DOCTOR OF PHILOSOPHY

The role of Serum- and Glucocorticoid kinase in the hormonal control of sodium transport in pulmonary epithelia

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Award date:
2011

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The role of Serum- and Glucocorticoid-kinase in the hormonal control of sodium transport in pulmonary epithelia

Gordon B. Watt

A dissertation submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Centre for Cardiovascular and Lung Biology

University of Dundee

July 2011

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Acknowledgements

Firstly I would like to thank the George John and Sheilah Livanos Charitable Trust for providing the funding, without which my PhD would not have been possible. I would also like to thank my supervisors, Dr Stephen Land and Dr Stuart Wilson for giving me the chance to work in the Centre for Cardiovascular and Lung Biology and for their advice and support throughout this project.

Particular thanks must go to Dr Michael Gallacher, who took the time to help me learn about patch clamping, and for also providing (one to many) "distractions" throughout my time in Dundee. I would also like to thank all the staff, past and present who have made my time here enjoyable.

Special thanks obviously go to my parents without whom I would not be around to have taken on this project and in particular my Mum who put up (financially and emotionally), with me moving back home for the duration of my write up. Special thanks go to all my friends, especially those out with science that kept asking "when are you going to get a proper job?" it never got old, not even once.

Finally I would like to thank my girlfriend Dr Kay Steel, for all her support, and maybe we will finally get to go on holiday after this! Anyway look at me still talking when there is science to do!

Declaration

I hereby declare that I, Gordon Bruce Watt, am the sole author of this thesis. The work, of which this thesis is a record, was carried out by myself, and all references cited have been consulted by myself. None of the work contained within this thesis has been previously accepted for a higher degree.

Signature of Candidate

Gordon Bruce Watt BSc

Supervisors Statement

I certify that Gordon Bruce Watt has fulfilled the conditions of ordinance 39 and of the relevant regulations, such that he is qualified to submit this thesis in application for the higher degree of Doctor of Philosophy.

Signatures of Supervisors

Dr Stephan C Land

Dr Stuart M Wilson

Abbreviations

AF1	Activation function 1
AF2	Activation function 2
APS	Ammonium persulphate
AVP	Arginine Vasopressin
ATI	Alveolar type I
ATII	Alveolar type II
BCS	Bovine Calf Serum
CO_2	Carbon Dioxide
cAMP	Cyclic Adenosine Monophosphate
CAP	Channel Activating Protease
CBP	cyclic adenosine monophosphate response-element binding protein
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
C_m	Membrane Capacitance
Dex	Dexamethasone
dH_2O	Deionised water
DMSO	Dimethyl Sulphoxide
EDTA	Ethlenediaminetetraacetic Acid
EGTA	Ethylene Glycol-bis-N, N, N', N'-tetraacetic Acid
ENaC	Epithelial Na^+ Chanel
FBS	Foetal Bovine Calf Serum
FDLE	Fetal Distal Lung Epithelial cells
Glucocorticoids	GCs
GR	Glucocorticoid receptor

hsp	Heat shock protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I_{Cl}	Cl^- Current
I_K	K^+ Current
I_{Na}	Na^+ Current
IRDS	Infant Respiratory Distress Syndrome
MC	Mineralocorticoid
MR	Mineralocorticoid Receptor
mTOR	Mammalian Target Of Rapamycin
mTORC1	mTOR Complex 1
mTORC2	mTOR Complex 2
NRDG1	N-myc Downstream Regulated Gene 1
Nedd4	Neural Precursor Cell-Expressed Developmentally Down-Regulated Protein 4
$NDMG^+$	N-methyl-D-Glucammonium
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
P_{CO2}	Partial Pressure of Carbon Dioxide
PDK1	Phosphoinositide-dependent Kinase 1
PI3K	Phosphoinositide-3-Kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PLC	Periciliary Liquid Layer
P_{O2}	Partial Pressure of Oxygen
R_a	Access Resistance

RDS	Respiratory Distress Syndrome
R_m	Membrane Resistance
SDS	sodium dodecyl sulphate
SGK1	Serum- and Glucocorticoid-Regulated Kinase 1
SRC	steroid receptor coactivator
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TJ	tight junctions
V_{Hold}	Holding Potential
V_m	Membrane Potential

Summary

In the hormonal control of sodium transport in the lung via glucocorticoids there is substantial evidence that supports a central role for serum- and glucocorticoid-kinase (SGK1). The activity of this kinase is dependent upon phosphorylation of two distinct residues by the target of rapamycin complex 2 (TORC2) and phosphoinositide-dependent Kinase 1 (PDK1), both of which are dependent upon Phosphoinositide-3-Kinase (PI3K). However, SGK1 knockout mice do not display any pulmonary abnormalities. Thus the role that SGK1 plays is not fully understood. In this thesis I have explored the role of this kinase in dexamethasone-induced ENaC activity in the H441 human bronchiolar cell model.

Dexamethasone-deprived cells do not display ENaC activity as there is no amiloride sensitive current in these cells. Groups of dexamethasone-treated H441 cells do display ENaC activity; however single cells do not display ENaC activity despite displaying an increase in current. Thus cell-cell contact is vital to the development of amiloride sensitivity. SGK1 activity does not mimic the electrophysiological effects of dexamethasone-stimulation as there is no amiloride sensitivity seen after ~3 hours despite a clear increase in SGK1 activity. Furthermore after ~24 hours stimulation, there is a clear amiloride sensitive current although SGK1 activity is comparable to that of dexamethasone-deprived cells. These findings further question whether SGK1 is required for dexamethasone-evoked ENaC activity.

Inhibition of PI3K, TORC2 and SGK1 abolished ENaC activity. However inhibition of TORC1 had no effect upon dexamethasone induced ENaC activity. Thus demonstrates that the maintenance of glucocorticoid induced ENaC activity, in pulmonary epithelium, is dependent upon the PI3K-TORC2-SGK1 signalling pathway. Furthermore PI3K plays a permissive, but critical role as its activity is required for SGK1 activity. However without SGK1 activity dexamethasone induced ENaC activity cannot be maintained.

Chapter 1 – General Introduction

The Respiratory system

All metabolic processes that take place in living organisms require energy in order to function. The most efficient way to provide this energy is through aerobic mechanisms that use oxygen (O_2) and produce carbon dioxide (CO_2). Uptake and secretion of these gases is therefore vitally important and is achieved through diffusion. Unicellular organisms can exchange these gases directly through the cell membrane with the external environment; however, in larger complex organisms it is not possible for every cell to be in contact with the external environment, therefore the development of specialised structures and organs, that could exchange O_2 and CO_2 for the whole animal, were vital for the evolution of large complex organisms.

Functions of the respiratory system

The primary function of the respiratory system is gas exchange. However, the act of inspiring air brings with it, its own set of problems, which the respiratory system has evolved to overcome.

Gas exchange

Air from the atmosphere during normal breathing is inspired through the nose and passes through the nasopharynx which performs the vital function of warming and humidifying the air. This process is important as, without it, body temperature would drop, thereby optimal temperature for enzymatic function would not be maintained. Furthermore inspired air has a much lower humidity than air in the respiratory system thus, without first being humidified, water vapour would move into the air from the lining of the respiratory system, thereby reducing airway surface hydration. By the time inspired air reaches the site of gas exchange, the alveoli, O_2 has a pressure of

~100mmHg which is lower that atmospheric O_2 which is ~159mmHg, because the partial pressure of O_2 (PO_2) in the alveoli is a combination of uptake and the supply. The pulmonary artery carries blood which has a low concentration of O_2 (PO_2 is ~40mmHg), to the lungs; therefore O_2 will move into the blood via diffusion. However O_2 must first dissolve into the fluid lining the alveoli before it can move via diffusion into the blood. This imposes a possible barrier, as Fick's law of diffusion states that the net rate of diffusion of a substance is inversely proportional to the thickness of what it must cross. This means that while the liquid lining of the alveoli is extremely important, the control of its depth is equally important as, if the depth were to increase, then the time taken for O_2 to move into the blood would greatly increase and could lead to insufficient gas exchange. The reverse is true for CO_2 where the PCO_2 is higher in the blood than in the alveoli and CO_2 is expelled. Excess fluid in the lung has been shown to cause inefficient gas exchange (Berger *et al*, 1996) and is a contributory factor in respiratory distress syndrome (RDS) which is a leading cause of death in premature and newborn infants (O'Brodovich, 1996). Therefore tight regulation of alveolar hydration is vitally important to the healthy function of the respiratory system.

Defence against infection

However hydration of the respiratory system is not just important at the level of gas exchange, the periciliary liquid layer (PCL) also needs to be tightly regulated so that efficient mucociliary clearance can take place. Mucus secreted into the airway lumen is constantly transported toward the pharynx, where it is then swallowed or expelled and has the purpose of trapping inhaled particles and potential pathogens. The upward movement is the result of beating cilia which propel the mucus towards the pharynx. However, this is reliant on the adequate hydration of the PCL (Boucher, 1999). This thin film of liquid is required so that the cilia can beat effectively and propel the

overlying, more viscous mucus towards the pharynx. Improper hydration of the PCL results in the mucus layer collapsing into the cilia and, due to the higher viscosity, the cilia are unable to beat effectively thereby impairing mucociliary clearance. Impaired mucociliary clearance is associated with increased incidence of lung infection and is particularly evident in cystic fibrosis, a genetic disorder that results in improper hydration of the airway surface liquid (ASL) which is the collective name for the PCL and mucus layer (Boucher, 2004).

Development

The process of lung development is highly dependent upon a fluid template which provides the distending pressure required for the formation of the lung. The closed vocal chords, larynx and nasopharynx help to prevent fluid escaping from the developing lung and therefore maintain the distending pressure (Brown *et al*, 1983; Fewell and Johnson, 1983). Further evidence to support this is that pathological states, such as oligohydramnios that lead to a decrease in distending pressure, result in hypoplastic lungs and lungs of a smaller volume (Moessinger *et al*, 1986; Wallen *et al*, 1990; Wallen *et al*, 1994) and the opposite also appears to be true as it has been reported that excess lung fluid results in larger and even hyperplastic lungs (Alcorn *et al*, 1977; Moessinger *et al*, 1990). Furthermore, it has been shown that surgically created congenital diaphragmatic hernia results in hypoplastic lungs and that by causing tracheal occlusion, using surgically implanted silicon balloons to restore hydrostatic pressure, the extent of hypoplasia can be minimised (Nelson *et al*, 2005). However, this study also found that this was improved by cyclical release of pressure every 47 hours. This may mimic foetal breathing movements, which appear to be required for normal lung growth (Wigglesworth and Desai, 1979). Gas exchange would be impossible if

this fluid remained at birth, therefore the regulated clearance is vital to the survival of the newborn, as excess fluid can contribute to respiratory distress (O'Brodovich, 1996). The hydration of the respiratory system is therefore dynamic throughout development, birth and adult life. Without regulation of the process underpinning hydration, the respiratory system would not be able to carry out its required functions, which would lead to disease states such as cystic fibrosis, pulmonary oedema, RDS and, eventually, death.

General Anatomy and physiology

In order to function efficiently the respiratory system has evolved a very specific anatomy and physiology that contains specialised epithelial cells that line the respiratory lumen. These cells act together to maintain sufficient hydration so that gas exchange, mucociliary transport and development are effective throughout foetal and adult life. One of the properties of these epithelial cells that enable them to maintain adequate hydration, is their ability to form selective barriers.

Ability to form a barrier

The formation of *zonulae occludentes* or tight junctions (TJ) by epithelial cells underlies their ability to form a sheet of cells that act as selective barriers. TJ form a belt-like area around the circumference of a cell, close to the apical border, where protein complexes from adjacent cells interact, thus creating a barrier that can prevent the movement of molecules through extracellular pathways. The formation of TJ naturally leads to the formation of polarity which underlines many of the vectorial transport systems in the body and results in an apical side and a basolateral side which are functionally different. The degree of "tightness" of epithelial tissue can vary and is thought to be attributed to differences in membrane proteins that form the tight

junctions (for review see Bauer *et al*, 2010), and this degree of "tightness" has been suggested to affect the permeability of epithelia to ions and macromolecules (Frömter and Diamond, 1972), thereby both the polarity and tightness of airway epithelia are vitally important in maintaining the healthy functioning of the respiratory system. The observation that cell-cell contact alters the electrical properties of airway epithelium (Brown *et al*, 2008) highlights the importance of cell contact in the ability of epithelial cells to form a barrier and carry out their required function.

Anatomy

The respiratory system has a very distinctive branching pattern from the trachea and can be divided into two main sections: the upper, conducting zone and the lower, respiratory acinus. The conducting zone consists of the larynx, trachea, bronchi and terminal bronchioles. As the name suggests these play no part in gas exchange and provide a low resistance pathway to the respiratory zone. The conducting airways also have an important role in defence against infection and it is in the upper airways that mucociliary transport occurs. The respiratory zone consists of the respiratory bronchioles, alveolar ducts and alveolar sacs and all are involved in gas exchange. The epithelia lining the airways act as specialised barriers that enable each part of the respiratory system to provide a specific function that is vital to its continued healthy functioning.

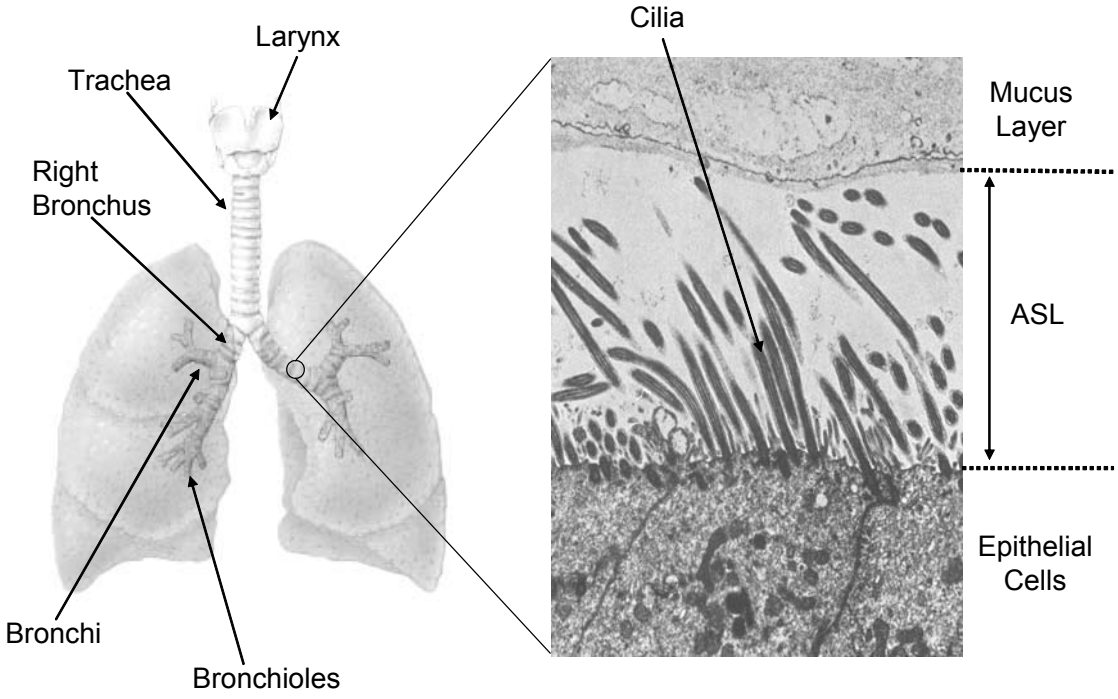


Figure 1.1 shows a diagram of the respiratory system. Highlighted are the conducting airways. The zoomed in view highlights the Airway surface liquid (ASL) and the mucus layer. Cilia can be clearly seen and beat in order to propel the mucus layer towards the larynx.

Cells of the airway epithelium

The epithelium of the trachea down to the terminal bronchi is lined with pseudostratified epithelium. In the upper airways the epithelium is ciliated so as mucociliary transport can take place. The epithelium contains mucous secretory cells known as goblet cells. These cells are found in the upper and lower respiratory tract and secrete mucins, which combine with other secretions from epithelial cells in the respiratory tract, to produce mucus which helps to protect the airway lining from mechanical and chemical damage and traps irritants and pathogens. Goblet cells have been shown to increase in number in response to irritants (for review see Rodgers, 2002).

Submucousal Glands

These glands are found in the upper airways and secrete mucins, antimicrobial proteins and fluid which provide some of the components of mucus. Due to the morphology of the gland, the fluid secretions help to flush out the macromolecule secretions. Fluid secretion helps to maintain the PCL required for mucociliary transport and secretion is stimulated by the vagus nerve (Haxhiu *et al*, 1990). Administration of acetylcholine to excised bronchi mimics the effect of vagus nerve stimulation on gland secretion (Quinton, 1979; Trout *et al*, 1998). However regulation of gland secretion is subject to control from other inputs such as ATP release from increased apical flow and hyposmotic stress (Guyot and Hanrahan, 2002). It is thought that ATP causes a rise in intracellular calcium levels which induces Cl^- secretion (Yamaya *et al*, 1996). Fluid secretion is driven by Cl^- and, to a lesser extent, by the secretion of bicarbonate, which is also secreted by submucousal glands. The primary function of bicarbonate is to act as a buffer to resist changes in pH which would affect enzyme activity.

The regulation of airway hydration to maintain efficient mucociliary transport is determined by the balance between fluid secretion via submucousal glands and the absorption of fluid by the epithelial cells lining the upper airways. The nature of these processes will be discussed later.

Another cell type found in the conducting airways is the Clara cell. These are cuboidal, non-ciliated cells found mainly in the bronchioles, although they are also found in the upper airways to a lesser extent (Broers *et al*, 1992). They have a role in mucus and surfactant production and secretion and also play an important role in neutralising toxins, via cytochrpme P-450 enzymes. Clara cell secretory protein is thought to have a protective role against the development of adenomas. For example mice lacking Clara cell secretory protein are more susceptible to developing lung tumorigenesis when

exposed to a carcinogen (Yang *et al*, 2004). Due to the distribution throughout the respiratory system, the role Clara cells play in airway hydration is likely to be minimal.

The respiratory zone contains the alveoli which is the site of gas exchange. In order for gas exchange to take place, the alveoli consist mainly of two types of epithelial cell: the squamous alveolar type I (ATI) pneumocyte and the cuboidal alveolar type II (ATII) pneumocyte that have specialised functions and morphology.

Alveolar type I cells

ATI cells only account for approximately 10% of the total cells in the normal human lung. However, due to their shape, thin flat squamous epithelium with cytoplasmic projections, they account for approximately 98% of the alveolar surface (Stone *el al*, 1992). This morphology, taking into account Fick's law, allows for effective diffusion of gas into and out of the blood supply. This also leads to the major obstacle into the study of this cell type as they are extremely fragile and isolation to create cultured ATI cells is very difficult to achieve. Initially the existence of these cells was debated until electron microscopy studies provided indisputable evidence for the existence of an epithelial lining consisting of ATI and ATII cells (Low, 1952). With advancements in techniques, much more has been learned about this cell type and its role in hydration. The discovery of the epithelial Na^+ channel (ENaC) in ATI cells has changed the original view that ATII cells are the main source of Na^+ transport that drives the absorption of fluid and suggests that ATI cells play a vital role in the transport of ions and thus hydration of the alveoli (Johnson *et al*, 2002; Bourke *et al*, 2005; Helms *et al*, 2006).

Alveolar type II cells

ATII cells are smaller than ATI cells and are cuboidal, with secretory granules called lamellar bodies that store the components of pulmonary surfactant. Surfactant is the name given to all substances that can reduce surface tension. The production of surfactant is extremely important as, without it, the muscular effort required to overcome surface tension in order to prevent the lung collapsing, would be exhaustive. Immaturity of the surfactant producing system is a contributing factor of RDS in premature infants (O'Brodovich, 1996). A great deal more is known about ATII cells as they are much easier to isolate and culture. Kikkawa and Yoneda were the first to propose a method of isolation for *in vitro* studies in 1974 (Kikkawa and Yoneda 1974). As well as roles in pulmonary surfactant production, ATII cells act as progenitor cells that replace damaged ATI cells in the event of lung injury (Evans *et al*, 1973; Kim *et al*, 2006) and provide a defence against infection as they synthesis immune effector molecules (Vanderbilt *et al*, 2003). ATII cells were thought to be the primary source of Na^+ transport in the alveoli and various studies have shown ATII cells to contain ENaC subunits using northern blot analysis, RT-PCR, immunoprecipitation and *in situ* hybridisation (Yue *et al*, 1995; Planes *et al*, 1997; Bove *et al*, 2010).

The accepted paradigm for fluid absorption in lung alveoli is that uptake of Na^+ generates osmotic gradients that fluid then follows passively. The bulk of Na^+ transport was thought to occur via ENaC in the ATII cells, with ATI cell providing a route for water via the aquaporin-5 channel (Nielson *et al*, 1997); however due to the large surface area of the ATI cells, it seemed likely that they might play a much more significant role in fluid absorption and a number of studies using isolated ATI cells and lung slice preparations support this view (Johnson *et al*, 2002; Bourke *et al*, 2005; Helms *et al*, 2006; reviews: Dobbs and Jonhson, 2007; Eaton *et al*, 2009).

Nature of ionic transport in relation to fluid secretion and absorption

Osmosis is the movement of water across a selectively permeable membrane, such as the plasma membrane of cells, from an area of high water concentration (or low solute concentration) to an area of low water concentration (or high solute concentration). Proteins that span the plasma membrane form channels that allow for the selective transport of ions. Large charged molecules can cause the uneven distribution of ions in what is known as the Gibbs-Donnan law; however, in most cases, the potential caused by this effect is not sufficient to account for the differences seen in ionic concentrations between two fluids, such as that between lung fluid in the developing foetus and that of the plasma (Adamson *et al*, 1969). The active transport of specific ions leads to the generation of osmotic gradients that either favours fluid secretion or absorption.

The Na^+/K^+ ATPase exchanger pumps out Na^+ in a ratio of three Na^+ ions out for two K^+ ions in. This helps to keep the intracellular concentration of Na^+ low (~10mM), therefore creating a chemical gradient that favours the uptake of Na^+ from the surrounding fluid. The ratio of Na^+ out to K^+ ions in means that there is a net loss of positive charge from the cell, resulting in a negative potential, thus the effect of the Na^+/K^+ ATPase is to generate an electrochemical gradient that favours Na^+ entry into the cell, for example via ENaC. In order to prevent depolarisation of the cell from build up of K^+ concentration, K^+ channels present in the membrane allow K^+ to "leak" out of the cell down the chemical gradient. The polarised nature of the respiratory epithelium means that vectorial transport of ions can be achieved. The Na^+/K^+ ATPase is located on the basolateral side of the cell and ENaC located in the apical membrane, thus the electrochemical gradient set up by the Na^+/K^+ ATPase favours the entry of Na^+ via the

apical ENaC channels. Thus the movement of Na^+ creates an osmotic gradient that fluid can follow passively, either through the paracellular pathway or via aquaporins.

Secondary Active Transport

The electrochemical gradient favouring Na^+ entry generated by Na^+/K^+ ATPase can provide the driving force for secondary active transport and it is this property that is thought to govern fluid secretion. Cl^- enters the cell via the basolateral Na^+, K^+, $2Cl^-$ co-transporter (NKCC) thereby elevating the intracellular Cl^- concentration, allowing Cl^- to exit via apically located Cl^- channels, therefore creating an osmotic gradient which water will follow. As in the Na^+ absorption model, K^+ channels located in the basolateral membrane help prevent depolarisation of the cell by allowing K^+ to exit. This model of Cl^- was first put forward by Silva *et al* in 1977 and is a form of secondary active transport (Silva *et al*, 1977).

