2 knock-down CCD have not been reported and reveal a tonic inhibition of ENaC function by PKD2 under basal conditions. Our study revealed that PKD2 is a novel regulator of the ENaCγ channel subunits out of the apical membrane back into the cytosol thus de-stabilising ENaC activity. Aldosterone treatment removes this tonic endocytosis of ENaC by phosphorylating PKD2 causing the kinase to be removed from the membrane and inhibiting the retrieval of ENaCγ back into the cytosol. In contrast, when PKD2 expression was suppressed by siRNA, the tonic retrieval of ENaC from the apical membrane was removed and ENaC expression was stabilised and enhanced at the membrane resulting in very large basal ENaC-mediated Na+ currents. Moreover, we uncovered a paradoxical inhibitory effect of aldosterone on ENaC Na+ currents in PKD2 knock-down CCD cells (figure 7.3).
Figure 7.3. Effects of PKD1 and PKD2 knock-down on basal and Aldosterone / ENaC activity. In wild-type M1 cells, aldosterone induces an increase in the $I_{\text{SC}}$ by phosphorylating PKD1 and SGK1 and initiating the insertion of ENaC into the apical membrane. Aldosterone also phosphorylates PKD2 which decreases the retrieval of ENaC back into the cytosol resulting in an increase in $I_{\text{SC}}$. When PKD1 is suppressed, ENaC insertion is decreased and aldosterone blunts this response from $I_{\text{SC}}$. Knocking down PKD2 in M1 cells has an inhibitory effect on the retrieval of ENaC from the apical membrane thus enhancing the stability of the channel. Both PKD1 and SGK1 are phosphorylated and the subsequent insertion of ENaC into the membrane increases the $I_{\text{SC}}$. Hormone stimulation of PKD2 knock down cells exhibit a blunted response in $I_{\text{SC}}$. This could be due to the phosphorylation of kinases such as ERK1/2 and an increase in $Ca^{2+}$ activity. It is possible that ENaC inhibitory signaling pathways have been unmasked.
We postulated that the reduction of ENaC activity following hormone treatment when PKD2 expression was suppressed and could be as a consequence of ubiquitination. However, upon investigation, it could not be determined if ubiquitination is responsible for the decrease in $I_{\text{SC}}$ as aldosterone did not increase the phosphorylation of Nedd4-2 when compared to both the untreated and vehicle controls. Furthermore, our study may have revealed a pleiotropic effect of aldosterone to stimulate and inhibit ENaC activity and Na+ absorption that is dependent on the expression status of PKD2.

Membrane-localised ENaC is subject to constant recycling. The inclusion of ENaC into the apical membrane is prerequisite for its ubiquitination and retrieval into the sub-apical pool or its degradation by the proteasome. Nedd4-2 interacts with ENaC through a C-terminal PY internalization motif to facilitate ENaC ubiquitination (450). The surface expression of ENaC may be equally regulated by de-ubiquitination by DUBs and ubiquitination by Nedd4-2 (451). However, evidence presented in this work provide an alternative hypothesis: the acute stimulatory effect of aldosterone is a PKD-dependent process that influences the trafficking dynamics of ENaCγ and other ENaC subunits and 2)

In this thesis we also report the successful development of both transient and stable PKD3 knock-down CCD cell lines and initiated studies on the role of this remaining PKD isoform in the regulation of ENaC under basal conditions and aldosterone treatment, . Similar to the previous two PKD isoforms, PKD3 is also expressed by principle cells of the distal nephron. Here we have confirmed the expression of PKD3 in the principle cells of the cortical collection duct. In our studies, aldosterone did not induce a redistribution of PKD3 ex vivo kidney tissue cryosections nor did aldosterone stimulate the rapid increase in PKD3 localisation in M1-CCD cells following treatment for 10 minutes with the hormone. This suggests that unlike the previous two PKD isoforms, PKD3 trafficking is not regulated by aldosterone. Therefore, it is possible that PKD3 does not play a role in the trafficking of ENaC channel subunits and the subsequent activity of the channel.

Our preliminary observations suggest that knocking down PKD3 using siRNA does not affect expression of ENaC channel subunits at the protein level, nor does it affect Nedd4-2 expression. One limitation of using siRNA to transiently knock down PKD3 is that we cannot use these cells for electrophysiological experiments to determine the
role of PKD3 in ENaC activity. By utilising state of the art techniques such as CRISPR, we have begun to characterise a PKD3 knock out in M1-CCD cells. In the CRISPR PKD3 knock-out M1CCD cells, aldosterone did not appear to enhance expression of ENaCγ at the apical membrane. Additionally, knocking out PKD3 using CRISPR did not affect the ability of these cells to form an epithelial monolayer which is in contrast to work outlined by Rozengurt in the airway epithelium (186). The stable PKD3 knock-out CCD cell line will be a valuable tool to advancing our understanding the role of all the three PKD isoforms in regulating basal and hormone-modulated Na+ reabsorption in the renal cortical collecting duct.