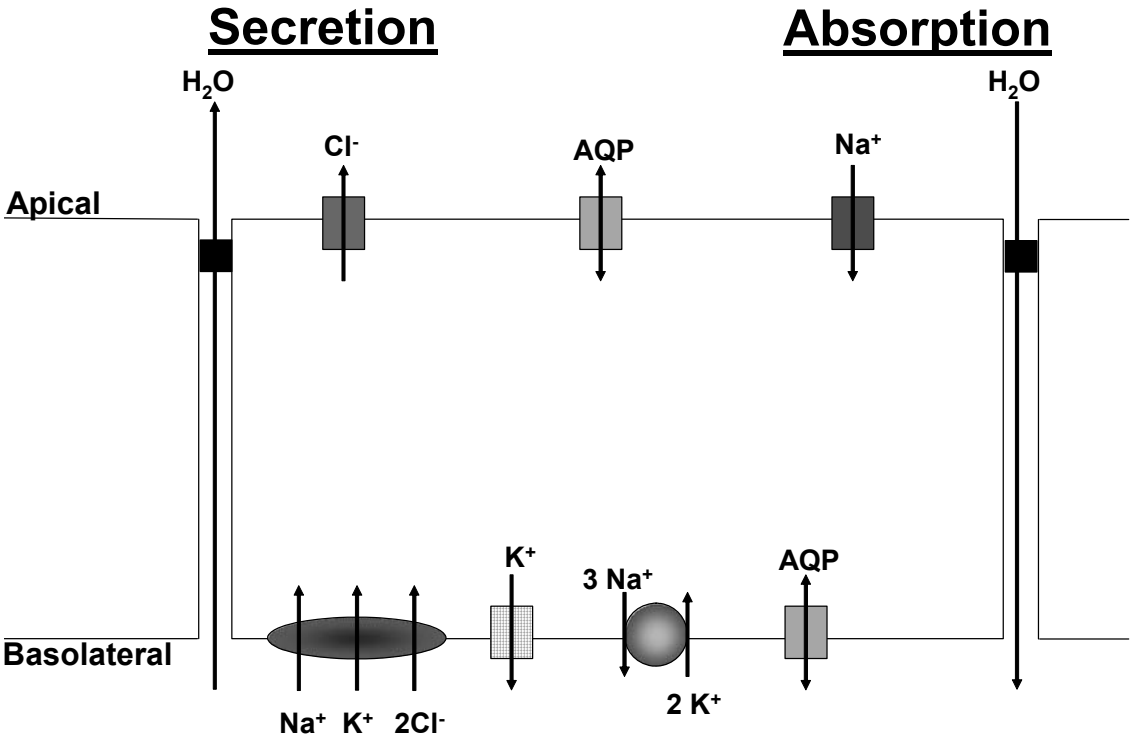


Figure 1.2 Simple diagram of fluid absorption and secretion. Na^+ / K^+-ATPase generates an electrochemical gradient that favours Na^+ entry via apical Na^+ channels, thus creating an osmotic gradient favouring fluid absorption via the paracellular pathway or via aquaporins (AQP) located in the membrane. K^+ exits the cell via basolateral K^+ channels to prevent depolarisation from K^+ build up. Cl^- enters the cell via the NKCC co-transporter, thus raising the intracellular [Cl^-] and Cl^- is secreted through apical Cl^- channels. This creates an osmotic gradient that favours the secretion of fluid through the paracellular pathway or possibly through aquaporins (AQP). K^+ channels help prevent depolarisation as in the model of Na^+ absorption. Thus the balance between Na^+ absorption and Cl^- secretion determines the hydration of the respiratory system.

The regulation of Na^+ absorption and Cl^- secretion and the balance between this is the major determinate of respiratory hydration and therefore the mechanism involved in their regulation is of critical importance. Furthermore the identification of the specific

channels involved is of equal importance so that strategies can be developed to treat diseases which result from impairment of these processes.

Importance of hydration in development

As discussed earlier, lung development needs a fluid template, required to provide distending pressure. The origin of this fluid was first assumed to be inhaled amniotic fluid. However, a study by Adamson and colleagues (Adamson *et al*, 1969) showed that there were differences between the compositions of the two fluids: the concentrations of phosphate and HCO^- were lower and Cl^- and H^+ were higher in lung liquid than in either amniotic or plasma filtrate and therefore could not be due to a mixing of amniotic and plasma filtrate. Furthermore the Gibbs-Donnan law could not explain this difference as there were low levels of protein in lung fluid. This lead them to the conclusion that the lung liquid must be the result of secretions by the foetal lung and not a mixture of amniotic and plasma filtrate (Adamson *et al*, 1969). Coupled with the observation that lung epithelium is very permeable to water (Normand *et al*, 1971), this lead to the theory that active transport of ions could be the driving force for fluid movement from the plasma to the lungs. Olver and Strang undertook work to study the movement of ions between the plasma and lung liquid in foetal lambs and to compare these results with the predicted movement of ions based on the electrochemical gradient in order to determine whether active transport of a specific ion or ions could be the determinate of the fluid secretions in the developing lung. This was achieved by adding radioactively labelled ions to either the lung liquid or into the blood stream via intravenous injection and then measuring samples taken from either the lung liquid or blood for radioactivity. Using this approach they were able to determine that Cl^- was actively transported and secreted into the lung lumen and that Na^+ follows passively. This movement of NaCl

creates an osmotic gradient that generates the liquid secretion into the lung (Olver and Strang, 1974). Furthermore they showed that potassium cyanide inhibited Cl^- transport, which leads to fluid absorption. They hypothesised that this could not account for the removal of liquid at birth as the rate of absorption was too slow to account for this, thus a change in the permeability of the lung epithelium would be needed to effectively clear the lungs of fluid in order for gas exchange to take place (Olver and Strang, 1974). This study, however, did not provide any evidence as to the identity of the ion channels involved in this mechanism.

Chloride channels involved in fluid secretion

It would seem unlikely that CFTR would be the major route for Cl^- secretion in the developing lung due to the lack of impaired lung development in patients with cystic fibrosis (Sturgess and Imrie, 1982). A more likely candidate is the ClC-2 channel of which there exists a number of studies providing evidence for the involvement of this channel. Murray *et al* have shown using immunohistochemistry that ClC-2 is expressed in the apical membrane of foetal lung epithelium and that expression decreases after birth (Murray *et al*, 1995). Furthermore the low pH of lung liquid reported by Adamson and colleagues (~6.3; Adamson *et al*, 1969) would appear to be a stimulus for Cl^- secretion as the ClC-2 channel has been shown to be activated by low pH (Schwiebert *et al*, 1998; Blaisdell *et al*, 2000). Blaisdell *et al* also showed that blockers of other Cl^- channels (CFTR, ClC-3 and 5) could not inhibit the increase in Cl^- transport seen with lowered pH, providing further evidence for the role of ClC-2 (Blaisdell *et al*, 2000). However not all data suggests a role for ClC-2, as this channel has a lower permeability to I^- than to Cl^-, which is the opposite to the ionic flux reported in Olver and Strang, 1974. Low pH activation of apical Cl^- would lead to a reduction in intracellular Cl^- concentration and it is therefore interesting to note that NKCC activation has been

linked to a reduction in Cl^- (Haas *et al*, 1995), therefore the low pH of the lung liquid itself may act as a mechanism to ensure the continual secretion of Cl^- and hence lung fluid. The NKCC would appear to be the most likely candidate for basolateral Cl^- entry. However, evidence from Ussing chamber experiment on *xenopus laevis* lungs has suggested that the HCO^-/Cl^- anion exchanger may be responsible for Cl^- uptake under basal conditions and that NKCC is only active under Cl^- gradient conditions (Berger *et al*, 2010), although differences between species may explain this.. There are relatively few studies in this area, therefore further investigation is needed to provide conclusive evidence for the identity of the channels involved in fluid secretion in the developing lung.

Fluid clearance (Birth, change from net secretion to absorption)

While fluid secretion is clearly important for the development of the lung, it needs to be cleared in order for respiration to begin. Initially it was thought that fluid clearance was the result of mechanical and Starling forces. However, studies on rabbits showed that lung wet weight decreased almost immediately before birth and is almost completed two hours after birth (Aherne and Dawkins, 1964). Furthermore caesarean section does not impede the removal of lung liquid as long as the onset of labour has commenced (Bland *et al*, 1980; Baines *et al*, 2000). This observation also suggests that the onset of labour is an important trigger for fluid clearance. This implies that another mechanism for the removal of lung liquid must be involved. There is an increased incident of RDS seen in elective caesarean section (Hales *et al*, 1993) and, along with the observation that the levels of adrenaline increase at birth (Lagercrantz and Bistoletti, 1977), these indicate a possible role for adrenaline as the trigger for fluid clearance. In 1983 Brown

et al observed that foetal lambs' sensitivity to adrenaline increased with gestational age, which caused a slowing of liquid secretion (before 130 days) that turns to liquid absorption (after 147 days) (Brown *et al*, 1983). Olver *et al* in 1986 confirmed these observations; however they also investigated ionic flux, using similar methods to that of Olver and Strang in 1974, and found that Na^+ is actively transported from the lung lumen to the plasma. Furthermore the use of a known Na^+ channel blocker, amiloride, prevented the adrenaline induced liquid absorption (Olver *et al* in 1986). These experiments therefore suggest that Na^+ flux could be the determining factor in driving liquid absorption. They went on to suggest that Na^+ and Cl^- transport were interlinked and that Cl^- transport was a result of co-transport with Na^+ driven by the Na^+/K^+-ATPase via, at the time, an unidentified transporter in the basolateral membrane. The opening of apical Na^+ channels would lead to Na^+ absorption also driven by Na^+/K^+-ATPase, which would cause the uptake of fluid due to a change in osmotic gradients. Later experiments, such as the administration of amiloride to the trachea of new born guinea pigs, which lead to the development of respiratory distress (O'Brodovich *et al*, 1990) further supported the view that Na^+ absorption via an apical Na^+ channel was responsible for fluid clearance. The effect of adrenaline appears to be mediated via cyclic adenosine monophosphate (cAMP) as administration of cAMP to the lungs of foetal lambs mimics its effects and this response can be blocked by amiloride (Walters *et al*, 1990).

The role of glucocorticoids and thyroid hormone

The rise in adrenaline is preceded by a rise in circulating glucocorticoids (GCs) and thyroid hormone before labour (Baines *et al*, 2000) which lead to the investigation of these hormones and the role they may play in the regulation of fluid clearance. Foetal thyroidectomy in lambs prevents the normal response to adrenaline and cAMP

analogues (Barker *et al*, 1988). This response is only reversed if thyroid hormone and GCs are administered together and not independently (Barker *et al*, 1990). This suggests that these hormones have different targets that are required for the response to adrenaline to take place. However it does not provide evidence as to the hormonal targets. A further study confirmed these results and through the use of a protein synthesis inhibitor, cycloheximide, determined that the effects of these hormones are mediated through a mechanism that requires the synthesis of new protein (Barker *et al*, 1991). However, again this could not provide the identity of the hormonal targets. Nevertheless, they did speculate that the likely targets could be the Na^+/K^+ ATPase and apical Na^+ channels (Barker *et al*, 1991). Further to this, Collett *et al* in 2002 demonstrated that isoproterenol, a β-adrenoceptor agonist, does not increase Na^+ conductance (G_{Na}) in foetal rat distal lung epithelial (FDLE) cells cultured in media containing no hormones. Exposure to both thyroid hormone and GCs, isoproterenol did cause an increase in G_{Na}, which was amiloride sensitive (Collett *et al*, 2002). These observations suggest a synergistic mode of action where GCs and thyroid hormone prepare the lung for the switch to an absorptive phenotype, while adrenaline activates Na^+ channels, which results in an increase in G_{Na}, through an amiloride sensitive Na^+ channel.

Other hormones involved in fluid clearance

Epinephrine, thyroid hormone and GC's are not the only hormones thought to be involved in the control of lung fluid clearance. Arginine vasopressin (AVP) and somatostatin are both thought to inhibit lung fluid secretion (Perks and Cassin, 1989; Albuquerque *et al*, 1998), whereas dopamine and serotonin are have been shown to activate Na^+ transport (Chua and Perks, 1998; Chua and Perks, 1999). This serves to stress the importance of lung fluid clearance to survival, as multiple levels of control

mean that if there is a problem with one mechanism, then other alternative mechanisms may still be able to initiate fluid clearance and therefore survival at birth is assured.

Sodium entry pathway

There is substantial evidence for the role of an apical Na^+ channel as the route of Na^+ absorption. However, the identity of the specific channel involved was not revealed until 1993 when Canessa and colleagues identified a novel cDNA sequence from rat colonic epithelial tissue that encoded for an amiloride sensitive Na^+ channel, which was termed α-ENaC (α-epithelial Na^+ channel) (Canessa *et al* 1993). α-ENaC was subsequently found in the kidney and lung (O'Brodovich *et al*, 1993; Voilley *et al*, 1994) and two other subunits were also identified: β-ENaC and γ-ENaC (Canessa *et al* 1994). However, when β-ENaC and γ-ENaC were expressed independently in *Xenopus* oocytes, they did not produce amiloride sensitive currents, although when co-expressed with α-ENaC an amiloride sensitive current that was 100 fold greater than that of those where only α-ENaC was expressed, was observed (Canessa *et al* 1994). It would therefore appear that fully functional ENaC is composed of three separate subunits, α-ENaC being the major pore forming subunit, while β-ENaC and γ-ENaC subunits conform selectivity. Further to this α-ENaC knockout mice die at birth due to insufficient fluid clearance, while β-ENaC and γ-ENaC knockout mice survive, although they do show reduced fluid clearance and develop respiratory problems as a result (Hummler *et al*, 1996). This not only adds further evidence for α-ENaC being the major pore forming unit, but also highlights the importance of ENaC in lung fluid clearance.

The ENaC channel

ENaC subunits share ~30% homology and have a large extracellular loop and two transmembrane domains that have intracellular N- and C-terminal domains (Canessa *et al*, 1994). The stoichiometry of ENaC is still under some debate, for example the crystal structure of the acid-sensing ion channel has been suggested to be a trimer. As this channel is part of the ENaC subfamily it has been suggested that a 1α, 1β and 1γ stoichiometry make up the ENaC channel (Jasti *et al*, 2007). However a 3α, 3β and 3γ stoichiometry has also been suggested by Staruschenko *et al*, 2004. However Staruschenko *et al* results only point to a stoichiometry where the channel contains two of each subunit and therefore only hints at the 3α, 3β and 3γ stoichiometry. Furthermore by using fluorescence resonance energy transfer to analyse channel stoichiometry, these results are reflective of the entire pool of ENaC and therefore the stoichiometry of active ENaC in the membrane may be difficult to determine. The 2α, 1β and 1γ stoichiometry would appear to be the most supported; however, despite similarities in techniques, there is some conflicting data with three studies (Firsov *et al*, 1998; Kosari *et al*, 1998; Anantharam and Palmer 2007) supporting a tetrameric stoichiometry, as another study, also using similar techniques, provided support for the 3α, 3β and 3γ stoichiometry (Snyder *et al*, 1998). These studies used co-expression of mutant and wild-type ENaC subunits and electrophysiological analysis to determine stoichiometry. These observations are dependent on a number of assumptions such as wild-type and mutants will express equally and associate randomly. If any of the assumptions made by these studies is incorrect then this will lead to a false interpretation of the results. Despite the differences in reported stoichiometry, the tetrameric arrangement would appear to be the most likely, as this arrangement seems to most closely resemble the biophysical properties of ENaC, a low conductance of ~5pS

with slow gating kinetics (Palmer and Frindt, 1986). A fourth ENaC subunit does exist, δ-ENaC, which may be involved in producing an amiloride sensitive channel (Bangel-Ruland *et al*, 2010).

Non ENaC like amiloride sensitive channels

Many electrophysiological studies of lung epithelial cells from FDLE have described an amiloride sensitive nonselective cation channel that poorly discriminates between Na^+ and K^+ and has a much higher conductance, ~21pS (Ito *et al,* 1997; Marunaka *et al*,1999), than that reported for the highly selective ENaC as proposed by Palmer and Frindt. However these differences could be due to differing combinations of ENaC subunits resulting from culture conditions. A study by Jain *et al* in 2001 demonstrated that culture conditions can impact significantly on the electrophysiological properties of cells. ATII cells were isolated from rat lungs and, when grown on a permeable membrane with an air interface in the presence of GC's, a channel with ENaC-like properties was observed (Jain *et al* 2001). However when these cells were cultured on glass coverslips or plastic (without an air interface in the absence of GC's), they displayed an amiloride-sensitive, nonselective channel. Thus differences in culture conditions may lead to differences in channel stoichiometry which impacts on channel properties, and could account for the differences in the reported channels. The expression of ENaC subunits appears to vary depending on gestational age (Helve *et al*, 2007). Therefore it could be that different subunit expression leads to different channel expression *in vivo* that is required at different stages of lung development and maturation.

Pseudohypoaldosteronism is a disease that results from mutations in the genes encoding for ENaC (Chang *et al*, 1996) and many patients develop a variety of symptoms such as

continual viral infections and wheezing and display an increased ASL volume (Kerem *et al*, 1999). However there is a delay from birth in the development of these symptoms and fluid clearance does not appear to be impeded thus suggesting that channels other than ENaC may be involved in fluid clearance. However the development of respiratory problems does suggest a role for ENaC.

Airway hydration in the adult lung

The regulated secretion of lung liquid is clearly important during foetal development and the subsequent absorption at birth is of equal importance. However the continued regulation of airway hydration is just as important throughout adult life. As discussed earlier, regulation of the ASL depth and the fluid lining the alveoli are critical in preventing infection and allowing efficient gas exchange. The importance of regulation is highlighted by the observation that mice models over-expressing β-ENaC display cystic fibrosis-like lung disease (Mall *et al*, 2004). Cystic fibrosis is a genetic disorder that is caused by impaired Cl^- secretion, is the most common genetic disorder among Caucasians (Quinton, 1989) and results in impaired mucociliary transport as a result of insufficient hydration. The defective Cl^- secretion is due to the mutation of the CFTR Cl^- channel (Riordan *et al*, 1989). Even though the ability to secrete Cl^- is unaffected, the increased Na^+ absorption caused by the over-expression of β-ENaC still resulted in cystic fibrosis-like lung disease (Mall *et al*, 2004). Furthermore increased Na^+ absorption has also been linked to compounding the effects of cystic fibrosis (Boucher, 1986).

This highlights the importance of regulated airway hydration in the adult lung; however as the increase in adrenaline seen at birth is not maintained throughout adult life, even though the Na^+ absorbing phenotype that it induces is maintained, this suggests other

factors must play a role, so that tight regulation of the ASL and alveoli lining must be observed throughout adult life. The next section describes some of the possible mechanisms involved.

Proteolytic cleavage

It has emerged that modulation of channel activity through proteolytic cleavage of ENaC subunits at the cell surface can modulate channel gating (Rossier, 2003; Hughey *et al*, 2004; Planes *et al*, 2009). Channel activating proteases (CAPs) have been shown to be expressed in tissues including the distal lung epithelium (Vallet *et al*, 2002) and to be contained in lung liquid secretions (Verghese *et al*, 2004; Planes *et al*, 2005). β-adrenergic agonists induce stimulation of lung fluid clearance, which was inhibited in mice lacking the CAP1 gene and was shown to be reversed with treatment of neutrophil elastase, a nonepithelial soluble serine protease (Planes *et al*, 2009). This could be taken as further evidence of the synergistic action of hormones in the control of alveolar fluid clearance, where GCs and thyroid hormone prime the lung for fluid clearance and epinephrine causes activation of ENaC in the membrane through proteolytic cleavage increasing channel activity.

Glucocorticoid-induced leucine zipper protein

Shi *et al* (2002) showed that ERK1/2 phosphorylation facilitated the interaction of βENaC and γENaC with Nedd4-2 (thought to be involved with targeting ENaC for degradation); furthermore ERK activation was shown to inhibit Na^+ transport (Robert-Nicoud *et al*, 2001; Shen and Cotton, 2003; Falin *et al*, 2005). These observations taken together suggest an alternative mechanism of ENaC regulation that may be SGK1 independent. A study in 2005 for the first time linked glucocorticoid-induced leucine zipper protein (GILZ) with a role in stimulating Na^+ transport by inhibiting ERK1/2

(Soundararajan *et al*, 2005). A number of isoforms of GILZ have been discovered however GILZ1 is the most potent in causing Na^+ transport stimulation (Soundararajan *et al*, 2007). It has been suggested that GILZ1 and SGK1 can act independently to increase ENaC surface expression although it would appear that they have a synergistic effect whereby GILZ aids the activity of SGK1 possibly by forming a regulatory complex that recruits SGK1 and facilitates the interaction of SGK1 with Nedd4-2 (Soundararajan *et al*, 2009). Further to this transfection of GILZ1 in the H441 cell line mimics the increase in Na^+ transport associated with GC treatment (unpublished observation, Michael Gallacher).

Cyclic adenosine monophosphate (cAMP)

Adrenaline is reported to act via cAMP (Walters et al, 1990), also arginine vasopressin (AVP) has been implicated in cAMP mediated ENaC regulation. AVP binds to V2 receptors and activates adenylate cyclase which synthesises cAMP from ATP. Increasing cAMP levels with AVP, phosphodiesterase inhibition or adenylate cyclase activation has been shown to increase Na^+ transport (Barbry and Hofman, 1997; Garty and Palmer, 1997; Yang *et al*, 2006). AVP receptors (V2) are expressed in the lung (Kaufmann *et al*, 2003) suggesting that cAMP could be involved in the regulation of Na^+ transport in the lung. This is further supported by the observation that forskolin, an adenylate cyclase activator, can increase amiloride sensitive Na^+ transport in a human lung cell line (Lazrak and Matalon, 2003; Ramminger *et al*, 2004). There is evidence that PKA, a downstream effector of cAMP, can phosphorylate Nedd4-2, therefore representing a possible convergence point for signalling pathways in the control of Na^+ transport (Snyder *et al*, 2004). Thus cAMP may affect ENaC expression in the membrane, although it has also been reported to increase channel open probability (Yang *et al*, 2006).

Partial pressure of oxygen

The first breath taken by newborns is thought to provide a stimulatory effect to increase Na^+ transport and evidence suggest that a change in oxygen concentration from ~3% (foetal) to ~21% (postnatal) may be a determining factor in this. In rat FDLE cells this change in concentration has been shown to increase Na^+ transport and also increase ENaC mRNA levels (Ramminger *et al*, 2000). However there is a discrepancy as Na^+ transport is increased before an increase in mRNA levels are seen (Pitkanen *et al*, 1996; Baines *et al*, 2001) and under hormone free conditions an increase in partial pressure of oxygen (P_{O2}) increases Na^+ transport without affecting the abundance of ENaC mRNA (Richard *et al*, 2003). Otulakowski *et al* in 2007 explained this by suggesting that GC's create a pool of ENaC mRNA that an increase in P_{O2} causes to be translated. Hypoxic conditions are known to reduce Na^+ transport (Vivona *et al* 2001; Planes *et al*, 1997; Wodopia *et al*, 2000), which is thought to be a contributing factor to the development of pulmonary oedema observed in high altitude sickness.

Lipopolysaccharide

LPS, which is present in the bacterial coat of many pathogens, causes the production of nitric oxide and tumour necrosis factor-alpha (TNF-α) as part of the inflammatory response to bacterial pathogens via the activation of nuclear factor-kappa B and the mitogen-activated protein kinase signalling pathway (Guha and Mackman 2001; Guillot *et al*, 2004). Both nitric oxide and TNF- α have been shown to inhibit ENaC expression in lung epithelial cells (Ding *et al*, 1998; Dagenais *et al*, 2006). More recent studies have provided further evidence for a role of LPS causing inhibition of ENaC via the ERK signalling pathway (Baines *et al*, 2010) and also suggest a role for LPS induced inhibition through a purinergic signalling pathway (Boncoeur *et al*, 2010). These

studies indicate a role of LPS that could cause the accumulation of fluid in the lung resulting in pulmonary oedema from bacterial infection.

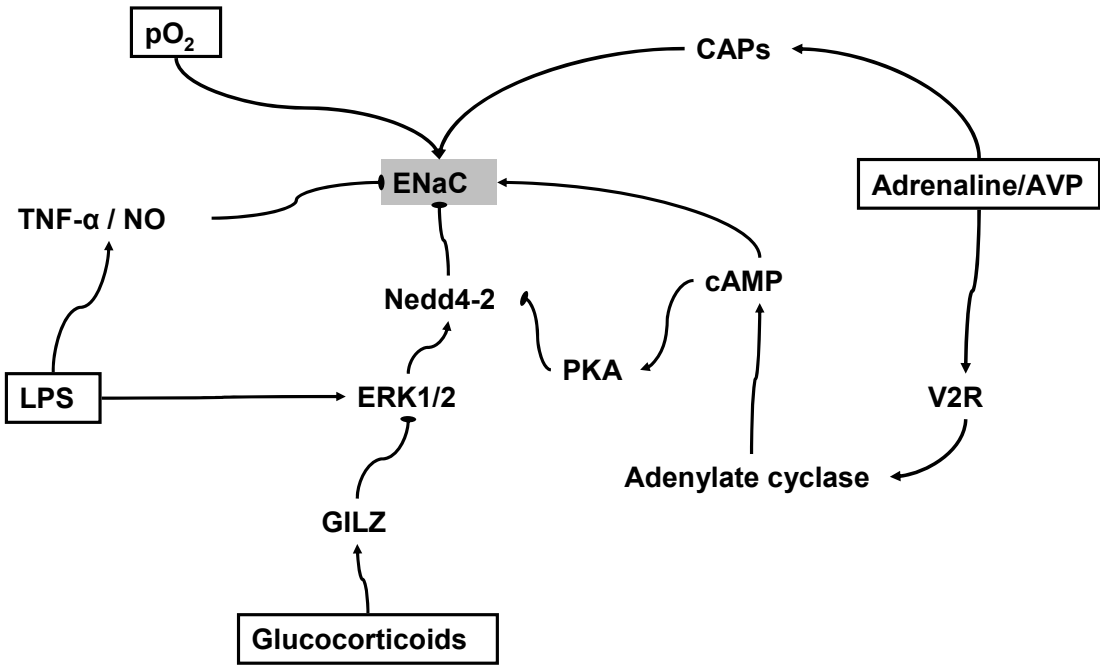


Figure 1.3 Diagram summarising inputs that can affect ENaC regulation. This only represents a few of the many inputs that can affect ENaC regulation. Blunt ended arrows represent inhibition/repression and arrows represent activation. Inputs are surrounded by a box. pO_2 = partial pressure of oxygen, CAPs = channel activating proteases, AVP = arginine vasopressin, cAMP = cyclic adenosine monophosphate, TNF-α, NO = nitric oxide, LPS = lipopolysaccharide, GILZ = glucocorticoid induced lucine zipper protein, PKA = protein kinase A, V2R = V2 receptor.

ENaC is subject to regulation from many other factors and this section does not cover them all. If there is an imbalance in these mechanisms then disease states can occur such as oedema from infection or altitude sickness. The most effective way of treating pulmonary oedema is the use of GCs, however despite this and extensive study, the exact mechanisms involved in GCs mediated fluid clearance is still unclear.

Glucocorticoids

GCs are a steroid hormone produced in the adrenal cortex and are lipophilic, thus enabling them to diffuse freely across the cell membrane. They have a wide range of effects throughout the body and the name glucocorticoid originates from the observation that they are involved with glucose metabolism (see table 1.1 for list of other effects). As was demonstrated earlier, GCs are important in mediating the switch to a Na^+ absorbing phenotype at birth. GCs are used clinically to treat pulmonary oedema and to improve lung maturation in pre-term infants. However there are concerns, especially in their use for treatment of RDS in premature infants, as it may lead to adverse effects on neuromotor and cognitive function as well as causing myocardial hypertrophy (Yeh *et al*, 2004; Zecca *et al*, 2001; Damsted *et al*, 2011). Therefore it is important to ascertain the mechanisms by which GCs exert their effects, as this may lead to improved treatment for patients.

Table 1.1 Functions of glucocorticoids.

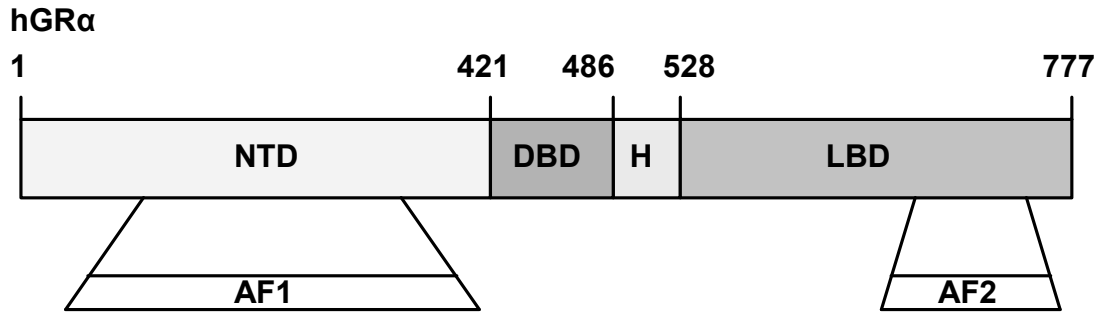
Role	Effects	References
Glucose metabolism	Stimulation of gluconeogenesis and the break down of adipose tissue. Inhibit glucose conversion to glycogen.	Christ-Crain *et al*, 2008 Buren *et al*, 2008
Immunity	Up-regulate anti-inflammatory proteins and down-regulate pro-inflammatory proteins.	Cutolo *et al*, 2011 Riccardi, 2010
Development	Important in lung maturation and also brain development such as terminal maturation and remodelling axons and dendrites.	Jauregui-Huerta *et al*, 2010 Yeh *et al*, 2004 Damsted *et al*, 2011
Memory	Shown to be involved in synaptic plasticity, is thought to aid memory formation.	Joels *et al*, 2009

Mechanisms of Glucocorticoid ENaC regulation

Glucocorticoid receptor

The human glucocorticoid receptor (GR) is part of the steroid/thyroid/retinoic acid nuclear receptor superfamily and was first isolated by expression cloning in 1985 by Hollenberg and colleagues (Hollenberg *et al*, 1985). They also discovered that alternative splicing leads to two isoforms of the GR; GRα and GRβ. Further splice variants have since been discovered and so far a total of five variants have been reported and only GRα and GRγ can bind GC (Oakley and Cidlowski 2011). Multiple start translation sites give rise to multiple isoforms of which a total of 8 have so far been reported (Oakley and Cidlowski 2011). The expression levels of the different isoforms differ depending on tissue type which may lead to unique target genes and therefore give rise to tissue specificity (Lu and Cidlowski, 2005). The GR has an amino-terminal or N-terminal domain, a DNA binding domain, hinge region and a ligand binding domain or the carboxyl terminal end. The ligand binding domain is not present in GRβ

and results in the inability of GRβ to bind steroids and appears to exert a dominant negative effect on the transcriptional activity of GRα (Kino *et al*, 2009). Within the N-terminal and the ligand binding domain are areas termed activation function 1 and 2 respectively (AF1 and AF2). AF1 is important in aiding interactions of the receptor, with molecules involved in forming a transcription complex; AF2 is important for receptor dimerisation and is involved in the binding of heat shock proteins (hsp) (Duma 2006).



Firgure 1.4 Diagram of glucocorticoid receptor. Shows structure of the human glucocorticoid receptor-α showing approximate location of activation function 1 and 2 (AF1 and AF2 respectively). NTD – N-terminal domain, DBD – DNA binding domain, H – hinge region, LBD – ligand binding domain or the carboxyl terminal end.

Glucocorticoid receptor alpha complex

GRα exists as a complex of proteins in the cytoplasm of cells which includes hsp90 (Pratt, 1993). Hsp90 prevents the translocation of the inactive GRα to the nucleus

(Cadepond *et al*, 1991) and ligand binding results in a conformational change resulting in the dissociation of hsp90 from the GRα complex thereby allowing translocation to the nucleus. The activated GC-GR forms a homodimer and binds to a GRE (Truss and Beato, 1993) on DNA. The GRE bound GR stimulates transcription by facilitating the formation of a transcription complex. AF1 and AF2 interact with cofactors to initiate the formation of this complex. AF1 and 2 attract steroid receptor coactivators (SRC's), which in turn recruits cyclic adenosine monophosphate response-element binding protein (CREB) and p300/CBP-associated factor as well as other cofactors (Nicolaids *et al*, 2010). This complex has histone acetyltransferase activity, which aids in the decondensation of chromatin at the site of the GRE, and aids in the recruitment of RNA polymerase II to form a transcription complex in order to cause upregulation of the target gene (Nicolaids *et al*, 2010). As the α-ENaC promoter contains a glucocorticoid response element (GRE) it would seem likely that GC would act via increasing α-ENaC transcription. This does seem to be the case as a number of studies have shown that GC cause upregulation of α-ENaC via reporter gene assays (McTavish *et al*, 2009; Otulakowski *et al*, 1999) and ribonuclease protection assay (Mick *et al*, 2001; Sayegh *et al*, 1999). GCs can also cause suppression of genes and it is thought this works in a similar manner but instead condenses chromatin, thereby preventing access and formation of a transcription complex to the gene (Ito et al, 2000). However suppression can also be due to increases in GRβ for example, tumour necrosis factor-alpha and interferon-gamma have been shown to cause an increase in GRβ without affecting GRα that is associated with GC resistance (Tliba *et al*, 2006).

Glucocorticoid receptor post-translational modifications

There is evidence to suggest that post-translational modifications can affect many properties of the GR such as: stability, target promoters, trafficking and even direct

interactions of the GR with other proteins, which may cause the transduction of a signalling cascade without effecting genomic expression. See table 1.2 for summary.

Table 1.2 lists reported post-translational modifications which are thought to affect the glucocorticoid receptor and therefore serves to highlight the complexity of GC signalling.

Post-translational Modification	Possible roles	References
Phosphorylation	Typically occurs after ligand binding. May affect turnover, trafficking, promoter specificity, cofactor interaction and nongenomic activation of signalling pathways.	Ismaili and Garabedian, 2004 Orti *et al*, 1993
Ubiquitination	Occurs after ligand binding. Can regulate motitility of GR and can promote rapid turnover of the receptor therefore decreasing activity.	Dennis and O'Malley, 2005
Acetylation	Occurs after ligand binding before nuclear translocation and deacetylation causes NF-κB suppression. May disrupt binding of GR to GRE's.	Ito *et al*, 2006 Nader *et al*, 2009
Sumoylation	Small ubiquitin-related modifier. May not be ligand dependent and may affect protein stability and transcriptional activation.	Le Drean *et al*, 2002

GC receptor regulation of Na^+ transport

It has also been suggested that serum- and glucocorticoid-regulated kinase 1 (SGK1) can also increase transcription of α-ENaC (Boyd and Naray-Fejes-Toth, 2005; Zhang *et al*, 2007). However these observations were obtained from studies in renal epithelia and McTavish *et al* found that SGK1 does not play a major role in α-ENaC transcription in pulmonary epithelium (McTavish *et al*, 2009). However there is a large body of evidence that does suggest a significant role of SGK1 in the control of Na^+ transport via alternative mechanisms.

Serum- and glucocorticoid-regulated kinase 1

SGK1 belongs to the AGC family of protein kinases and was originally cloned from rat mammary tumour cells in 1993 (Webster *et al*, 1993) and as the names suggests is regulated by GCs and serum. However SGK1 regulation is subject to control from a wide variety of stimulators and inhibitors (for review see; Lang *et al*, 2009) including mineralocorticoids. Two further isoforms of the *sgk* gene have been reported *sgk2* and *sgk3* and these do not appear to be regulated by GCs (Kobayashi *et al*, 1999), although both have been reported to be involved in ENaC regulation (Friedrich et al, 2003). However in *sgk3* knockout mice there is no effect on NaCl excretion (McCormick *et al*, 2004). SGK1 has been shown to increase ENaC activity and also contains a GRE in the 5' flanking end of the *sgk1* gene which is upregulated by GCs.

Activation of SGK1

As with many AGC kinases, SGK1 activation is dependent on the phosphorylation of a threonine residue (Thr^{256} for SGK1) in the T-loop of the kinase and a serine residue (Ser^{422} for SGK1) within the hydrophobic motif. The activity of SGK1 has been shown to be dependent on phosphoinositide-3-kinases (PI3K) (Kobayashi and Cohen, 1999; Park *et al*, 1999). SGK1 is first phosphorylated on the hydrophobic motif by the mammalian target of rapamycin complex 2 (mTORC2) (Garcia-Martinez and Alessi, 2008). The identity of the kinase responsible for this has been the subject of debate with suggestions that mTORC1 was in fact responsible for hydrophobic motif phosphorylation of SGK1 (Hong *et al*, 2008). However Garcia-Martinez and Alessi disputed these findings and show SGK1 activity in the presence of the specific mTORC1 inhibitor rapamycin which is consistent with other studies (Kobayashi and Cohen, 1999; Park *et al*, 1999). This view is supported by a more recent study

suggesting mTORC2 activity is required for hydrophobic motif phosphorylation of SGK1 and that this is required for ENaC mediated Na^+ transport (Lu *et al*, 2010). It would therefore seem likely that mTORC2 is in fact the kinase responsible for hydrophobic motif phosphorylation. The phosphorylation of the hydrophobic motif promotes the interaction of SGK1 with phosphoinositide-dependent kinase 1 (PDK1) resulting in the phosphorylation of the activation loop of SGK1 (Biondi *et al*, 2001) and confers activity. This mode of action where phosphorylation of the hydrophobic motif of an AGC kinase thus, in turn making it the target for phosphorylation of its T-loop, is thought to be important for a number of the AGC kinases.

Target of rapamycin (TOR)

TOR exists as two complexes; TORC1 and TORC2, consisting of different associated proteins. TORC1 contains the regulatory associated protein of TOR (Raptor) (Kim *et al*, 2002) and G-protein β-subunit like protein, also known as LST8 (Kim *et al*, 2003). TORC1 also contains proline-rich PKB substrate 40 kDa. However there is some debate as to its role in TORC1 function, as it has been shown to block TORC1 access to its substrates (Wang *et al*, 2007). It has also been described as a substrate for TORC1 phosphorylation (Fonseca *et al*, 2007; Oshiro *et al*, 2007). The best characterised substrates of TORC1 are involved in both cell growth and proliferation; eukaryotic initiation factor 4E-binding protein 1, (Hara *et al*, 1997) and p70 S6K (Fingar *et al*, 2004). Phosphorylation of p70 S6K at residue Thr^{389} is often used to assay for TORC1 activity (Land and Tee, 2007; Mansley and Wilson, 2010). Inhibition of TORC1 is achieved using rapamycin which has been described as an exquisitely specific inhibitor of TORC1 (Bain *et al*, 2007) and acts by forming a complex with FKBP12 which targets TORC1 (Heitman *et al*, 1991). TORC2 on the other hand is composed of; rapamycin insensitive companion of TOR (Rictor) (Sarbassov *et al*, 2004), LST8,

mSin1 (Jacinto *et al*, 2006) and proline-rich protein 5 (Woo *et al*, 2007). TORC2 is acutely insensitive to rapamycin treatment; however prolonged treatment inhibits assembly of the complex (Sarbassov *et al*, 2006). Partly due to a lack of specific inhibitors of TORC2, much less is known about its specific roles, however the development of specific inhibitors of TOR (Thoreen *et al*, 2009; Feldman *et al*, 2009) has given new insights into the role this kinase plays.

SGK1 activation results in increased ENaC trafficking

The ubiquitin ligase protein, neural precursor cell-expressed developmentally down-regulated protein 4 (Nedd4) contains WW domains that can bind to PY motifs of other proteins. All three ENaC subunits have PY motifs and Nedd4 (specifically Nedd4-2) has been shown to bind to these motifs (Staub 1996; Snyder *et al*, 2002). Thus Nedd4-2 targets ENaC for ubiquitination and degradation. SGK1 also contains PY motifs and interacts with Nedd4-2 causing phosphorylation which in turn recruits 14-3-3 protein which reduces Nedd4-2 interaction with ENaC (Bhalla *et al*, 2005; Ichimura *et al*, 2005) possibly by causing steric hindrance or by inducing a conformational change in the WW domains of Nedd4-2 resulting in the inability to bind to other PY motifs. Many studies have linked the ability of SGK1 inhibiting Nedd4-2 to the regulation of ENaC (Bhalla *et al*, 2005; Ichimura *et al*, 2005; Snyder *et al*, 2002; Wiemuth *et al*, 2010) which has lead to the development of the theory that SGK1 regulates ENaC activity by increasing the trafficking and expression of ENaC in the membrane which has been demonstrated in *Xenopus oocytes* (Alvarez de la Rosa *et al*, 1999). Furthermore, preventing ubiquitination of ENaC leads to increase in the stabilation of the channel in the membrane further supporting increased Na^+ transport (). A negative feedback loop has been described as SGK1 phosphorylation of Nedd4-2 causes Nedd4-2 mediated

degradation of SGK1. This may aid in the fine regulation of Na^+ transport (Zhou and Snyder, 2005).

Counter argument for SGK1 involvement

Despite the evidence for the involvement of SGK1-Nedd4-2 pathway, there is evidence that would appear to dispute this central role of SGK1 as the main mechanism in the control of Na^+ transport. There is evidence that would suggest that SGK1 does not interact or interacts very weakly with Nedd4-2 (Henry *et al*, 2003; Rauh *et al*, 2006). However Rauh *et al* suggested this was due to species differences and that the interaction between SGK1 and Nedd4-2 is only strong enough to be detected in those studies where constructs of human origin are used, a view that is supported by Wiemuth *et al*, 2010. The strongest evidence that SGK1 is not involved, or at least that other factors play an important role in the regulation of Na^+ transport, is the observations made from *sgk1* gene knockout mice (*sgk1*$^{-/-}$). These mice do not display any functional abnormalities and are viable (Wulff *et al*, 2002; Grahammer *et al*, 2006; Rexhepaj *et al*, 2006; Fejes-Toth *et al*, 2008), unlike mice lacking functional ENaC which die due to severe respiratory distress (Hummler *et al*, 1996). However *sgk1*$^{-/-}$ do show impairment of renal Na^+ retention although this only becomes apparent during salt deficiency (Wulff *et al*, 2002). This cannot be explained by compensation through enhanced SGK3 activity as double knockout mice display a similar phenotype to *sgk1*$^{-/-}$ mice (Grahammer *et al*, 2006). It would seem unlikely that increased SGK2 activity could account for this as transcript levels are not increased in the double knockout mice (Grahammer *et al*, 2006). It would be expected that *sgk1*$^{-/-}$ mice would not be viable if SGK1 was the sole regulator of ENaC trafficking to the membrane. Therefore these studies demonstrate that the role of SGK1 is still not fully understood and also point to a

SGK1 independent mechanism of ENaC regulation. Adding to this, the observation that *sgk1*$^{-/-}$ mice do not show disruption of ENaC activity in colonic epithelium (Rexhepaj *et al*, 2006) suggests that renal and colonic ENaC regulation may involve different mechanisms. It is therefore not unreasonable to postulate that the role played by SGK1 in the regulation of pulmonary ENaC may also differ from that reported in other tissues.

Aldosterone

In response to decreases in blood volume and pressure aldosterone, a mineralocorticoid (MC) is released from the adrenal cortex and causes an increase in Na^+ absorption which leads to fluid uptake (Booth *et al*, 2002). Aldosterone exerts its effects in the kidney and specifically the distal nephron, where the final adjustments are made to the reabsorption of fluid in order to maintain blood volume and pressure. Aldosterone enters the epithelial cells lining the distal nephron and acts in much the same way as glucocorticoids, however bind to the mineralocorticoid receptor (MR) which then translocates to the nuclease and can bind to DNA to cause either expression or suppression of genes (Booth *et al*, 2002). Furthermore in common with the GR the sterioid bound MR has been reported to be involved in exerting its effects via protein-protein interactions (Stockand, 2002). It is important to note that both the MR and GR receptors can be bind either MC or GC, however, they have higher affinities for their respective namesake. Thus it is therefore possible that increased levels of aldosterone seen in *sgk1* gene knockout mice (Wulff *et al*, 2002) could provide a compensating mechanism to bring about fluid clearance. GILZ has been shown to be an aldosterone-induced protein (Robert-Nicoud *et al*, 2001) and along with the observation that MR is present in the lung (Keller-Wood *et al*, 2005) adds weight to this theory.

Insulin

Insulin is a metabolic hormone composed of 51 amino-acids and is released in response to increased levels of blood-glucose and stimulates the uptake of glucose. The inability of the body to produce or its tendency to become resistant to insulin results in diabetes mellitus (type 1 and 2 respectively). Hyperinsulinemia is a condition associated with type 2 diabetes whereby the levels of circulating insulin are increased and this condition is often associated with hypertension. A possible explanation for this is that insulin has been shown to increase Na^+ transport in renal epithelial by increasing the number of active channels in the apical membrane (Blazer-Yost *et al*, 1998; Blazer-Yost *et al*, 2004) and by increasing the open probability of channels (Staruschenko *et al*, 2007; Pochynyuk *et al*, 2007) in an amiloride sensitive fashion. This leads to increased fluid retention which results in raised blood pressure.

Possible mechanism of action of insulin

PI3K catalyses the formation of phosphatidylinositide second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3) from the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2). Insulin-induced Na^+ transport in A6 cells was shown to be mainly the result of increased channel density (Blazer-Yost *et al*, 1998). Using confocal fluorescence microscopy Blazer-Yost and colleges were able to show that ENaC and PI3K were co-localised and that insulin stimulation resulted in the translocation of this "complex" to the lateral membrane followed by translocation to the apical membrane in A6 cells. This was thought to be dependent upon PI3K as LY294002 prevented the translocation of the complex to the lateral membrane and inhibited Na^+ transport (Blazer-Yost *et al*, 2003). They speculated that the lack of formation of PI3K second messengers was the cause of this and later they were able to

show again using confocal fluorescence microscopy that the distribution of PIP_3 followed the translocation pathway of the ENaC-PI3K complex and therefore came up with a model that predicted that ENaC insertion into the membrane was a result of changes in lipid composition that favoured the insertion of ENaC channels (Blazer-Yost *et al*, 2004). Furthermore there is evidence that $PIP_{2/3}$ can alter channel kinetics (Staruschenko *et al*, 2007; Pochynyuk *et al*, 2007). It has been suggested they are able to augment ENaC channel activity through directly binding to ENaC subunits and modulating channel kinetics (Pochynyuk *et al*, 2007). It is likely that a combination of these mechanisms is responsible for insulin-induced Na^+ transport. However the effects are also very rapid and are more indicative of acute regulation of ENaC, furthermore these studies were carried out in renal epithelia. Moreover LY294002 was used to inhibit PI3K and this compound is now known to exert non-specific effects and its use as an effective PI3K inhibitor has been recommended to be discontinued (Bain *et al*, 2007). Nevertheless they do suggest a possible mechanism of action for insulin / PI3K mediated control of ENaC activity that is independent of SGK1 and therefore could explain why *sgk1* gene knockout mice do not display any overt lung phenotype.

Therefore a possible role for insulin / PI3K in the control of Na^+ transport in the lung is feasible and has received much less attention. It has been reported that insulin could augment the GC mediated increase in Na^+ transport seen in pulmonary epithelium however replacing external Na^+ with a nominally impermeant cation ($NMDG^+$) in insulin treated cells had no effect on membrane currents recorded from single H441 cells thus indicating that Na^+ transport was not involved. However lowering external Cl^- caused a hyperpolarising response and further investigation with a non-specific Cl^- channel blocker confirmed the response to insulin was a result of a Cl^- current rather than an increase in Na^+ transport (Brown *et al*, 2008). However this observation was

made from recordings of single H441 cells which have different electrical properties from that of H441 cells growing in contact with other cells (Brown *et al*, 2008). Furthermore insulin has been linked to Na^+ transport in the foetal lung (Hagiwara *et al*, 1992) and insulin does appear to improve gas diffusion in diabetic patients (Guazzi *et al*, 2002a; Guazzi *et al*, 2002b).

Closing remarks

Despite the extensive research into the hormonal regulation of ENaC and its involvement in lung fluid clearance and maintenance of the ASL / PLC, there are still many points of contention. One of the main areas of debate surrounds the role of SGK1 as, although there is clear evidence for a role in ENaC regulation, the observation that *sgk1*$^{-/-}$ mice are viable would strongly suggest otherwise, especially given that ENaC knockout and GR knockout results in death due to a failure to clear the lungs of fluid. Thus the role of SGK1 is not fully understood and requires further examination. Investigation into the molecular mechanisms that underlie GC regulated Na^+ transport has, in part, been hindered by the lack of specific pharmacological inhibitors of the kinases thought to be involved. However this situation has now changed with the development of specific inhibitors for PI3K, mTOR and SGK1, which are thought to be part of a kinase signalling pathway that is central to GC regulated Na^+ transport. By utilising these inhibitors it should be possible to selectively inhibit each kinase and, using an electrophysiological approach to determine what role these kinases play, if any, in GC regulated Na^+ transport in lung epithelia.

Aims/hypothesis

The aim of this thesis is to answer the question: is SGK1 required in the regulation of ENaC activity by glucocorticoids in pulmonary epithelium?

By using specific kinase inhibitors this should allow the determination of a number of other important questions, which are:

- Do glucocorticoids act via the PI3K – mTOR – SGK1 pathway?
- Can phosphatidylinositide second messengers maintain ENaC activity independently of SGK1?
- Can PKB maintain ENaC activity independently of SGK1?
- Is SGK1 activity dependent upon TORC2?

Thus the main hypothesis of this thesis is: SGK1 activity is vital to the hormonal control of ENaC activity via glucocorticoids in pulmonary epithelium.