Work presented in this thesis suggests that aldosterone can rapidly phosphorylate PKD isoforms in a renal cell model for ADPKD (WT 9-12 cells). As these results are from a small dataset of results, they will require replicates in order to be sure that our observation thus far is an effect of aldosterone phosphorylating PKD isoforms. Further investigation into the role these kinases play in regulating transporter activity in ADPKD is necessary.

To date, there is no single unifying mechanism that is related to the normal function of the polycystin proteins to the pathology that is developed in their absence. However, several studies have claimed that the progression of kidney cystic disease is centred on disconcertion of two fundamental processes. Epithelial cells lining the cyst seem to proliferate excessively and these cells lead to secretion rather than absorption of fluid and electrolytes. Hence there are many efforts that aim to develop small molecule therapies for ADPKD that can target either of these imbalances (Chang and Ong, 2008; Harris and Torres, 2009; Patel et al., 2009).

Our data reveal novel molecular mechanisms for PKD1 and PKD2 in regulating the membrane trafficking and stability of ENaC and Na+/K+-ATPase pump in the distal nephron. The work presented in this thesis takes into the account the importance of understanding the role of aldosterone activation of PKD isoforms in the handling of Na+ before we can reflect on how it may contribute to the regulation of ENaC and Na+/K+-ATPase pump in patients with ADPKD. Moreover, we have further hypothesised that aldosterone can stimulate ENaC resulting in fluid retention in cystic epithelial cells and this response could be coupled to the initiation of PKD-mediated signalling events. In order to test this hypothesis going forward, WT 9-12 cells can be...
cultured in spheroids while immunocytochemistry coupled with confocal microscopy can be used to determine the effect of aldosterone on cyst expansion. Additional experiments are required in order to elucidate the response of PKD isoform phosphorylation and activation in response to aldosterone. The results outlined in this thesis are encouraging and suggest that PKD isoforms are sensitive to aldosterone in WT 9-12 cells. To date, there is nothing in the literature on aldosterone-induced PKD activity in a model of ADPKD which provides a novelty to this work. A further line of investigation would be to use immunohistochemistry to investigate whether archived kidney samples from patients with ADPKD express the PKD isoforms or polymorphisms and to what degree with the stage of the disease. We can also implement gene-editing techniques such as CRISPR to knock out PKD isoforms in WT 9-12 cells and measure its effect on ENaC and Na⁺/K⁺-ATPase pump activity. This model would also provide a platform to explore whether a dysregulation of ENaC activity is mediated by PKD isoforms thus leading to hypertensive effects observed in patients with ADPKD.

7.2. Future perspectives

This thesis provides ample material to further develop our understanding of the role of PKD isoforms in the regulation of ENaC. Using the PKD2 knock down CCD cell line, this work could be progressed by investigating at the role of PKD2 in the activity of Na⁺/K⁺-ATPase pump. The work presented in this thesis requires additional experiments before any real conclusions can be drawn. Going forward, we could also explore other possible routes of activation of PKD2 such as through PKA. Functional assays could be carried out by mounting hormone treated wild-type and M1-CCD PKD2 knock down cells in Ussing chambers and antagonising PKA while measuring the cAMP response to forskolin. Using pharmacological interventions coupled with Western blotting, we could block different downstream targets of aldosterone and measure the effect this has on phosphorylation of PKD2 in vitro.

Our preliminary work using the CRISPR generated PKD3 knock out in M1-CCD cells provides us with a novel platform to pursue an investigation into teasing out the role of this kinase. Western blotting can be used to determine whether aldosterone can
induce the rapid phosphorylation of PKD3 in M1-CCD cells. Using the PKD3 knock out CCD cell line, we can determine the role of PKD3 in modulating both $I_{SC}$ and amiloride-sensitive $I_{ENaC}$ by conducting Ussing chamber experiments. The PKD3 knock out can be used to explore the role of PKD3 in terms of trafficking ENaC channel subunits. The effects of aldosterone on the redistribution of ENaCy in M1-CCD PKD3-deficient cells is a hot topic to pursue to put the role of PKD3 in context with the two ther PKD isoforms. Additionally, it would be interesting to investigate the potential role of PKD isoforms in the miss-trafficking of ENaC and Na$^+$/K$^+$-ATPase subunits to the opposite membranes in our model of ADPKD. Knocking down these isoforms either by shRNA or the CRISPR method described in this thesis could aid in correcting this incorrect targeting of these transporters to the apical and basolateral membranes.

7.3. Conclusion

The essential roles of PKD isoforms in different biological systems are becoming more understood with the identification of novel substrates for this family of kinases that interact with other kinases and transcription factors that can modulate intracellular trafficking. These PKD isoform regulated processes could fine-tune the specificity of MR-dependent transcriptional events and this family of kinases could act as important intermediaries between rapid signalling events stimulated by aldosterone and the transcriptional processes to regulate renal electrolyte balance. In conclusion, this thesis reports novel rapid 'non-genomic' roles for PKD isoforms to regulate both basal activities and aldosterone ENaC and Na$^+$/K$^+$-ATPase pump activities in renal CCD cells.
Chapter 8

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