Chapter 2 – Materials and Methods

The H441 cell model

All experiments were performed using the H441 human bronchiolar epithelial cell line (American Type Culture Collection). This cell line is derived from cells from a pericardial effusion from a male patient suffering a pulmonary papillary adenocarcinoma (Gazdar *et al*, 1990). These cells have been shown to display peripheral airway cell characteristics of bronchiolar Clara cell and exhibit the ability to form adherent monolayers that are suitable for cell culture (Gazdar *et al*, 1990). However Clara cells are not thought to play a significant role in fluid clearance or the maintenance of the ASL, despite this they are still a useful model to investigate the activation of ENaC by GC. There exists a large body of evidence that demonstrates the ability of the synthetic glucocorticoid hormone, dexamethasone, to induce a sodium (Na^+) absorbing phenotype in these cells that is consistent with the co-expression of α-, β- and γ-ENaC (Lazrak and Matalon, 2003; Ramminger *et al*, 2004; Clunes *et al*, 2004; Brown *et al*, 2008; Gallacher *et al*, 2009). H441 cells are therefore a useful model cell line in which to investigate the pathways that underlie hormonal control of the induction and maintenance of Na^+ transport in pulmonary epithelium.

I. H441 Cell Culture

Cells were kept in an incubator at 37 °C at 5% CO_2 and grown in 75cm^2 flasks (Greiner bio-one) until confluent in RPMI 1640 media supplemented with: 8.5% fetal bovine serum, 8.5% bovine calf serum (Gibco), 2mM glutamine, 5µg/ml transferrin, 5ng/ml selenium, 0.2% antibiotic (antimycotic solution, sigma).This media will be referred to as "complete media" throughout this thesis. When cells reached confluence they were detached using trypsin/EDTA (Gibco). A small aliquot of the cell suspension was mixed with trypan blue in a 1:1 ratio and a hemocytometer (Immune systems) was used

to calculate the number of cells per ml of solution. This allowed accurate seeding of cells so as to maintain the cell line and to seed appropriate densities of cells for experimental use.

For patch experiments cells were seeded out into 6 well cell culture plates (Corning Incorporated) containing 3-4 round glass cover slips and maintained in complete media in a humidified incubator at 5% CO_2 and 95% air at 37°C until cell attachment had occurred (~2-3 hours). The complete media was then replaced with a media where the foetal bovine serum and bovine calf serum were replaced with 8.5% dialysed foetal bovine serum with a molecular weight cut off of at 10 000kDa, in order to remove hormones and growth factors that may affect the pathways under investigation. Insulin was also added to this media (20nM). The dialysed media was supplemented with insulin as H441 cells were easier to maintain in culture with this hormone present, however it would appear, at least in single H441 cells, insulin induces a chloride conductance (Brown *et al*, 2008). Therefore future experiments should be undertaken with this in mind and should address these issues. Furthermore insulin is a potent activator of PI3K, therefore it would be interesting to remove this hormone from the dialysed media and examine the effects of this upon the results obtained in this thesis. In most cases dexamethasone (dex) was added to the dialysed media (0.2 μM). Cells were maintained for 18-48 hours in a humidified incubator at 5% CO_2 and 95% air at 37°C.

For Western blot experiments cells were seeded out into 6 well cell culture plates and maintained in complete media until ~80% confluence was reached. Complete media was then replaced with dialysed media and replaced back into the incubator overnight.

For 18-24 hour dex-treatment dex was added at this point. The following day appropriate hormones and / or inhibitors were added. Western blots were carried out using the Bio-Rad mini trans-blot cell system.

II. List of Reagents and solutions used for western blot

Resolving gel

373mM tris (pH8.8), 10% acrylamide/bis-acrylamide (Bio-Rad), 65.6mM ammonium persulphate (APS), 3.45mM SDS and 0.12% of N,N,N',N'-tetramethylethylenediamine (TEMED)

Stacking gel

123mM Tris-HCl (pH6.8), 4% acrylamide/bis-acrylamide, 65.6mM APS, 3.45mM SDS and 0.12% TEMED

Running Buffer

0.19M glycine, 24.7mM tris and 1.7mM SDS

Transfer buffer

0.19M glycine, 24.7mM tris and 20% methanol

Tris Buffered Saline (TBS)

20mM tris, 136mM NaCl and 0.1% tween can be added to give TBST

Blocking buffer

5% non fat milk in TBST

Stripping buffer

130mM glycine, 10mM tris, 2% tween, pH to 2.2 using concentrated HCl

Lysis buffer

10mM monopotassium phosphate, 1mM EDTA, 10mM $MgCl_2$, 50mM glycerophosphate, 5mM EGTA, 0.5% nondent P-40, 0.1% Brij 35, 1mM sodium orthovanadate. One protease inhibitor tablet was added per 25mls of lysis buffer.

Loading Dye

32 mM Tris HCl, 12.5% glycerol, 1% SDS, 0.05% Bromophenol Blue

TBS

10mM Tris, 150mM NaCl and 0.1% Tween-20 can be added to make TBS-T

Table 2.1 List of the primary antibodies used in Western experiments.

Primary Antibody	Source	Company	Dilution Factor	Molecular Weight (kDa)
NDRG1 - Phospho	Sheep	Cohen group	1:150	~ 46-48
NDRG1 - Total	Sheep	Cohen group	1.515	~ 46-48
Akt (PKB)	Rabbit	Cell Sig	1:1000	60
Phospho-PKB (Ser473)	Mouse	Cell Sig	1:1000	60
Phospho-PKB (Thr308)	Rabbit	Cell Sig	1:1000	60
PRAS40 – Total	Rabbit	Cell sig	1:1000	40
Phospho – PRAS40 (Thr246)	Rabbit	Cell sig	1:1000	40

III. Collection of protein samples for western blot

Media from experimental cells was removed and cells were washed 2 times in ice cold PBS. 150 µls of lysis buffer was added to the cells, and vigorous scraping was applied

to aid cell lysis. The resulting slurry was transferred into ice cold eppendorf tubes and was sonicated for 10 seconds and then centrifuged for 10 minutes at 14000 rpm at 4°C. The supernatant was transferred into fresh eppendorf tubes. Bradford assay was then performed to determine the protein content of each sample.

Bradford assay

Bradford protein assay was developed by Mariorn Bradford and allows for quick quantification of protein in a sample (Bradford, 1976). Coomassie Brilliant Blue G forms a complex with the proteins in a sample which causes a shift in the absorption maximum of the dye from 465 to 595nm, the amount of absorption is proportional to the protein present.

Protein standards were prepared from a 100 µg/ml stock of BSA dissolved in deionised H_2O at 0, 1.25, 2.5, 5 and 10 µg/ml. Each sample was diluted 100 fold in deionised H_2O. 180 µls of the protein standards and samples were mixed with 20 µls of Bradford reagent (Bio-rad, UK) and the amount of absorbance of each standard or sample solution was read at 595nm on a MRX microplate reader (Dynatech laboratories, UK). All standards and samples were carried out in duplicate. The absorbance values from the known standards were plotted in a line graph using Microsoft Excel. The protein concentrations of the unknown samples were calculated using the equation of the line ($y = mx + c$), where m is the gradient of the line and c is where the line intercepts the y-axis.

Polyacrylamide gel electrophoresis (PAGE)

Protein samples were fractionised using sodium dodecyl sulphate (SDS) -PAGE. Each gel was made fresh and contained an upper 4% acrylamide stacking gel and a lower 10% acrylamide resolving gel. Glass plates were clamped into the gel casting assembly

and the resolving gel was poured into the caster and butanol was overlaid and the gel left to polymerise for ~30minutes. The butanol is washed off and stacking gel is poured into the caster. Gel combs were added and the gels left to polymerise for ~30 minutes.

Separation and transfer of proteins

Gels casts were placed in the electrode tank and the inner and outer chambers were filled with running buffer. Samples were defrosted and warmed for 5 minutes at 60°C before being loaded into the lanes produced by the combs. A rainbow molecular weight marker (Bio-Rad, UK) and biotinylated molecular weight marker (New England Biolabs, UK) were also loaded. The proteins were fractionised at 200V for ~40 minutes until they had reached the bottom of the gel. Proteins were transferred onto nitrocellulose membrane, pore size 0.45 μm, by placing them in the transfer cassette and immersing them in transfer buffer and run at 100V for ~75 minutes.

Western blotting

Membranes were washed for 5 minutes in TBST and then blocked for 1 hour using the blocking buffer. Membranes were then incubated in primary antibody overnight on at roller at 4°C. Primary antibodies were diluted in blocking buffer to the appropriate concentration, see table 2.1. Membranes were then washed 3 times in TBST for 7 minutes per wash. Membranes were next incubated in HRP-conjugated secondary antibody diluted in blocking buffer for 1 hour on a roller at 4°C (for NDRG1$^{Thr346/356/366}$ blots secondary antibody was incubated at room temperature). Membranes were washed 3 times for 7 minutes per wash in TBST. Bands were then visualised by combining ECL solutions 1 and 2 in a 1:1 ratio and applied to the membrane. Each membrane was placed in a sealed plastic bag and exposed to x-ray film (Konica, UK) and developed using the Compact X4 developer (Xograph imaging systems, UK).

Stripping

Blots using antibodies for NDRG1$^{Thr346/356/366}$, PKB^{Ser473} and PKB^{Thr306} were stripped off the membrane using stripping buffer. After proteins were visualised the membranes were washed 2 times in TBST for 5 minutes per wash and then washed twice for 30 minutes per wash in stripping buffer. Membranes were then washed in phosphate buffered saline (PBS) for 10 minutes before being washed 3 times in TBST for 10 minutes per wash. Membranes were then blocked for 1 hour in blocking buffer and then re-probed using antibodies raised against the total protein for NDRG1 or PKB.

Inhibition of kinases

The role of phosphatidylinositol 3-kinase (PI3K), mammalian target of rapamycin (mTOR) and serum- and glucocorticoid-regulated Kinase 1 (SGK1) were investigated using specific inhibitors. For PI3K inhibition PI-103 (Merk Chemicals) was used at 0.5μM. Inhibition of mTOR was achieved using Torin1 at 0.1μM provided by the Sabatini lab (Cambridge, Massachusetts, USA). As both PI-103 and Torin1 inhibit both mTOR complex 1 and mTOR complex 2 (mTORC1 and mTORC2 respectively), a specific inhibitor of mTORC1, rapamycin (Sigma), was used at 0.1μM. SGK1 inhibition was achieved using GSK 650394 (Tocris Bioscience) at 10μM. Stock solutions were made of each inhibitor by dissolving in the appropriate volume of dimethyl sulphoxide (DMSO) which were then aliquoted and stored at -20°C.

After at least 18 hours exposure to dexamethasone, cells were pre-treated with a single inhibitor for three hours before patching. Only results obtained between 3-4 hours after pre-treatment with inhibitor were used. While every effort was made to treat only cells in which a successful recording had been made from dexamethasone-treated cell, the nature of the patch clamp technique did not always allow for this.

IV. Electrophysiology

Cells were continuously superfused with a "control" solution that mimics the conditions of the extracellular fluid (ECS) at a rate of approximately 3mls/min. ECS contained (mM): NaCl (140), $CaCl_2$ (2.5), KCl (4.5), Glucose (5), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 10) and $MgCl_2$ (1). The pH was then adjusted to 7.4 using NaOH giving a final Na^+ concentration of 144.4mM. The pipette filling solution contained: NaCl (10), KCl (18), K-gluconate (92), $MgCl_2$ (0.5), ethylene glycol tetraacetic acid (EGTA, 1), HEPES (10) and the pH adjusted to 7.2 with KOH bringing the final K^+ concentration to 113mM. Amphotericin-B (Sigma) was made up in dimethyl sulphoxide (DMSO) to 10mg/ml and then diluted to 40µg/ml in pipette filling solution. Amiloride hydrochloride hydrate (Sigma) was dissolved in water to give a stock of 10mM and was then diluted in ECS and the Low Na^+ solution to a final concentration of 10µM. Where cells had been pre-treated with an inhibitor, this was added to the superfusing solutions to the appropriate concentration. All experiments were carried out at room temperature.

Patch Clamp

The patch clamp technique is a method by which it is possible to record the activity of single or multiple ion channels depending upon the configuration used. The patch clamp technique involves bringing a glass fire polished microelectrode, filled with an appropriate filling solution (pipette filling solution), into close contact with a target cell. A gentle suction is applied to generate negative pressure in order to form a tight seal between the electrode and the cell membrane. This seal is often referred to as a giga-ohm (GΩ) seal as the electrical resistance between the cell and electrode is in excess of one GΩ. The formation of this seal is vital as the high electrical resistance means that

there will be very little current leak between the electrode and pipette therefore reducing any error as a result of current leak.

All patch clamp experiments in this thesis were measured using the perforated patch clamp technique. This involves the addition of an antibiotic to the pipette filling solution (amphotericin B) which perforates the membrane under the patch. This has the advantage over conventional whole cell recording as it allows control over the internal ionic concentrations of the cell without causing dialysis of the cell content. However the access resistance (R_a) tends to be higher using perforated patch clamp which will introduce an error into the recordings as voltage applied to the cell will drop further over the higher R_a. To overcome this problem the signal that is applied to the cell is compensated, and is known as series resistance compensation and will be discussed later in this chapter. The majority of recordings were obtained from groups of H441 cells containing approximately 3-6 cells. Data obtained from single H441 cells is clearly identified where appropriate.

Basic electrophysiological principles

The cell must be thought of as an electrical circuit and how ion channels, the membrane and ionic movement are represented equate to the properties of an electrical circuit are crucial to this understanding. Charge is a property that allows something to be affected by an electrical field, for example, ions in solution. Voltage is the separation of charge which produces an electric field, for example intracellular ions are separated by the cell membrane from ions in the ECS. Current is the movement of charge which therefore relates to the movement of ions. Resistance is the opposition to the flow of charge and since ion channels permit the flow of ions they therefore provide a resistance, as such ion channels can be thought of as resistors when thinking about the cell as a circuit. Capacitance is the ability to store charge and as ions cannot cross the cell membrane,

they accumulate on either side of the membrane and therefore act in the manner of a capacitor. Thus a model of a cell can be represented as a resistor-capacitor circuit (RC circuit) and is shown in figure 2.1.

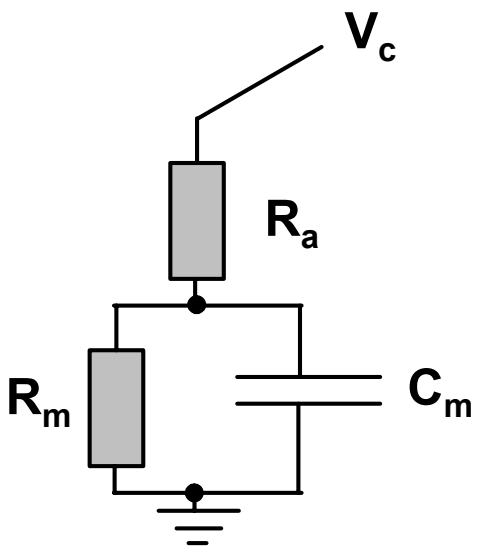


Figure 2.1 RC model of cell-attached patch. R_m is the membrane resistance and C_m is the membrane capacitance. The resistance provided by the remaining membrane in the patch, the pipette resistance, solution resistance and the resistance of the connecting circuitry is known as the access resistance (R_a). V_c represents the voltage command than can be sent by the experimenter to the cell. However as R_a and R_m are in series the V_c will drop across this resistance introducing a series resistance error.

Series resistance compensation

In patch clamp recordings the access resistance is in series with the membrane resistance (as seen in figure 2.1) therefore the voltage will drop over this. The result is

that when a voltage is applied to the cell, it will drop over the R_a and R_m and therefore the initial command will not be applied to the cell resulting in a voltage error in the recordings. In order to minimise this error, series resistance compensation was applied using the Axopatch 200B amplifier, which applies a compensated signal to the cell thus reducing the error. The average R_a across all experiments is 33.2 MΩ, if we take the lowest value for R_m which was ~0.5 GΩ then the average voltage drop would be ~6.5% of the initial V_c. However ~50% compensation was achieved therefore the average voltage drop would be ~3.25% of the original V_c and this was assumed to be negligible.

Liquid junction potential

A liquid junction potential (LJP) is the voltage that is generated by two solutions that come into contact with each other and arises due to flux of ions between the two solutions. The differences in the mobility and concentrations of ions between the solutions effects the potential generated. When the pipette comes into contact with the bath solution an LJP is generated, thus introducing a voltage error which needs to be corrected for. The Clampex software suit contains a programme for working out the LJP (JPCalc; Barry, 1994) and was found to be 12.6mV for solutions used in this thesis. All values reported for V_{hold} and V_{rev} have been corrected for this. A salt bridge filled with 3M KCl / 4% agar was used to ground the bath solution so as to reduce the effect of the solutions changes that take place during experiments on the reference electrode potential (Barry and Lynch, 1991)

Experimental design

Glass coverslips containing growing cells were placed into a perfusion bath of ~1ml volume that was mounted on a Nikon Diaphot inverted microscope. The perfusion system allowed the changing of solutions by superfusion using a pinch valve system

(Scientific Instruments ALA-VM8, ALA Scientific Instruments inc, USA). Borosilicate glass microelectrode pipettes (Clark Electromedical Instruments, UK) were fire polished to 1-1.8MΩ using a Flaming/Brown micropipette puller (P-97, Stutter Instruments co., USA). A GΩ seal was formed by bringing the glass microelectrode into close contact with the target cell using the Burleigh PCS-5000 micromanipulator (Burleigh, USA) and applying a gentle suction. Experiments were only performed after the formation of a stable GΩ seal and successful perforation of the membrane achieved. A successful perforation was determined by a fall in R_a to a stable value which was typically around 32 MΩ. However there was some degree of variability in this value.

Recording Protocol

Membrane currents (I_m) were recorded from cells held under voltage clamp in the perforated patch configuration. In all experiments a voltage ramp was applied (-113mV to +87mV, 2 s) which is repeated four times and the I_m associated with this is recorded and averaged. R_a and C_m were monitored throughout experiments and minor change applied as necessary, and all data is derived from experiments in which these parameters remained stable. Plots are constructed to show the relationship between V_{hold} and I_m. Downward deflections represent a depolarising inward current and upward deflections a hyperpolarising outward current. The reversal potential is the value of V_{hold} at which I_m is zero (V_{rev}). The amiloride sensitive current (I_{amil}) was established by recording under control conditions and then switching to the solution containing amiloride and ~30 seconds later a second recording was made. The current recorded in the presence of amiloride was digitally subtracted from the current recorded under control conditions in order to isolate the amiloride sensitive component of the total current. Conductance (G) was estimated by regression analysis of the I_m - V_{hold} plots.

Equilibrium Potentials

All equilibrium potentials quoted in this thesis were determined using the Nernst equation which is given as:

$$(1.1)\quad E_s = 2.303(RT/z_sF)\log_{10}([S]_o/[S]_i)$$

Where R is the gas constant (8.31 $JK^{-1}mol^{-1}$), T is the temperature in Kelvin (K), z_s is the valency of the ion (S), F is the Faraday constant ($96.5x10^3$ $Cmol^{-1}$) ans $[S]_o$ is the extracellular ion concentration and $[S]_i$ is the intracellular ion concentration. If we use Na^+ as an example under standard conditions in this thesis this gives:

$$(1.2)\quad E_{Na} = 2.303\,(((8.31\ JK^{-1}mol^{-1})(295.15))/1(96.5x10^3\ Cmol^{-1}))\log_{10}$$

$$(1.3)\quad ([144.4mM]_o/[10mM]_i)$$

$$(1.4)\quad E_{Na} = 58.5mV\ \log_{10}([144.4mM]_o/[10mM]_i)$$

$$(1.5)\quad E_{Na} = 67.8mV$$

All equilibrium potentials were worked out in a similar manner and for E_K was -82mV and E_{Cl} was -42mV. It is important to note that this equation is only for single ions.

Statistics

All data are presented as mean ± standard error of the mean (SEM) and values of n refer to the number of independent experiments. Statistical significance between recordings under ECS and ECS + amiloride was determined using Students paired *t*-test. Where applicable, data were analysed using one-way analysis of variance (ANOVA), i.e. in cases where more than two groups were compared, as multiple *t*-tests results in a large type two error which results in the false rejection of the null hypothesis. Bonferroni post-hoc test was performed, for example to determine the statistical significance of the effects of an inhibitor when compared to the control group.

Chapter 3 - Properties of H441 cells

Introduction

The regulated absorption of fluid in the respiratory system is vital for fluid clearance at birth and efficient functioning throughout adult life, which develops late in gestation and is dependent on an increase in circulating GCs (Baines *et al*, 2000). It is clear that GCs are important in the switch to a Na^+ absorbing phenotype and this is demonstrated as they are used clinically to treat RDS and pulmonary oedema. However, despite this and a large body of work, the mechanisms by which GCs act are still not fully understood (as discussed in the introduction).

The H441 human bronchiolar cell line has been used as a model to investigate the mechanism of action of GCs to induce Na^+ transport. Work on this cell line has shown that H441 cells display an amiloride sensitive apical G_{Na} in cells grown as confluent monolayers that is induced by dexamethasone (Sayegh *et al*, 1999; Itani *et al*, 2002; Lazrak and Matalon, 2003; Ramminger *et al*, 2004; Thomas *et al*, 2004; Albert *et al*, 2008). However differences exist between the studies such as the size of currents recorded: approx 7.5 (Sayegh), 10 (Itani) and 40 $\mu A/cm^2$ (Ramminger). However these differences were assumed to arise from slight differences in culture methodology. A more significant difference was observed as Lazark and Matalon, 2003 reported that H441 cells formed resistive monolayers and expressed basal amiloride sensitive Na^+ transport, the opposite reported in Ramminger *et al*, 2004. A relatively simple explanation for this discrepancy lies in the difference as to what both studies define as control conditions. A serum containing a poorly defined mix of hormones and growth factors was added to the control medium (Lazark and Matalon, 2003) whereas the other study used a serum that was dialysed to remove hormones and growth factors (Ramminger *et al*, 2004), therefore it is likely that the basal amiloride sensitive Na^+

transport was a result of activation by hormones/growth factors in the serum. A subsequent study from Ramminger supported this as patch clamp recordings from H441 cells only display an amiloride sensitive Na^+ conductance in the presence of dexamethasone (Clunes *et al*, 2004). It would also appear that cell contact is vital for the formation of a Na^+ selective, amiloride sensitive conductance as only nonselective Na^+ currents have been observed in single H441 cells (Brown *et al*, 2008; Gallacher *et al*, 2009). Thus it is clear that H441 cells have an endogenous amiloride sensitive Na^+ current that can be activated by dexamethasone and it would appear that this conductance can be acutely regulated by cAMP. Exposing cells to compounds that raise intracellular cAMP levels resulted in an increase in observed currents (Ramminger *et al*, 2004; Clunes *et al*, 2004) and increased both channel open probability and the number of active channels (Lazark and Matalon, 2003). Thus it would appear that H441 cells are a useful model to investigate the activation of Na^+ transport via GCs as they fit well with the model that GCs "prime" the lung for the switch to a Na^+ absorbing phenotype and that cAMP can acutely regulate the channel. However, as slight differences in culture conditions may affect channel properties and expression, it is therefore important to first characterise the dexamethasone induced increase in Na^+ transport even though the culture conditions are similar to that reported in previous studies (Clunes *et al*, 2004; Brown *et al*, 2008; Gallacher *et al*, 2009). It is important to note that the H441 cells were not grown in an air-liquid interface or on permeable supports which has also been reported to alter ion channel expression (Jain *et al*, 2001)

SGK1 activation requires the phosphorylation of two distinct residues in order to confer activity (Biondi *et al*, 2001), which is dependent on PI3K activity (Kobayashi and Cohen, 1999; Park *et al*, 1999; Biondi *et al*, 2001). However there is little work into the

activity of either SGK1 or PI3K in H441 cells in the response to GCs. Furthermore much of the evidence for the role of these kinases comes from studies in renal epithelia, therefore it is important to investigate this in pulmonary epithelia such as the H441 cell line. Studies that have looked to address this have transfected H441 cells with mutant forms of SGK1 and PI3K that are either constitutively active or catalytically inactive and because of this approach focus on single cells that only display a nonselective conductance (Brown *et al*, 2008; Inglis *et al*, 2009). As such the activity of SGK1 and PI3K has not been fully investigated in this cell line. NDRG1 is phosphorylated by SGK1 at $Thr^{346/356/366}$ and not by other related kinases such as PKB/S6K1 or RSK1 (Murray *et al*, 2004; Murray *et al*, 2005). Thus antibodies raised against phosphorylated NDRG1-$Thr^{346/356/366}$ can be used to monitor SGK1 activity (Murray *et al*, 2004) although this study was carried out in HeLa cells and *sgk1* knockout mice. This approach has since been used as an assay for SGK1 activity in mouse cortical collecting duct cells (Mansely and Wilson, 2010a) and in H441 cells (Inglis *et al*, 2009; McTavish *et al*, 2010). I therefore utilised this approach to further characterise the role of SGK1 in the response to GC treatment. A similar approach can be applied to assay for PI3K as the phosphorylation of PKB-Ser^{473} has been shown to be a reliable read out for PI3K activity (Bayascas and Alessi, 2005) and has been used to assay for PI3K activity in mouse cortical collecting duct cells (Mansely and Wilson, 2010a). Using these approaches I designed experiments to characterise the activity of both SGK1 and PI3K activity in H441 cells.

Results

The aim of this section is to establish if dexamethasone could induce an amiloride sensitive current in H441 cells and whether this is affected by cell-cell contact. To establish this patch clamp recordings were taken from single and groups of cells. In order to better understand the molecular mechanisms involved, Western blot analysis was used to determine PI3K and SGK1 activity via phosphorylation status of PKB-Ser^{473} and NDRG1-$Thr^{346/356/366}$ respectively.

Properties of single H441 cells

Figure 3.1 shows the I_m-V_{hold} relationship and the values of V_{rev} derived from these recording of single H441 cells superfused with the standard ECS control solution and ECS + amiloride (ECS + Amil., 10 μM). The C_m for single cells was 28.3 ± 3.2pF which is similar to that reported in Brown *et al*, 2008 and Gallacher *et al*, 2010. In the absence of dexamethasone, exposure to 10 μM amiloride had no effect on currents recorded and also no effect on the vales of V_{rev}. In the presence of dexamethasone (0.2 μM, 18-36 h), 10 μM amiloride had no effect on currents recorded or the values of V_{rev}. However the magnitude of the current at negative values of V_{hold} is larger. There is a slight difference in the values of V_{rev} between cells in the presence and absence of dexamethasone. However this was determined to be non significant (one-way ANOVA with Bonferroni post hoc).

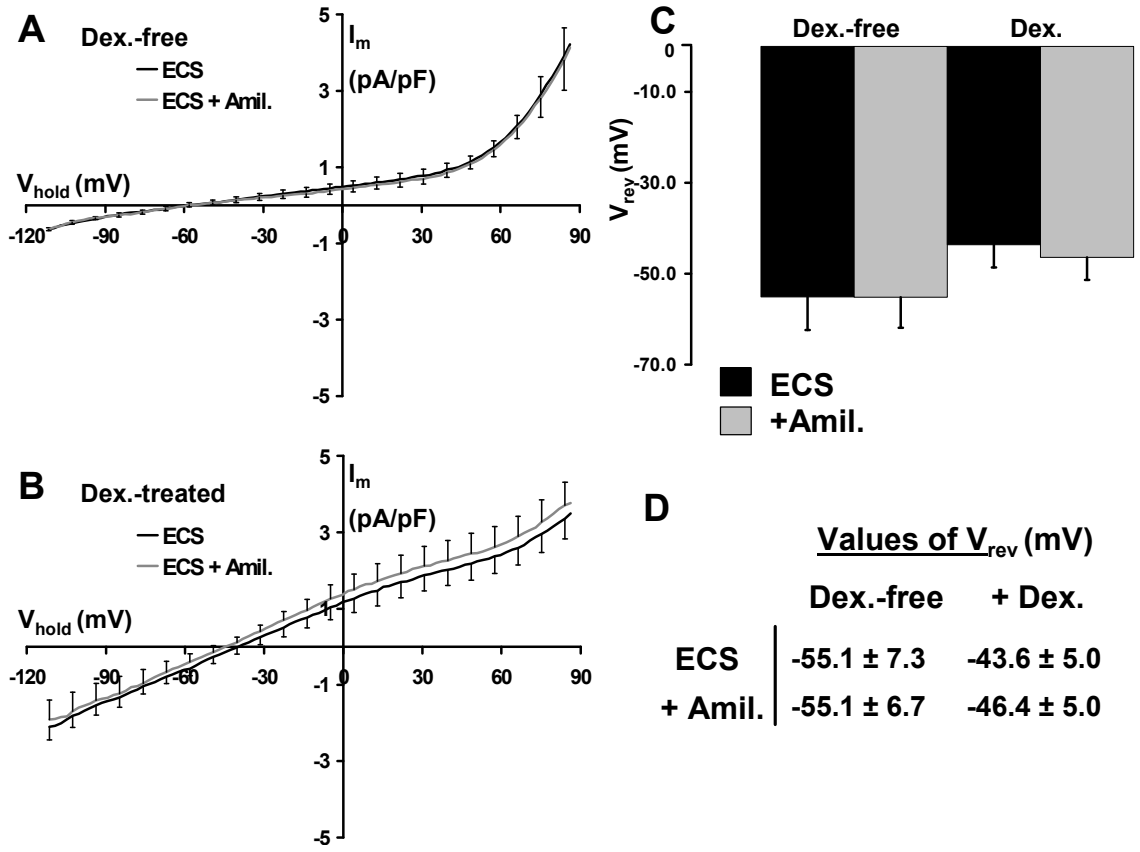


Fig. 3.1. Properties of single cells. (A) Relationship between membrane current (I_m) and holding potential (V_{hold}) quantified in glucocorticoid-deprived (n = 4, C_m = 27.5 ± 3.4 pF, R_a = 14.3 ± 1.0 MΩ) cells during exposure to the standard extracellular solution (ECS) and after ~30s exposure to 10 μM amiloride (+Amil.). (B) Equivalent data from dexamethasone-treated (0.2 μM, 18-36 h) cells (n = 6, C_m = 28.8 ± 5.2 pF, R_a = 25.8 ± 3.5 MΩ). (C) Values of V_{rev} derived by analysis of the data in A and B. Table (D) shows the values for V_{rev}.

Properties of groups of H441 cells

Figure 3.2 shows the I_m-V_{hold} relationship and the values of V_{rev} derived from these recordings in groups of H441 cells superfused with ECS and ECS + amiloride (ECS + Amil., 10 μM). The C_m for groups of cells was 43.9 ± 4.4 pF. In the absence of dexamethasone, exposure to 10 μM amiloride had no effect on currents recorded and also no effect on the vales of V_{rev}. In contrast to single cells, dexamethasone (0.2 μM, 18-36 h) caused depolarisation and subsequent exposure of 10 μM amiloride caused a hyperpolarising response and a reduction in the magnitude of the inward current flowing at negative values of V_{hold}.

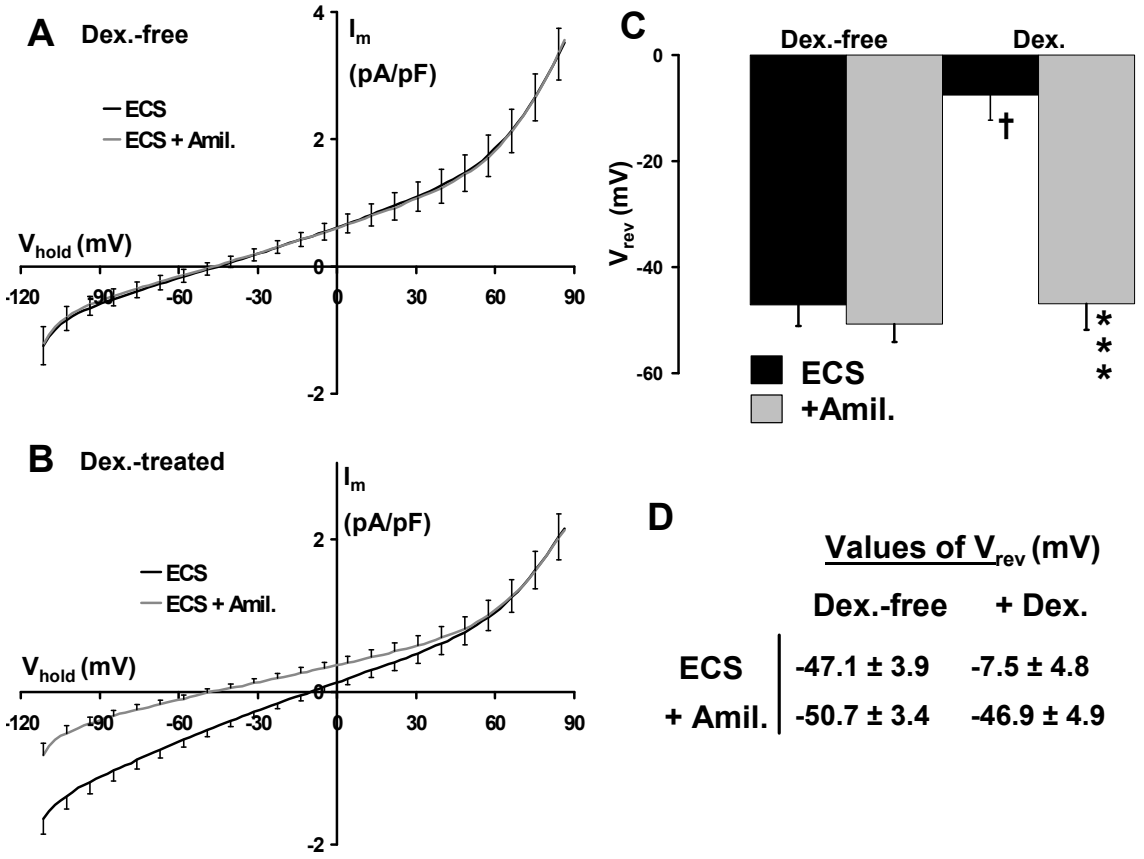


Figure. 3.2. Properties of groups of cells. (A) (n = 12, C_m = 41.0 ± 4.9 pF, R_a = 22.2 ± 4.1 MΩ) cells during exposure to ECS and after ~30s exposure to 10 μM amiloride (+Amil.). (B) Equivalent data from dexamethasone-treated (0.2 μM, 18-36 h) cells (n = 9, C_m = 47.6 ± 8.0 pF, R_a = 28.9 ± 3.1 MΩ). (C) Values of V_{rev} derived by analysis of the data in A and B; asterixes denote a statistically significant effect of amiloride (***, $P < 0.001$, Student's paired t-test), cross denotes a statistically significant effect of dexamethasone (†, $P < 0.05$, one-way ANOVA with Bonferroni post hoc test). Table (D) shows the values for V_{rev}.

Differences between groups and single cells

Figure 3.3 shows further analysis of the data in figure 3.1 and 3.2 to obtain the I_{amil} (see methods) and the current at -82mV (I_{-82}). In single cells there is negligible I_{amil} in dexamethasone-deprived and dexamethasone-treated cells. The I_{-82} in single dexamethasone-deprived cells is very low whereas the I_{-82} for dexamethasone-treated cells is larger although not amiloride sensitive. Thus dexamethasone causes an increase in the magnitude of the I_{-82}. Under dexamethasone-deprived conditions in single cells there is negligible I_{amil} in groups of cells however there is a clear I_{amil} in groups of cells that have been treated with dexamethasone. Furthermore this reverses close to the predicted value for E_{Na} (+67.8 mV) indicating a high degree of Na^+ selectivity. Dexamethasone causes an increase in the magnitude of the I_{-82} compared to dexamethasone deprived cells and in contrast to single cells this is amiloride sensitive.

Table 3.1 values of I_{-82} derived from data in figure 3.1 (single cells)

	Dex.-Free (pA/cell)	**Dex.-treated (pA/cell)**
ECS	-7.6 ± 1.7	-42.7 ± 8.2
+ Amil.	-8.2 ± 2.0	-39.1 ± 14.2

Table 3.2 values of I_{-82} derived from data in figure 3.2 (groups of cells)

	Dex.-Free (pA/cell)	**Dex.-treated (pA/cell)**
ECS	-16.5 ± 5.6	-34.3 ± 4.7
+ Amil.	-14.8 ± 5.2	-9.9 ± 2.4

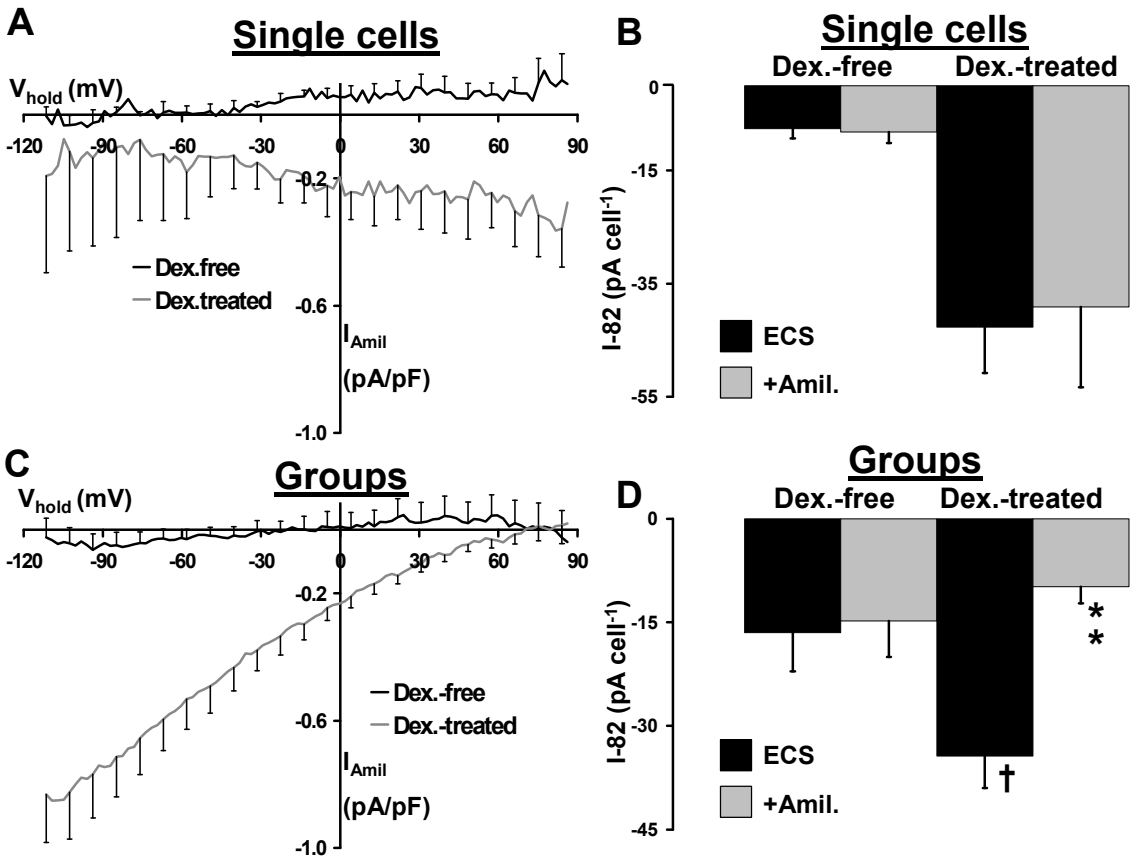


Figure. 3.3. Differences between groups and single cells. (A) Analysis of the amiloride sensitive component (I_{amil}) of the total membrane current for single cells derived from data shown in figure 3.1. A and B (n = 4, dex.-free; n = 6 dex.-treated). (B) Current flowing at -82mV derived from data in figure 3.1. A and B. Effect of dexamethasone was not found to be significant however it did approach significance (P = 0.054, one-way ANOVA with Bonferroni post hoc test) (C) Analysis of I_{amil} of total membrane current for cells in groups derived from data shown in figure 3.2 A and B (n = 12, dex.-free; n = 9 dex.-treated). (D) Current flowing at -82mV, derived from data in figure 3.2. A and B; asterixes denote a statistically significant effect of amiloride (**, P < 0.01, Student's paired t-test). Cross denotes a statistically significant effect of dexamethasone (†, P < 0.05, one-way ANOVA with Bonferroni post hoc test)

Conductance in groups of H441 cells

As the aim of this work was to investigate ENaC regulation the data from groups of H441 cells was further analysed in order to determine the conductance. Figure 3.4 shows the conductance derived from data in figure 3.2. Dexamethasone (18-36 h, 0.2 μM) clearly increases the conductance in H441 cells and this is reduced by exposure to 10 μM amiloride. Dexamethasone free cells have a similar conductance to that of dexamethasone-treated cells that have been exposed to amiloride thus indicating that the increase in conductance is due to amiloride sensitive channels.

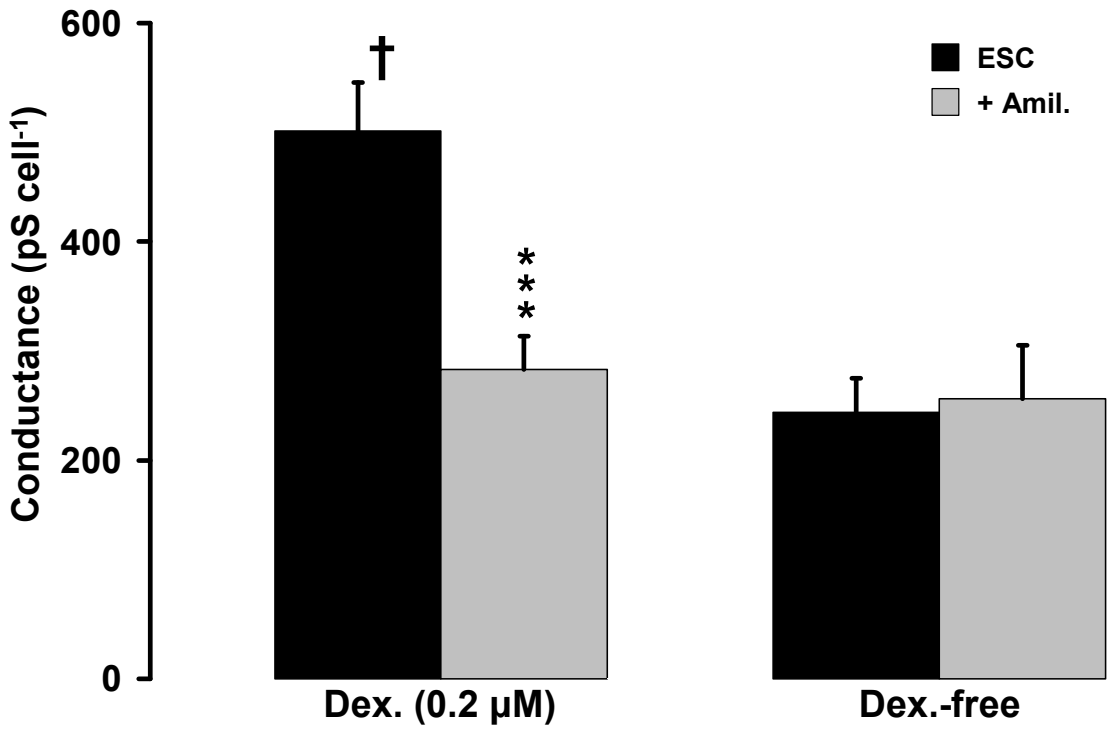


Figure 3.4. Conductance derived from dex.-treated (n = 9) and dex.-free (n = 12) cells. Asterixes denote a statistically significant effect of amiloride (***, $P < 0.001$, Student's paired t-test), cross denotes a statistically significant effect of dexamethasone (†, $P < 0.001$, one-way ANOVA with Bonferroni post hoc test)

Pooled I_{amil} data

All results from cells exposed to dexamethasone (0.2 μM 18-36 h) were pooled and the I_{amil} plotted. The purpose of this was to confirm that dexamethasone caused the induction of a highly Na^+ selective current. Figure 3.5 shows the pooled data of the I_{amil} from all cells exposed to dexamethasone (0.2 μM 18-36 h) used in this thesis and the C_m was 53.8 ± 4.1 pF. The reversal potential is 66.7 ± 3.1 mV which is extremely close to the predicted value of E_{Na} which is 67.8 mV indicating a very high degree of Na^+ selectivity.

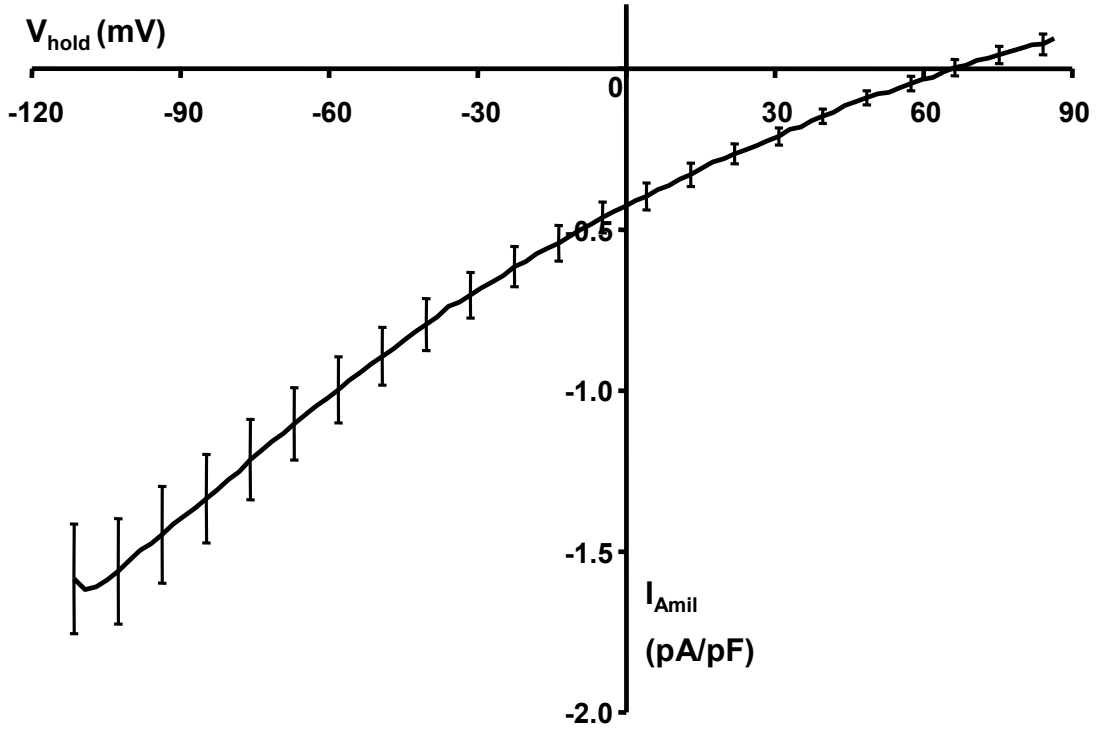


Figure 3.5 Pooled I_{amil} data for all control dex-treated recordings. Pooled data of I_{amil} of the total membrane current for all dex-treated cells (0.2 μM, 18-36 h) recorded for this thesis (n = 31)

Does dexamethasone treatment alter the activity of PI3K and SGK1?

Western blot analysis was carried out in order to determine the effects of dexamethasone treatment upon the activity of PI3K and SGK1. Figure 3.6 shows the effects of dexamethasone-treatment on SGK1 activity as monitored by NDRG1-Thr$^{346/356/366}$ phosphorylation and PI3K activity as monitored by PKB-Ser^{473} phosphorylation both acutely (0.2 μM, 0-6 h) and in cells that have been exposed to dexamethasone for 24 hours. Acute dexamethasone-treatment increases the abundance of NDRG1-Thr$^{346/356/366}$ phosphorylation without affecting the total abundance of NDRG1. This increase is clearly apparent after 1 hour dexamethasone-treatment and persists for 6 hours although a decline is apparent by this time point. Dexamethasone has no marked effect upon PKB-Ser^{473} phosphorylation or total PKB. As patch clamp experiments are routinely carried out after 18-36 hours dexamethasone treatment, the phosphorylation status of both NDRG1 and PKB were examined after 24 hours exposure to dexamethasone.

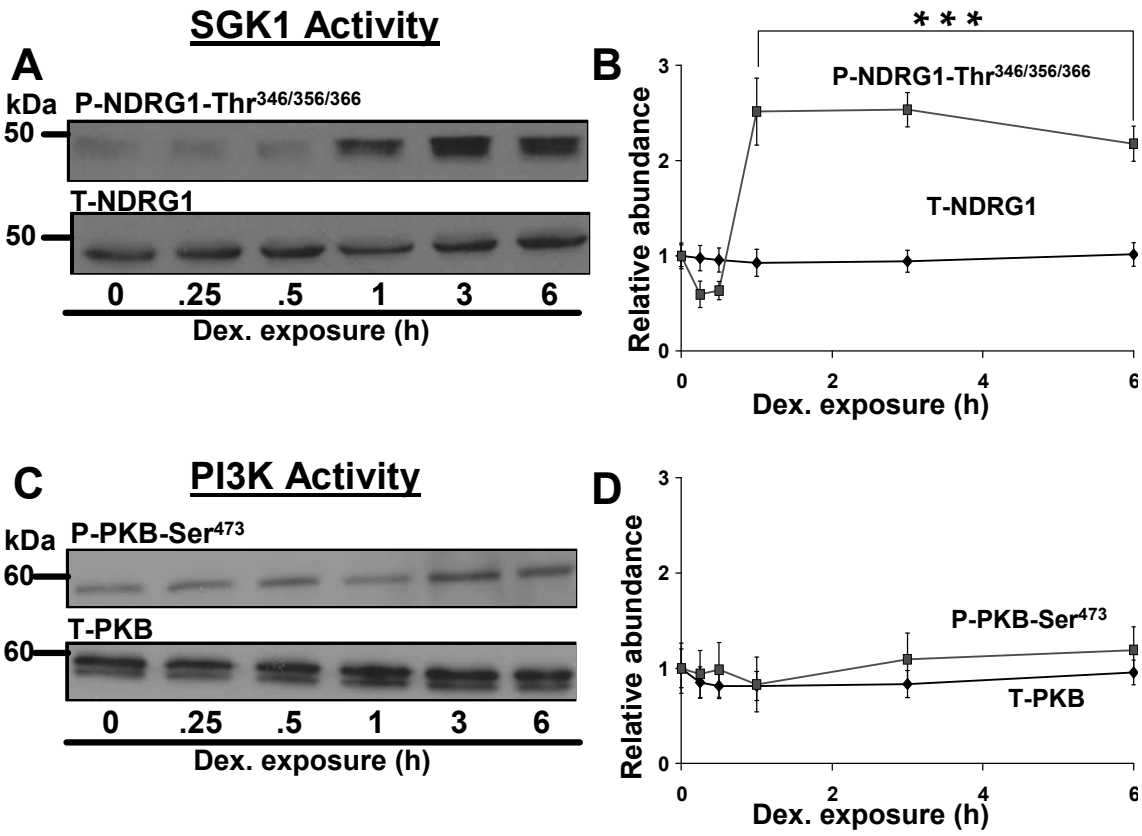


Figure. 3.6 Endogenous protein phosphorylation in dex-treated cells. (A) Typical western blot showing the effects of dex-treatment (0.2 μM, 0-6 h) on the abundance of NDRG1-Thr$^{346/356/366}$ phosphorylation and total NDRG1. (B) Densometric analysis of pooled data from 6 experiments. (C) Typical western blot showing the effects of dex-treatment (0.2 μM, 0-6 h) on the abundance of PKB-Ser^{473} phosphorylation and total PKB. (D) Densometric analysis of pooled data from 6 experiments. Asterixes denote a statistically significant effect of dexamethasone ($P < 0.001$, one-way ANOVA)

Acute vs. 24 hour dex treatment upon PI3K and SGK1 activity

As patch clamp experiments were undertaken after ~24 hours exposure to dexamethasone a series of experiments were designed to examine the effects of this treatment upon activity of PI3K and SGK1. A 3 hour time point was chosen as a positive control as this typically gave the largest response. Figure 3.7 confirmed that acute dexamethasone-treatment (3 h) increased the abundance of NDRG1-Thr$^{346/356/366}$ phosphorylation, however after 24 hours exposure to dexamethasone NDRG1-Thr$^{346/356/366}$ phosphorylation was essentially identical to that of those measured in dexamethasone-deprived cells. Dexamethasone exposure had no effect upon -Ser^{473} phosphorylation either acutely or after 24 hours exposure.

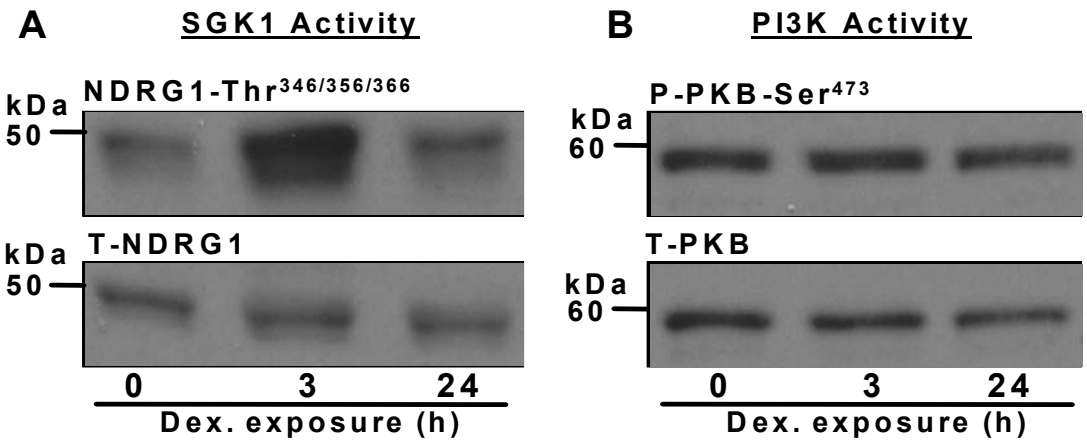


Figure. 3.7 Effect of dex.-treatment upon endogenous protein phosphorylation. Typical western blot showing the effect of short term (3 h) and long term (24 h) dex treatment upon the cellular abundance of (A) NDRG1-Thr$^{346/356/366}$ phosphorylation and total NDRG1 and (B) PKB-Ser^{473} and total PKB; essentially identical data was obtained from 4 experiments.

Does acute increase in Dex-evoked SGK1 activity induce ENaC activity

Figure 3.8 shows the I_m-V_{hold} relationship and the values of V_{rev} from dexamethasone-deprived cells, dexamethasone-treated cells (0.2 μM, 18-36 h) and acute exposure to dexamethasone (0.2 μM, 3-4 h).The C_m for all recordings was 42.4 ± 2.8 pF. There was no effect upon exposure to 10 μM amiloride in dexamethasone-deprived cells or the values of V_{rev}. 18-36 hours exposure to dexamethasone resulted in a depolarisation that was reversed by 10 μM amiloride. Three hour dexamethasone exposure did not result in depolarisation and there was no effect upon exposure to 10 μM amiloride. The values of V_{rev} were similar to those of dexamethasone-deprived cells.

Table 3.3 values of V_{rev} derived from data in figure 3.8

	Dex.-Free (mV)	Dex. (3 h) (mV)	Dex. (18-36 h) (mV)
ECS	-64.4 ± 4.2	-60.8 ± 7.2	-7.9 ± 7.3
+ Amil.	-64.6 ± 3.1	-61.3 ± 7.2	-65.8 ± 8.3

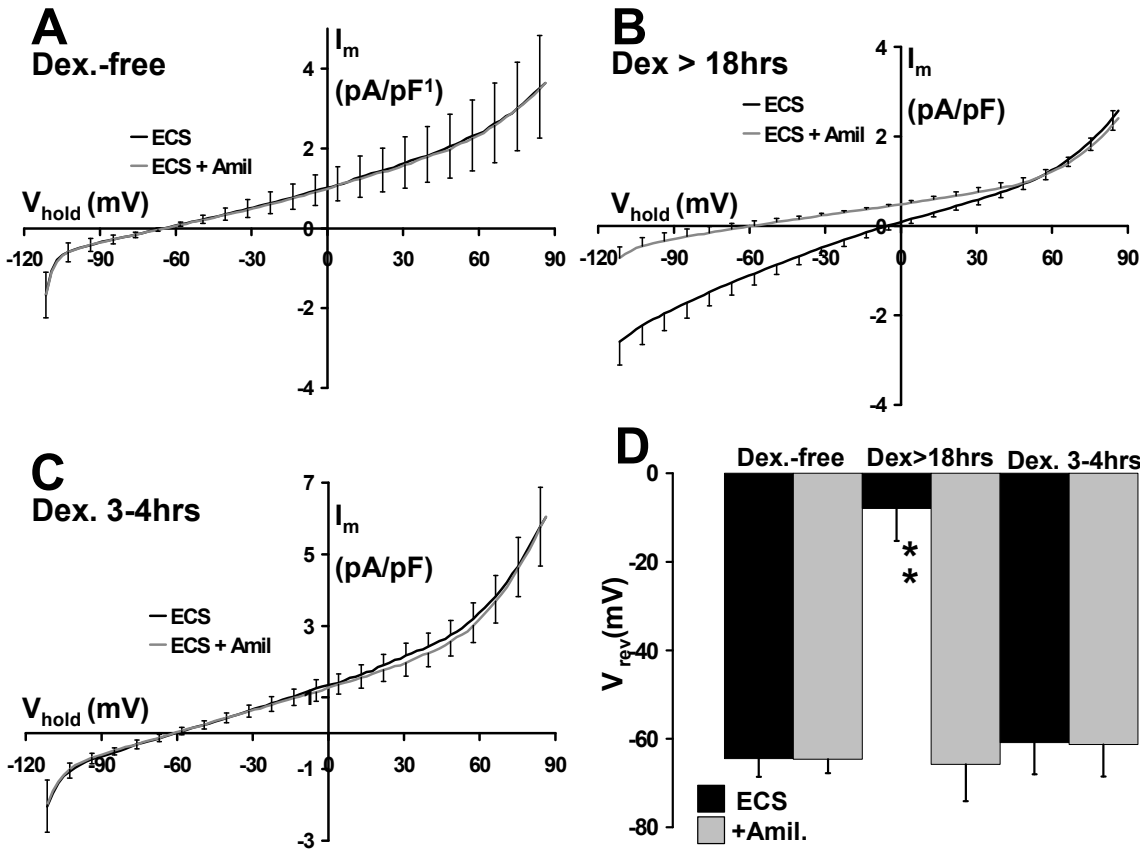


Figure 3.8. Effect of 3 hour vs. 18-36 hour dex-treatment. (A) Relationship between membrane current (I_m) and holding potential (V_{hold}) quantified in glucocorticoid-deprived cells (n = 3, R_a = 30.7 ± 9.1 MΩ). (B) Equivalent data from dex-treated (0.2 μM, 18-36 h) cells (n = 5, R_a = 19.3 ± 2.1 MΩ). (C) Equivalent data from dex-treated (0.2 μM, 3-4 h) cells n = 5, R_a = 30.7 ± 4.8 MΩ). (D) Values of V_{rev} derived from data in A, B and C; asterixes denote a statistically significant effect of amiloride (**, P < 0.01, Student's paired t-test).

Acute dexamethasone treatment does not evoke ENaC activity

Figure 3.9 shows further analysis of the data in figure 3.8 to obtain the I_{amil} and the I_{-82}. There is no I_{amil} in dexamethasone-deprived or acute exposure to dexamethasone. Conversely in cells exposed to dexamethasone for 18-36 hours there is a clear I_{amil} that reverses at 49.9 ± 4.5 mV which is close to E_{Na} indicating a high degree of Na^+ selectivity. Analysis of the I_{-82} shows that dexamethasone-deprived and acute exposure to dex have little effect on the current whereas longer exposure to dexamethasone causes a clear rise of the I_{-82} which is reversed by amiloride.

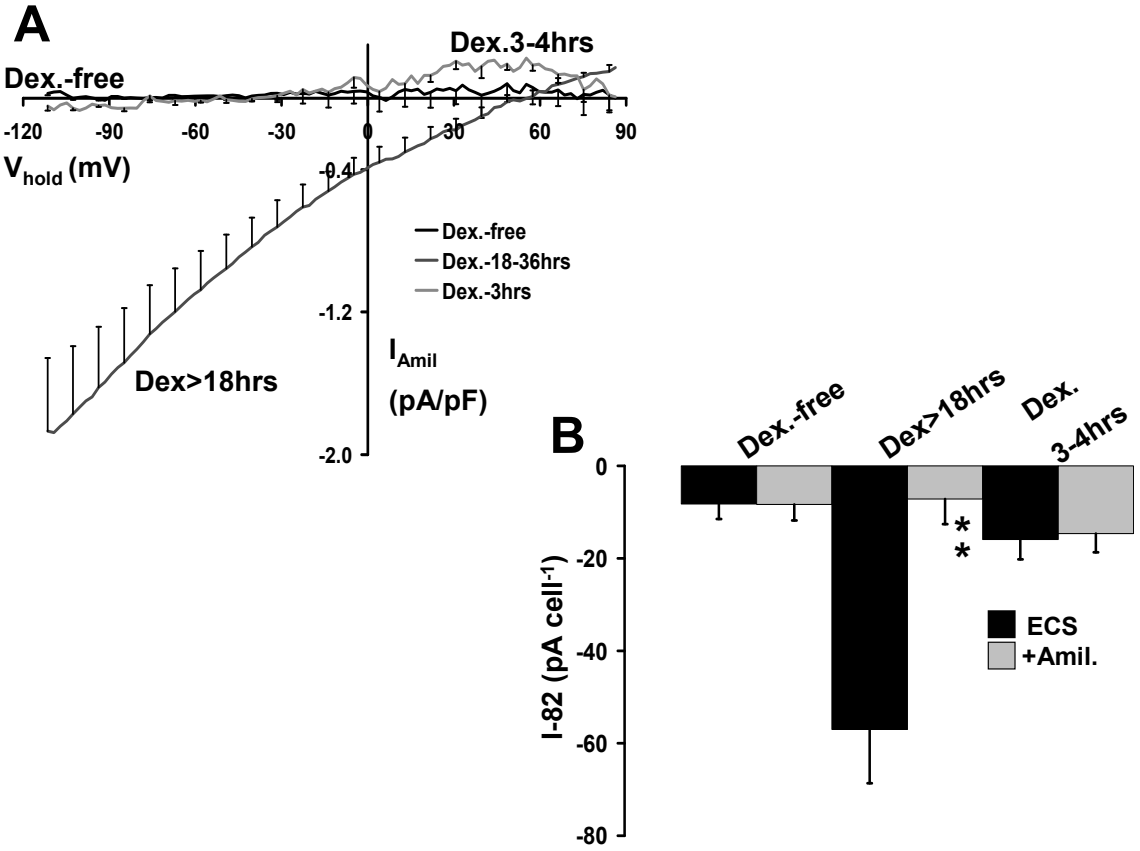


Fig. 3.9. I_{amil} of 3 hour vs. 18-36 hour dex-treatment. (A) Analysis of the I_{amil} of the total membrane current for cells derived from data shown in fig. 4.5. A, B and C. (B) Current flowing at -82mV derived from data in fig. 4.5. A, B and C; asterixes denote a statistically significant effect of amiloride (**, $P < 0.01$, Student's paired t-test).

Discussion

The data presented in this chapter confirms that dexamethasone induces an ENaC like current in H441 cells, however it also demonstrates that SGK1 activity does not correlate with this ENaC activity. Furthermore PI3K activity is not affected by dexamethasone treatment and as this is reported to be required for SGK1 activity then this data questions the role that both SGK1 and PI3K play in the induction and maintenance of GC induced ENaC activity.

Glucocorticoid-induced current in H441 cells

The data clearly demonstrates that single H441 cells do not display an I_{amil}, however dexamethasone does cause a slight depolarisation in the values of V_{rev} (~10 mV) and also causes an increase in the magnitude of the currents recorded and this is in agreement with previous studies of single H441 cells (Brown *et al*, 2008; Gallacher *et al*, 2009). In these studies currents were recorded under an additional experimental solution where the external concentration of Na^+ was lowered from 144.4 mM to 10 mM by replacing Na^+ with the nominally impermeant cation N-methyl-D-glucammonium, which resulted in a hyperpolarising response thereby indicating that the depolarisation was a result of Na^+ transport. Furthermore the currents recorded from single H441 cells were shown to be sensitive to lanthanum (Gallacher *et al*, 2009), which is a well known blocker of nonselective cation channels. Therefore due to the similarity between the data presented in this thesis and the data from Brown *et al*, 2008 and Gallacher *et al*, 2009 it would seem highly likely that dexamethasone treatment causes the induction of a nonselective cation conductance. The expression of nonselective and selective sodium currents have been previously observed in H441 cells grown as monolayers (Shlyonsky *et al*, 2005; Albert *et al*, 2008), A549 cells and

isolated rat ATII cells (Ito *et al,* 1997; Marunaka *et al*, 1999; Jain *et al* 2001). Interestingly one of these studies has reported an effect of cell-cell contact in H441 cells on the expression of nonselective currents. Amiloride sensitivity was not observed in low resistance monolayers but was seen in high resistance monolayers thus indicating that the degree of "tightness" or the formation of tight junctions influences the biophysical properties of H441 cells (Shlyonsky *et al*, 2005). While electrophysiological experiments were carried out under subconfluent conditions, groups of H441 cells clearly display an I_{amil} that reverses close to E_{Na} therefore indicating a high degree of Na^+ selectivity that is only present with dexamethasone-treatment. The expression of a highly selective Na^+ current is associated with the expression of α-ENaC, β-ENaC and γ-ENaC subunits (Canessa *et al* 1994; Kosari *et al*, 1998; Anantharam and Palmer 2007) and as such the results here confirm previous observations that dexamethasone exposure results in an ENaC like conductance in H441 cells that is dependent on cell-cell contact (Clunes *et al*, 2004; Brown *et al*, 2008; Gallacher *et al*, 2009).

The observation that dexamethasone increases the conductance in H441 cells in an amiloride sensitive fashion is further evidence of ENaC like activity. However there is an underlying conductance which is likely attributed to Cl^- transport. Brown *et al* (2010) reported that the V_{rev} in hormone-deprived single H441 cells was -80mV which was almost identical to E_K (-82 mV) and that lowering either $[Na^+]_o$ or $[Cl^-]_o$ had no effect upon this. However raising $[K^+]_o$ resulted in a depolarising response therefore they concluded that the dominant ionic conductance in hormone-deprived cells is K^+ transport. However they also found that in single insulin-treated H441 cells that the V_{rev} was -60mV and this difference was the result of a Cl^- conductance. As the V_{rev} of single and groups of dexamethasone-free cells reported in this thesis is almost identical

to that reported by Brown *et al* (2010), it is likely that there is a Cl^- conductance present, as V_{rev} has shifted towards E_{Cl} (-42 mV) and this is the likely cause of the conductance in dexamethasone-free cells and the amiloride insensitive conductance.

The role of SGK1 and PI3K

Acute dexamethasone treatment clearly causes an increase in the phosphorylation of NDRG1-$The^{346/356/366}$ thereby indicating increased activity of SGK1. This result was expected as SGK1 is known to be activated by GCs (Webster *et al*, 1993; Lang *et al*, 2009). Increased activity could be seen after one hour dexamethasone exposure and remained elevated for at least six hours although a slight decline was evident at this time point and this result accords well with that reported in Inglis *et al*, 2009. SGK1 activity is dependent on PI3K-TORC2 phosphorylation of its hydrophobic motif (Ser^{422}) and as such PI3K activity was hypothesised to increase with dexamethasone exposure. However this is clearly not the case as there is no discernible affect on PKB-Ser^{473} phosphorylation thus indicating that PI3K activity is not increased in dexamethasone-treated cells. Thus it would appear that the role of PI3K is a permissive one and this has been reported in kidney epithelium where dexamethasone activated SGK1 without affecting PI3K or TORC2 activity (Mansley and Wilson, 2010b). While these results indicate the activity of SGK1, they cannot determine the abundance of SGK1 and it is therefore important to note that dexamethasone has been shown to increase the abundance of SGK1 (Wang *et al*, 2001; Gonzalez-Rodriguez *et al*, 2007), thus it is possible that increased activity of SGK1 is a result of increasing levels of SGK1 and that PI3K-TORC2 confer catalytic activity and therefore does not require further activation of PI3K.

It was observed that after 24 hour treatment SGK1 activity had returned to the levels observed in dexamethasone-deprived cells. This result was unexpected as by this time

there is clear activation of the amiloride sensitive Na^+ current. This indicates that increased activity of SGK1 is not required to maintain the amiloride sensitive Na^+ current, rather, it may act as a type of biological "switch" whereby a transient increase in activity of SGK1is permissive for the cellular response to dexamethasone. This chronic decrease in SGK1 activity was documented in Inglis *et al*, 2009, where SGK1 activity was actually observed to fall below that seen in the control cells. This discrepancy may be explained as insulin was not present in the media used in this study thereby possibly lowering the activity of PI3K and therefore lowering SGK1 activity. This also brings into question the role that SGK1 plays in the activation of the amiloride sensitive Na^+ current in lung epithelium.

Acute dexamethasone-treatment upon the electrophysiological properties

As SGK1 activity is highest after three hours treatment with dexamethasone, we explored where or not there was activation of the amiloride sensitive Na^+ current at this time point. However the data clearly shows (see fig 3.8 and 3.9) that dexamethasone has no effect on the electrophysiological properties of H441 cells after three hours. This brings into question the role that SGK1 plays in the maintenance and induction of Na^+ transport in lung epithelium, a view that is supported by the lack of any functional abnormalities in *sgk1* gene knockout mice (Wulff *et al*, 2002; Grahammer *et al*, 2006; Rexhepaj *et al*, 2006; Fejes-Toth *et al*, 2008) and suggests some redundancy in the SGK1 mediated control of ENaC.

Conclusions

These findings support previous studies that show GCs can induce Na^+ currents in H441 cells and that cell-cell contact plays a vital role in the biophysical properties of the cell.

Most importantly they demonstrate that H441 cells display an inducible "ENaC like" Na^+ conductance in response to GCs and are thus is in agreement with previous studies that this cell line is a useful model in which to investigate the molecular mechanism underlying ENaC activity in lung epithelium. While it is reported that PI3K is vital to confer catalytic activity of SGK1, dexamethasone does not appear to affect the activity of this kinase therefore suggesting its role is permissive. Furthermore the increased SGK1 activity in response to dexamethasone is not in accordance with an increase in Na^+ transport as there appears to be a time discrepancy between the two. This result brings into question the role of SGK1 in the induction and maintenance of Na^+ transport in H441 cells thus suggesting that dexamethasone may act via alternative signalling pathways. In order to further explore the role of PI3K and SGK1 we designed experiments using novel specific inhibitors to examine the effects of PI3K/TORC2 and SGK1 inhibition on the response to dexamethasone. The results of these experiments are discussed in chapters five and six.

Chapter 4 – The role of PI3K

Introduction

The role that SGK1 plays in the induction and maintenance of the Na^+ absorbing phenotype of lung epithelium is unclear as there is no discernible lung phenotype in *sgk1* gene knockout mice, both histology and function were unaffected (Wulff *et al*, 2002). Whereas it was expected that *sgk1* knockout would result in a failure to clear lung fluid at birth. The results presented in chapter four of this thesis further question the role of SGK1, as when activity is highest, there is no ENaC activity (see figure 3.7 and 3.8). However there is evidence that PI3K may work independently of SGK1 to induce Na^+ transport. For example it has been shown that PKB isoforms can mediate the effect of insulin on ENaC in rat thyroid cells (Lee *et al*, 2007). This study concluded that both SGK1 and PKB activity were required to maintain basal levels of Na^+ transport as siRNA directed against PKB and SGK1 reduced amiloride sensitive Na^+ transport, although there was a more pronounced effect when siRNA directed against both kinases were transfected together (Lee *et al*, 2007). Nevertheless this does provide evidence of SGK1 independent control of Na^+ transport. Furthermore PI3K may affect ENaC through its second messengers PIP_2 and PIP_3 which have been shown to be involved in ENaC trafficking (Blazer-Yost *et al*, 2004) and to affect channel gating kinetics (Pochynyuk *et al*, 2007). However this study found that while acute increases in PIP_2 could increase channel open probability, chronic exposure had no effect, leading to the conclusion that PIP_2 is required to confer constitutive channel activity that can be altered via other modulators (Pochynyuk *et al*, 2007). Thus PI3K activity could be able to confer ENaC activity independently of SGK1 via this mechanism. However, as cells were maintained in media containing corticosteroids, this possibility remains experimentally unverified.

Many studies have used pharmacological inhibitors of PI3K such as LY294002 and wortmannin, however these inhibitors were shown to inhibit mTOR at concentrations similar to those used to inhibit PI3K and they do so by inactivating the PI3K p85-p110 catalytic domain (Brunn *et al*, 1996). However both compounds act in a different manner: LY294002 acts as a competitive inhibitor of ATP and is reversible (Vlahos *et al*, 1994), whereas wortmannin acts as a non competitive inhibitor (Okada et al, 1994) by covalently modifying a lysine residue in the p110 catalytic domain (Wymann *et al*, 1996). Furthermore a study into protein kinase inhibitors confirmed that both wortmannin and LY294002 have non-specific effects (Bain *et al*, 2007). Although wortmannin was found to have fewer non-specific effects than LY294002, it is unstable in aqueous solution and therefore its use is limited. Both LY294002 and wortmannin inhibit other kinases aside from PI3K that have been implicated in ENaC regulation. Casein kinase 2 has been reported to be involved with ENaC regulation by inhibiting the Nedd4-2 pathway (Shi *et al*, 2002; Bachhuber *et al*, 2008). Wortmannin was also shown to inhibit smooth muscle myosin light chain kinase, which may contribute to ENaC trafficking (Tokuda *et al*, 2002). Thus care should be taken when interpreting results obtained from the use of these compounds. A study in 2006 showed that a novel PI3K inhibitor, PI-103, displayed a much more selective inhibition of PI3K and at lower concentrations than LY294002; however they also found that it inhibited mTOR (Fan *et al*, 2006). Bain *et al*, 2007 found that PI-103 did not inhibit either casein kinase 2 or smooth muscle myosin light chain kinase or other members of the PI3K superfamily and therefore suggest that use of LY294002 be discontinued and PI-103 used as its replacement. However as PI-103 also inhibits mTORC1 the highly specific mTORC1 inhibitor rapamycin should be used in parallel to make sure the effects of PI-103 are due to PI3K, and not mTORC1, inhibition (Bain *et al*, 2007).

There are studies that have explored the role of PI3K in Na^+ transport in H441 cells (Brown *et al*, 2008; Gallagher *et al*, 2009; McTavish *et al*, 2009, Inglis *et al*, 2009). Transfection of a constitutively active PI3K subunit (CD2-p110α) did not induce Na^+ transport in hormone deprived cells but did augment the response to dexamethasone thus suggesting that PI3K activity alone is not sufficient to induce Na^+ transport in single H441 cells (Brown *et al*, 2008; Gallagher *et al*, 2009). Nevertheless PI3K activity was thought to be important as LY294002 completely abolished Na^+ transport in single H441 cells (Gallagher *et al*, 2009; Inglis *et al*, 2009). Furthermore it would appear that PI-103, a more specific and potent inhibitor of PI3K (Fan *et al*, 2006; Bain *et al*, 2007), does not cause complete inactivation of SGK1 activity as monitored by NDRG1 phosphorylation (McTavish *et al*, 2009) thus suggesting that SGK1 activity may not be fully dependent on PI3K. However as the electrophysiological properties of the cells were not investigated in this study, the effect of PI3K inhibition on Na^+ transport could not be determined.

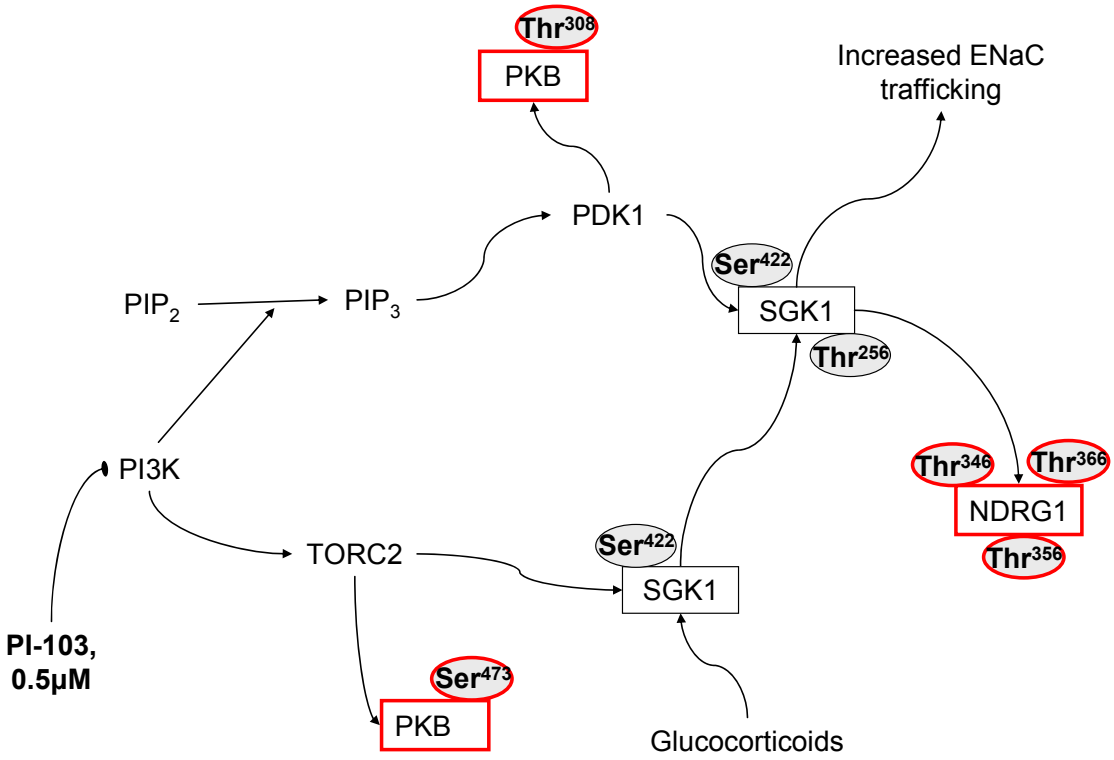


Figure 4.1 Diagram showing predicted pathway of Glucocorticoid ENaC regulation. Phosphorylation of PKB at the Ser^{473} residue can be used to assay for TORC2 activity while phosphorylation at the Thr^{308} residue can be used to assay for PDK1 activity. As both these kinases are PI3K dependent then phosphorylation of these residues indicates PI3K activity. Similarly NDRG1 phosphorylation can be used to assay for SGK1 activity.

PI3K is thought to activate SGK1 through a signalling pathway involving TORC2 (Garcia-Martinez and Alessi, 2008; Lu *et al*, 2010); however it has also been shown that TORC2 is the kinase responsible for hydrophobic motif phosphorylation of PKB-Ser^{473} (Sarbassov *et al*, 2005). Therefore while phosphorylation of PKB-Ser^{473} can be used as an assay for PI3K activity, it also indicates the activity of TORC2 and has been used for this purpose (Thoreen *et al*, 2009; Lu *et al*, 2010; Mansley and Wilson, 2010a; Kuehn *et al*, 2011). Phosphorylation of PKB-Ser^{473} is thought to enhance PDK1 dependent phosphorylation of the activation loop of PKB (Thr^{308}) (Scheid *et al*, 2002; Yang *et al*,

2002; Sarbassov *et al*, 2005) possibly by providing a docking site for PDK1. As PDK1 is dependent on PI3K activity, PKB-Thr^{308} phosphorylation can therefore be used to determine PDK1/PI3K activity. Therefore by blotting for both PKB-Ser^{473} and PKB-Thr^{308}, PI3K activity can be monitored and also allows for distinction between inhibition of mTORC2 and PDK1. However care must be taken when interpreting results as PKB-Ser^{473} phosphorylation affects PDK1 dependent phosphorylation of PKB-Thr^{308} (Sarbassov *et al*, 2005; Lu *et al*, 2010). Using this approach I designed experiments to look at the effect of PI-103 on PI3K activity and the subsequent effects on the dexamethasone-induced ENaC activity in H441 cells, in order to determine whether this is truly dependent upon PI3K.

Results

The aim of this section was to determine the role of PI3K in the maintenance of the amiloride sensitive current induced by dexamethasone. This was achieved by recording from groups of H441 cells that had been exposed to dexamethasone for 18-36 hours (control) and control cells that had been exposed to 3-4 hours PI-103 after dexamethasone treatment (18-36 h). Western blot analysis was used to determine that inhibition of PI3K was successful, by phosphorylation status of two downstream substrates (PKB-Ser^{473} and PKB-Thr^{308}) and the effect on SGK1 activity (NDRG1-$Thr^{346/356/366}$). As PI-103 is known to inhibit mTORC1, further experiments were carried out using the specific mTORC1 inhibitor rapamycin to determine whether any affects of PI-103 were the result of mTORC1 inhibition rather than PI3K inhibition.

Effect of PI3K inhibition on the dexamethasone induced depolarisation

Figure 4.2 shows the I_m-V_{hold} relationship from recordings of H441 cells that had been exposed to control conditions (dex: 0.2 μM, 18-36 h) and after 3-4 hours exposure to PI-103 (0.5 μM). Initial recording was made under ECS control solution and then ECS + amiloride (ECS + Amil., 10 μM). Bathing solutions for PI-103 treated cells contained 0.5 μM PI-103, and recordings were made from them. The C_m for control cells was 55.3 ± 10.8 pF and C_m for PI-103-treated cells was 46.2 ± 9.3 pF. As observed in chapter 4 dexamethasone causes depolarisation and a hyperpolarising response is seen upon application of amiloride. PI-103-treatment abolishes the depolarisation in response to dexamethasone and there is no effect of amiloride.

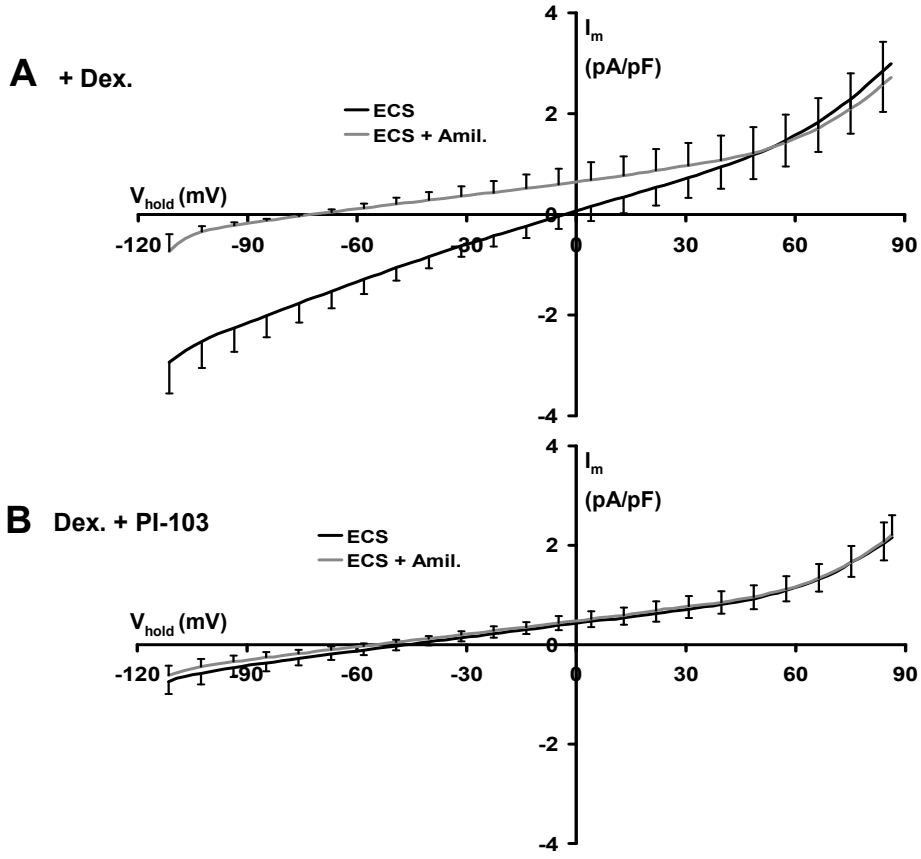
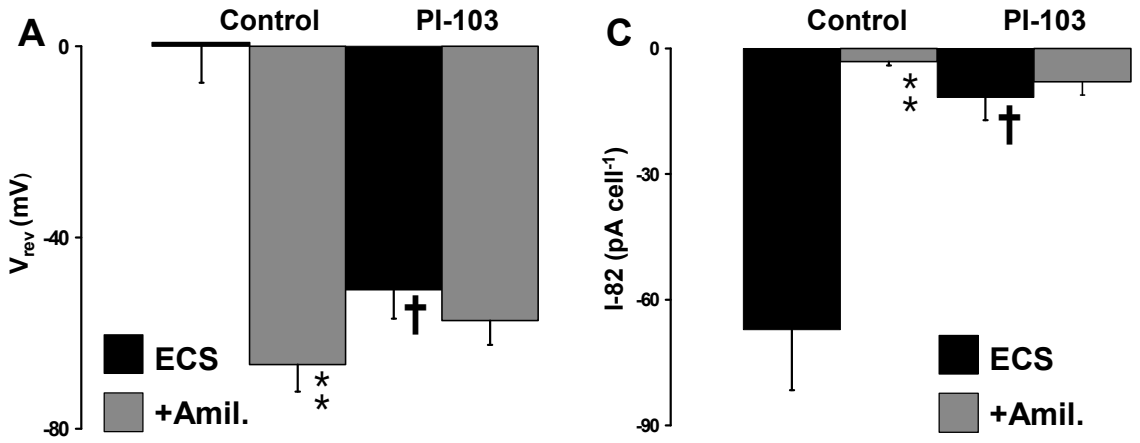


Figure 4.2 Dexamethasone and PI-103 treated cells. (A) Relationship between membrane current (I_m) and holding potential (V_{hold}) quantified in dex.-treated cells (0.2 μM, 18-36 h, n = 5) during exposure to ECS and ECS + amiloride (10 μM). (B) Equivalent data from control cells that have been exposed to 3-4 hours of PI-103 (0.5 μM, n = 6) after dex.-treatment.

Effect of PI3K inhibition on V_{rev} and I_{-82}

Figure 4.3 shows the values of V_{rev} and the I_{-82} derived from data in figure 4.2. Dexamethasone (0.2 μM, 18-36 h) causes depolarisation and exposure to 10 μM amiloride causes a hyperpolarising response. However exposure to PI-103 (0.5μM, 3-4 h) abolishes the dexamethasone induced depolarisation and there is no effect of amiloride on the value of V_{rev}. PI-103 reduces the magnitude of the I_{-82} and is amiloride insensitive, whereas under control conditions there is clear amiloride sensitivity to the I_{-82}.



B

Values of V_{rev}

	Control	PI-103
ECS	0.4 ± 8.1	-51.0 ± 6.0
+ Amil.	-66.6 ± 5.6	-57.4 ± 5.1

D

Values of I_{-82}

	Control	PI-103
ECS	-67.1 ± 14.4	-11.7 ± 5.4
+ Amil.	-3.2 ± 0.9	-8.0 ± 3.2

Figure 4.2 V_{rev} and I_{-82} for dex-treated and PI-103 treated cells. (A) and (B) values of V_{rev} derived by analysis of the data in figure 4.2 A and B (control n = 5; PI-103-treated n = 6). (C) and (D) current flowing at -82mV, derived from data in figure 4.2. A and B (control n = 5; PI-103-treated n = 6). Asterixes denote a statistically significant effect of amiloride (*, $P < 0.05$, **, $P < 0.01$, Student's paired t-test), cross denotes a statistically significant effect of PI-103 (†, $P < 0.001$, one-way ANOVA with Bonferroni post hoc test).

Effect of PI3K inhibition on the amiloride sensitive current

Figure 4.4 shows further analysis of the data in figure 4.1 to obtain the I_{amil} for control cells and cells treated with PI-103. In control cells there is a clear I_{amil} that reverses at 51.1 ± 4.0 mV which is similar to the predicted value for Na^+ therefore indicating a high degree of Na^+ selectivity. Exposure to PI-103 (0.5μM, 3-4 h) completely abolishes the I_{amil}.

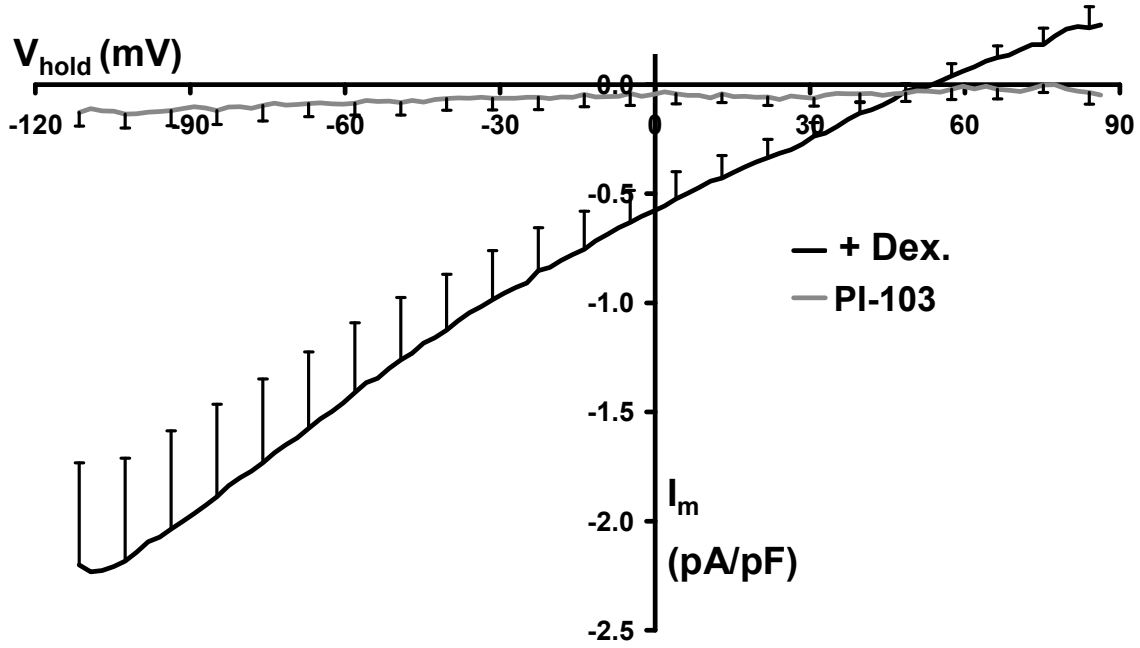


Figure. 4.4. I_{amil} for dex-treated and PI-103 treated cells. (A) Analysis of the amiloride sensitive component (I_{amil}) of the total membrane current for control cells (n = 5) and PI-103 treated cells (0.5 μM, n = 6) derived from data in figure 4.2 A and B.

Effect of PI3K inhibition on endogenous protein phosphorylation

Figure 4.5 shows the effects of dexamethasone-treatment and PI3K inhibition on SGK1 activity as monitored by NDRG1-Thr$^{346/356/366}$ phosphorylation and PI3K activity as monitored by PKB-Ser^{473} and PKB-Thr^{308} phosphorylation. Acute dexamethasone-treatment increases the abundance of NDRG1-Thr$^{346/356/366}$ phosphorylation whereas, after 24 hours exposure to dexamethasone, NDRG1-Thr$^{346/356/366}$ phosphorylation was essentially identical to that of those measured in dexamethasone-deprived cells. PI-103-treatment completely abolishes NDRG1-Thr$^{346/356/366}$ phosphorylation, furthermore total NDRG1 remains unaffected. Acute and longer term dexamethasone-treatment has no effect on either PKB-Ser^{473} or PKB-Thr^{308} phosphorylation. However PI-103-treatment abolishes phosphorylation of both PKB-Ser^{473} and PKB-Thr^{308} while total PKB is unaffected.

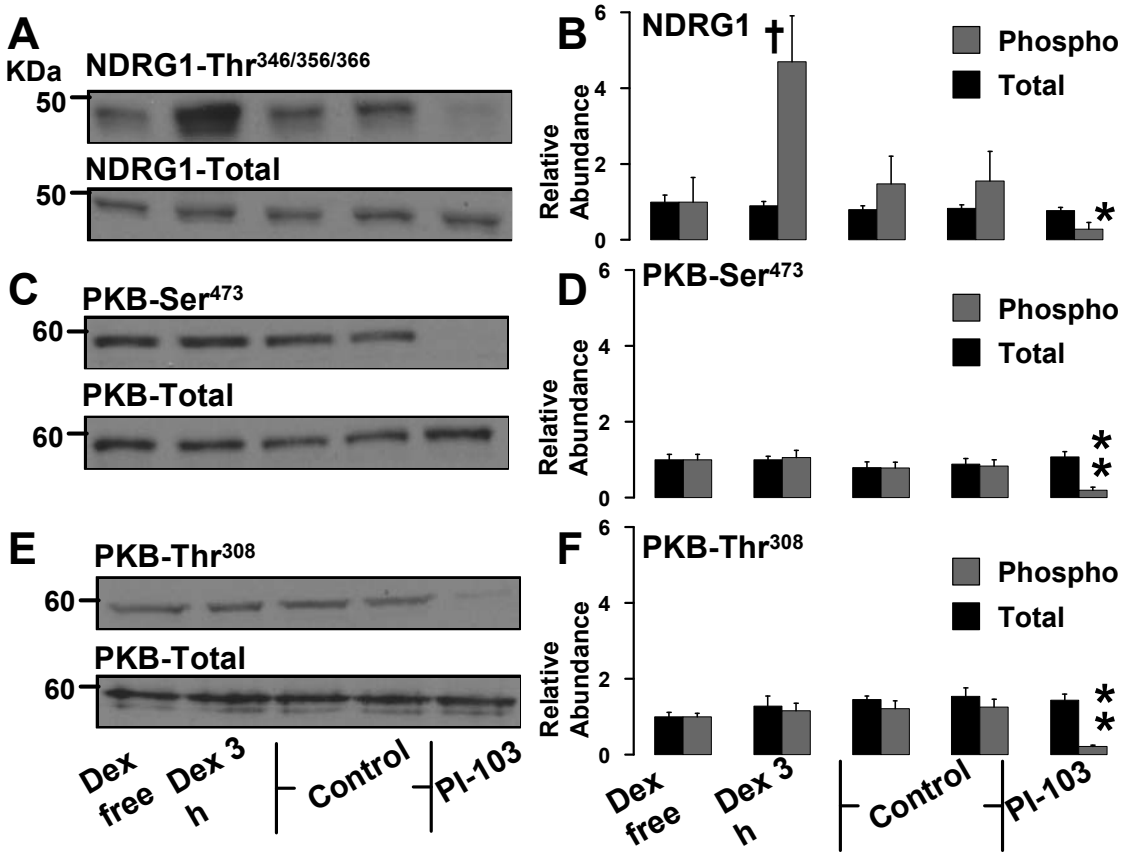


Figure 4.5 Effect of PI-103 upon endogenous protein phosphorylation. Cells were maintained in dexamethasone-free medium and then exposed to dexamethasone for 3 hours, ~24 hours or ~24 hours with 3 hours exposure to PI-103 (0.5μM) and then subjected to western blot analysis to determine phosphorylation and total protein abundance of NDRG1 and PKB. Shown are typical blots representative of 4 such experiments (A) NDRG1-Thr$^{246/256/366}$ / Total-NDRG1 (C) PKB-Ser^{473} / PKB-total (E) PKB-Thr^{308} / PKB-total. Densometric analysis of 4 such experiments are shown in (B), (D) and (F). Cross indicates statistically significant effect of dexamethasone (†, $P < 0.05$ one way ANOVA), asterixes denote a statistically significant effect of PI-103 (* $P < 0.05$, **, $P < 0.01$ one way ANOVA).

Effect of mTORC1 inhibition

As PI-103 has been shown to inhibit mTOR, parallel experiments were run using rapamycin which is an exquisitely specific inhibitor of mTORC1 (Bain *et al*, 2007). Figure 4.6 shows the I_m-V_{hold} relationship from recordings of H441 cells that had been exposed to control conditions (dex: 0.2 μM, 18-36 h) and after 3-4 hours exposure to rapamycin (0.1 μM). Initial recording was made under ECS control solution and then ECS + amiloride (ECS + Amil., 10 μM). Recordings made from rapamycin-treated cells the bathing solutions contained 0.1 μM rapamycin. The C_m for control cells was 67.4 ± 13.4 pF and C_m for rapamycin-treated cells was 79.3 ± 19.3 pF. Dexamethasone causes depolarisation and a hyperpolarising response is seen upon application of amiloride. Rapamycin-treatment does not abolish the depolarisation in response to dexamethasone and there is a hyperpolarising effect of amiloride.

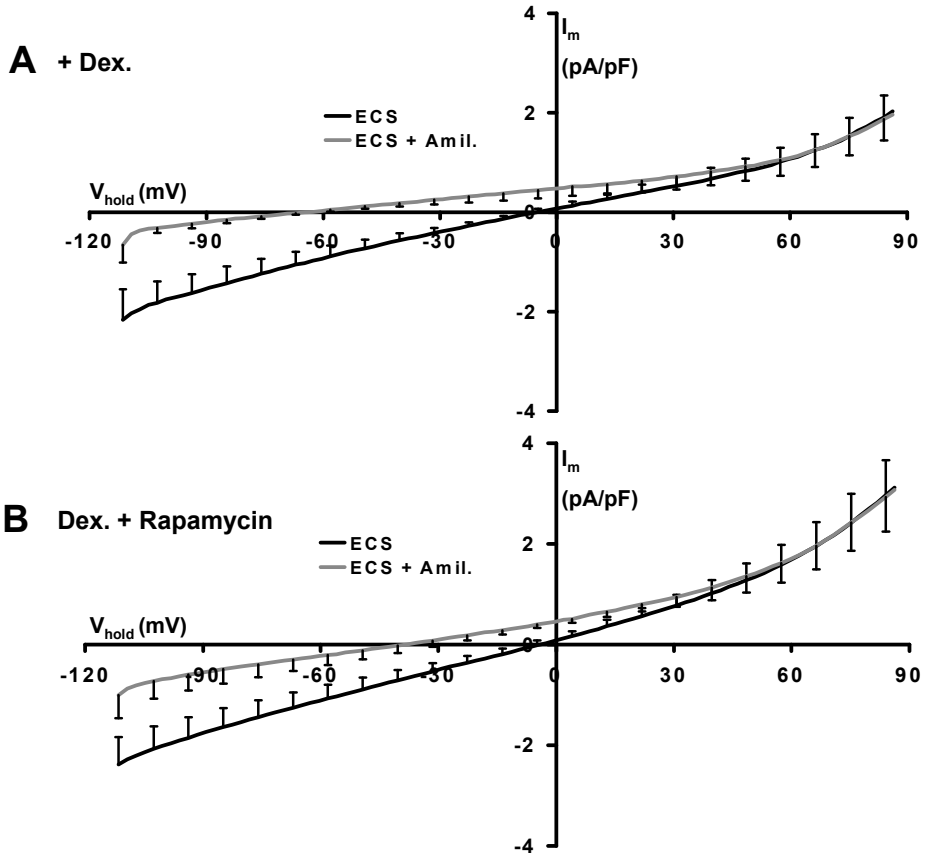
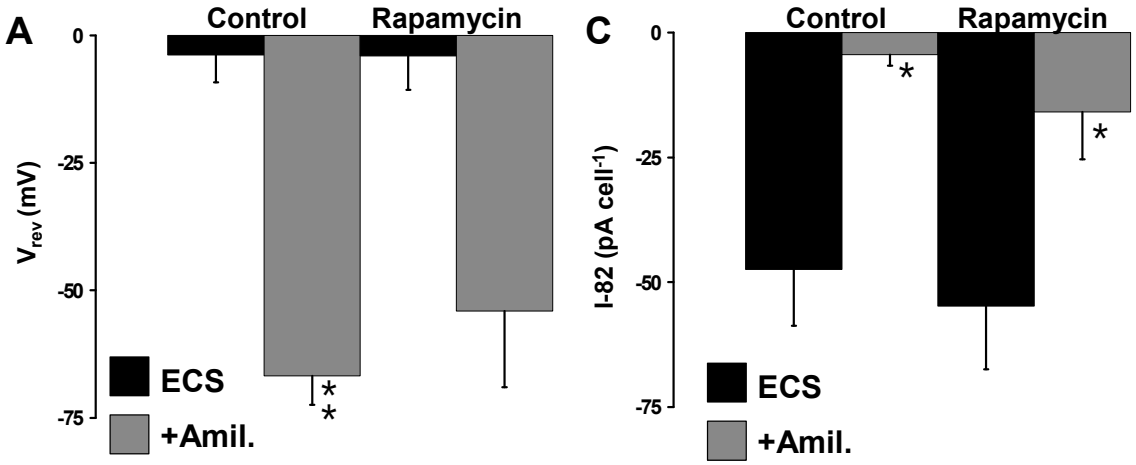


Figure 4.6 Dexamethasone and rapamycin treated cells. (A) Relationship between membrane current (I_m) and holding potential (V_{hold}) quantified in control cells (0.2 μM, 18-36 h, n = 4) during exposure to ECS and ECS + amiloride (10 μM). (B) Equivalent data from control cells that have been exposed to 3-4 hours of rapamycin (0.1 μM, n = 5).

Effect of mTORC1 inhibition on V_{rev} and I_{-82}

Figure 4.7 shows the values of V_{rev} and the I_{-82} derived from data in figure 4.5. Dexamethasone (0.2 μM, 18-36 h) causes depolarisation and exposure to 10 μM amiloride causes a hyperpolarising response. Exposure to rapamycin (0.5μM, 3-4 h) does not abolish the dexamethasone induced depolarisation and there is a hyperpolarising response to amiloride on the value of V_{rev}. Although this response is not significant, it does tend towards significance (P = 0.0576, students paired t-test). Rapamycin does not have a significant effect on the values of V_{rev} or I_{-82} (ANOVA with Bonferoni post hoc test).



B

Values of V_{rev}

	Control	Rapamycin
ECS	-3.9 ± 5.3	-4.0 ± 6.7
+ Amil.	-66.8 ± 5.7	-54.1 ± 14.9

D

Values of I_{-82}

	Control	Rapamycin
ECS	-47.5 ± 11.3	-54.8 ± 12.7
+ Amil.	-4.4 ± 2.2	-15.9 ± 9.5

Figure 4.7 V_{rev} and I_{-82} for dex- and rapamycin treated cells. (A) and (B) values of V_{rev} derived by analysis of the data in figure 4.1 A and B (control, n = 4; rapamycin, n = 5). (C) and (D) current flowing at -82mV, derived from data in figure 4.6. A and B (control, n = 4; rapamycin, n = 5). Asterixes denote a statistically significant effect of amiloride (*, $P < 0.05$, **, $P < 0.01$, Student's paired t-test). Whilst the effect of amiloride was found not to be significant in rapamycin treated cells, it did approach significance ($P = 0.058$, student's paired t-test).

Effect of mTORC1 inhibition on the amiloride sensitive current

Figure 4.8 shows further analysis of the data in figure 4.1 to obtain the I_{amil} for control cells and cells treated with rapamycin. In control cells there is a clear I_{amil} that reverses at 58.4 ± 10.4 mV. Furthermore there is an obvious I_{amil} in rapamycin-treated cells that reverses at 62.0 ± 5.4 mV. Both reverse at values similar to the predicted value for Na^+ therefore indicating a high degree of Na^+ selectivity.

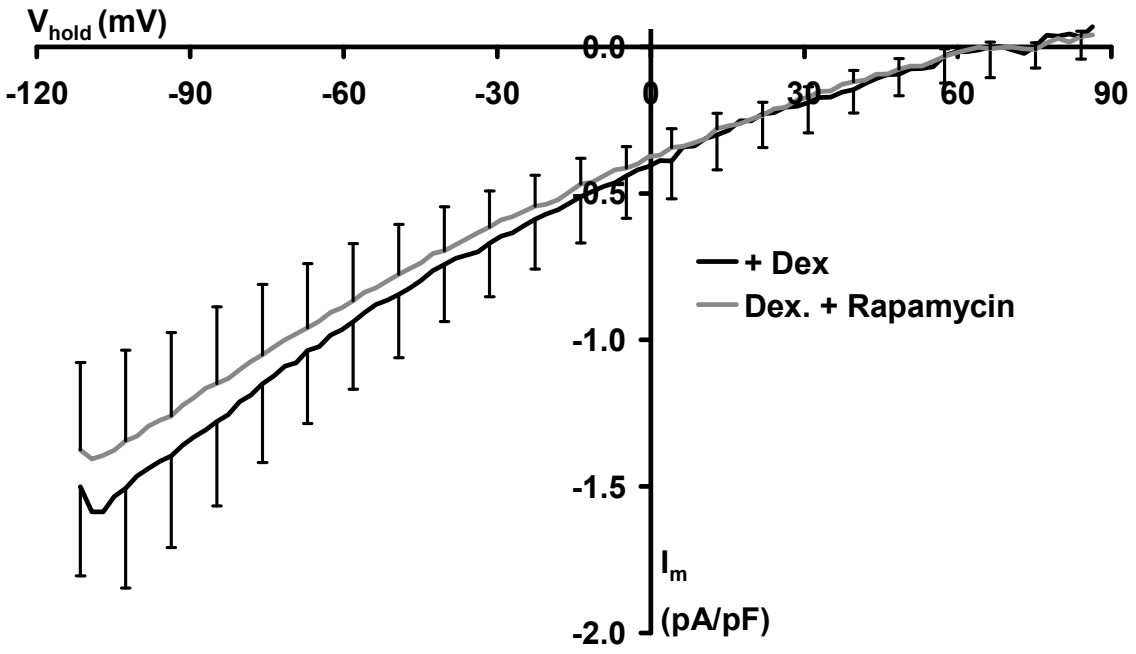


Figure. 4.8. I_{amil} for dex- and rapamycin treated cells. (A) Analysis of the amiloride sensitive component (I_{amil}) of the total membrane current for control cells (n = 4) and rapamycin treated cells (0.1 μM, n = 5) derived from data in figure 4.6 A and B.

Effect of mTORC1 inhibition on endogenous protein phosphorylation

Figure 4.9 shows the effects of dexamethasone-treatment and mTORC1 inhibition on SGK1 activity as monitored by NDRG1-Thr$^{346/356/366}$ phosphorylation, mTORC2 activity as monitored by PKB-Ser^{473} and PI3K activity as monitored by PKB-Thr^{308} phosphorylation. After 24 hours exposure to dexamethasone NDRG1-Thr$^{346/356/366}$ phosphorylation had not increased compared to hormone free. Rapamycin-treatment has no effect on NDRG1-Thr$^{346/356/366}$ phosphorylation, furthermore total NDRG1 remains unaffected. Rapamycin has no effect on either PKB-Ser^{473} or PKB-Thr^{308} phosphorylation and total PKB is unaffected.

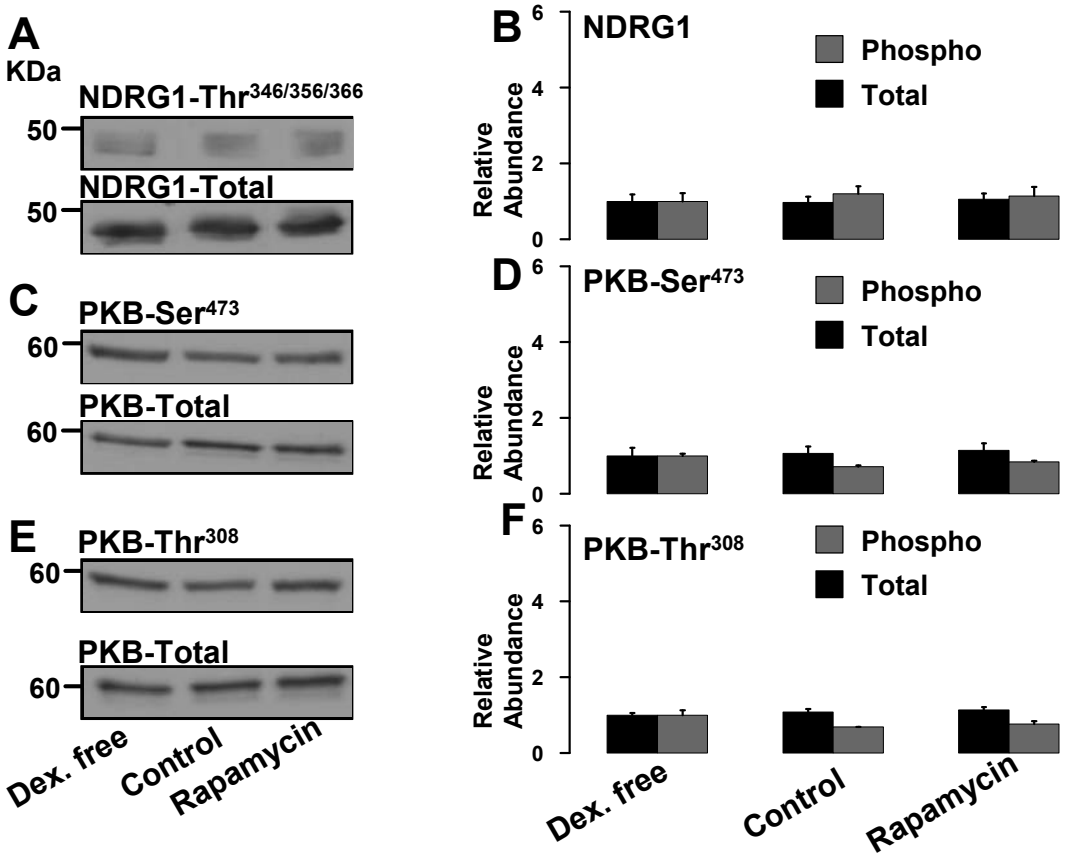


Figure 4.9 Effect of rapamycin on endogenous protein phosphorylation. Cells were maintained in either dexamethasone-free media, exposed to dexamethasone for ~24 hours (control) or control cells with 3 hours exposure to rapamycin (0.1 μM) and then subjected to western blot analysis to determine phosphorylation and total protein abundance of NDRG1 and PKB. Shown are typical blots representative of 4 such experiments (A) NDRG1-Thr$^{246/256/366}$ / Total-NDRG1 (C) PKB-Ser^{473} / PKB-total (E) PKB-Thr^{308} / PKB-total. Densometric analysis of 4 such experiments are shown in (B), (D) and (F).

Effect of PI-103 and rapamycin on conductance

To further examine the effects of PI-103 and rapamycin on the biophysical properties of H441 cells the conductance was determined by further analysis of the data obtained from figures 4.1 and 4.5. Figure 4.10 clearly shows that dexamethasone-treatment (18-36 h, 0.2 μM) increases the conductance and this is sensitive to 10 μM amiloride (A) and (B). PI-103 (0.5 μM, 3-4 h) completely abolished the effect of dexamethasone however rapamycin (0.1 μM, 3-4 h) had no significant effect.

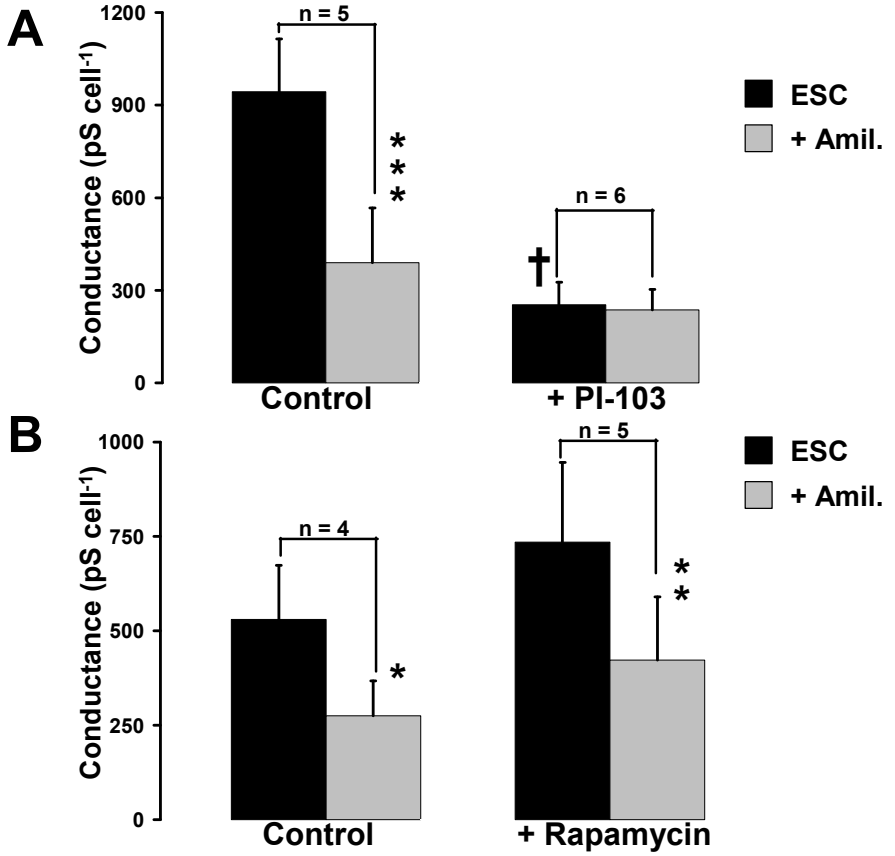


Figure 4.10 Effect of PI-103 and rapamycin upon conductance. Further analysis of the data obtained from figures 4.2 (A) and 4.6 (B); asterixes denote a statistically significant effect of amiloride (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Student's paired t-test), cross denotes a statistically significant effect of PI-103 (†, $P < 0.001$, one-way ANOVA with Bonferroni post hoc test).

Discussion

The data further confirm the observation that dexamethasone increased SGK1 activity and that after ~24 hours exposure, activity dropped to the level seen in dexamethasone-free cells. The PI3K assay was expanded upon to include PKB-Thr^{308} phosphorylation, thus allowing the determination of the phosphorylation status of two downstream substrates, which confirms dexamethasone has no effect upon PI3K activity, thus strengthening the case of a permissive role for PI3K. Treatment with PI-103 completely abolishes the phosphorylation of both PKB residues and therefore is consistent with the view that PI-103 completely inhibits PI3K (Fan *et al*, 2006; Bain *et al*, 2007). PI-103 treatment also abolishes SGK1 activity and this result was expected as SGK1 activity has been shown to be dependent on PI3K activity (Kobayashi and Cohen, 1999; Park *et al*, 1999; Biondi *et al*, 2001). However, this result is in contrast to another study which showed that PI-103 did not fully inhibit SGK1 activity as NDRG1 phosphorylation was not abolished in H441 cells (McTavish *et al*, 2009). The reason for this discrepancy is unclear as the cells were treated in a similar fashion, although as PI3K activity was not assayed in McTavish *et al*, (2009), the possibility that PI-103 did not fully inactivate PI3K cannot be ruled out. However it is clear from the data presented here that PI-103 causes inhibition of PI3K and SGK1 activity. Therefore I looked at the effect of PI-103 on the dexamethasone-induced amiloride sensitive current and found that PI-103 abolished the dexamethasone induced amiloride sensitive current; this agrees well with the theory that PI3K is vital for the induction and maintenance of Na^+ transport in lung epithelium. PI3K inhibition has been shown to abolish Na^+ transport in H441 cells (Inglis *et al*, 2009); however LY294002 was used and therefore these results may have been due to the non-specific effects of LY294002. The results presented here therefore

confirm that PI3K activity is important for the maintenance of dexamethasone induced ENaC activity in lung epithelium.

As PI-103 is known to inhibit TORC1 (Bain *et al*, 2007) the effects of the highly specific TORC1 inhibitor, rapamycin, were explored. Rapamycin-treatment did not mimic the effects of PI-103 on NDRG1 or PKB phosphorylation, and it had no effect upon the electrophysiological properties of dexamethasone-treated cells. Therefore these results are in agreement with those of Lu *et al*, 2010 that report no role for TORC1 in the induction and maintenance of ENaC activity irrespective of PI3K activity.

Interestingly PI-103 does not completely abolish the conductance seen in H441 cells. There is a clear amiloride-insensitive conductance, which is likely, in part, due to insulin stimulated Cl^- transport (Brown *et al*, 2008). PI-103 did not abolish the amiloride-insensitive component, thus suggesting that insulin is acting through a mechanism independent of PI3K. However, as this was not investigated, this could not be experimentally verified. Rapamycin-treatment on the other hand had no significant effect upon the conductance and this accord well with the previous results that mTORC1 is not involved in dexamethasone-induced Na^+ transport.

Conclusions

These data confirm previous studies, which suggest PI3K is vital to dexamethasone-induced ENaC activity. However building on this, the results on this chapter exclude a role for TORC1 in the GC-mediated increase in Na^+ transport in the H441 cell line. These results cannot determine whether TORC2 / SGK1 are part of the pathway involved in dexamethasone-induced ENaC activity. Indeed the finding that PI-103 inhibits both SGK1 and PKB activity is to be expected as both of these kinases are

thought to be dependent upon PI3K. As PI3K was fully inactivated, the effects of PI-103 could be a result of PKB or $PIP_{2/3}$ inhibition. We therefore designed further experiments using novel inhibitors of mTORC1 / 2 and SGK1, Torin1 and GSK650394 respectively (Thoreen *et al*, 2009; Sherk *et al*, 2008; Mansley and Wilson, 2010b) that would allow for inhibition of these kinases while leaving PI3K activity unaffected therefore addressing the role of both TORC2 and SGK1 in the maintenance of GC-induced ENaC activity in lung epithelium. The results of these experiments are presented in the next chapter.

Chapter 5 – The role of TORC2 and SGK1

Introduction

The results presented in chapter four support the theory that PI3K is vital to the maintenance of Na^+ transport, however further investigation is required to determine the exact signalling mechanisms that take place. Recent evidence would suggest that ENaC activity is dependent on TORC2 phosphorylation of the hydrophobic motif of SGK1 (Lu *et al*, 2010). This provides a docking site for PDK1 thereby promoting interaction of SGK1 with PDK1 causing phosphorylation of the activation loop of SGK1 and conferring activity (Biondi *et al*, 2001). As discussed in chapter one SGK1 is thought to cause phosphorylation of Nedd4-2 which increases the trafficking and expression of ENaC in the membrane. However there are other mechanisms of action that may contribute to the hormonal regulation of ENaC activity that are dependent on PI3K such as the phosphatidylinositide second messengers (PIP_2 and PIP_3) and/or PKB (Blazer-Yost *et al*, 2004; Pochynyuk *et al*, 2007; Lee *et al*, 2007; Diakov *et al*, 2010). This, taken with the observations that *sgk1* gene knockout mice are viable, suggests alternate mechanisms are involved in the control of ENaC. The lack of specific inhibitors of TORC2 and SGK1 has in part hindered the investigation into the role these kinases play. The development of novel small molecule inhibitors that are specific for mTOR or SGK1 may provide an effective way to inactivate these kinases (Thoreen *et al*, 2009; Sherk *et al*, 2008) without causing inhibition of other closely related kinases involved in the proposed signalling pathway.

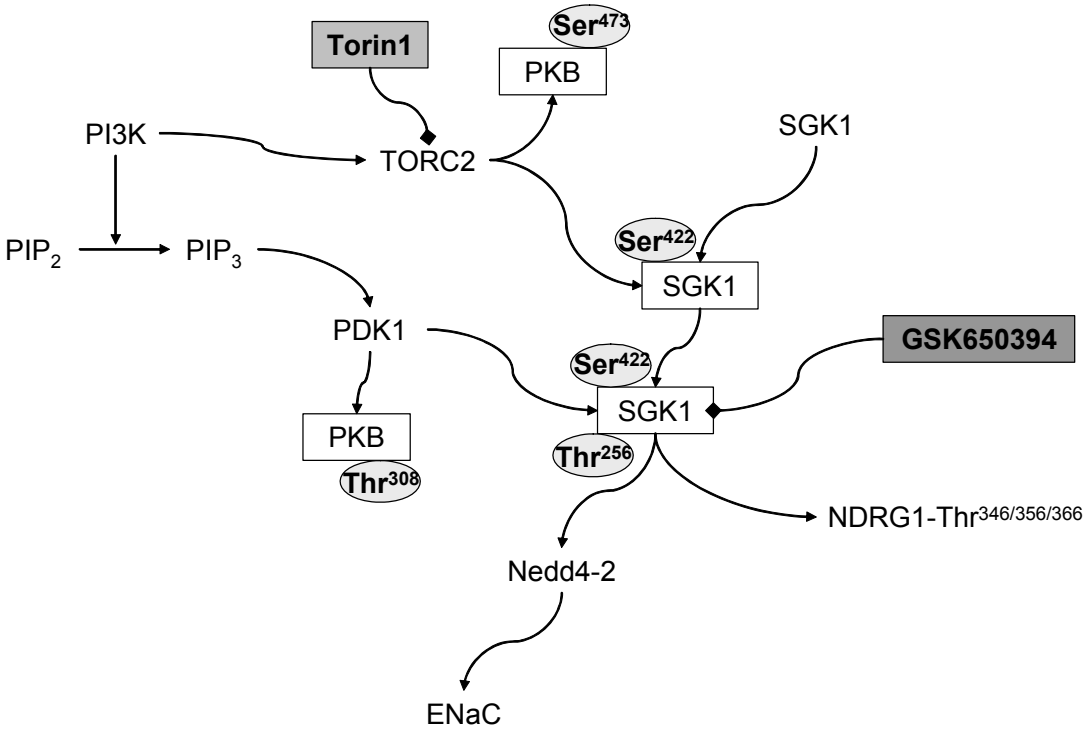


Figure 5.1 Diagram of proposed signalling pathway involved in ENaC regulation. Thus if SGK1 is vital to the regulation and maintenance of ENaC then Torin1 and GSK650394 would be expected to inhibit the glucocorticoid-evoked amiloride sensitive current.

The development of Torin1 would seem to provide an effective way of inhibiting TORC2 in the absence of a truly specific TORC2 inhibitor. Torin1 has been shown to be an ATP-competitive TOR inhibitor as its effects are abolished by increasing ATP concentration, additionally no significant non-specific effects were found (Thoreen *et al*, 2009). In order to test the effects of Torin1 on PI3K activity, PKB-Thr^{308} phosphorylation was used in this study. However a problem of this approach arises due to TORC2 being responsible for PKB-Ser^{473} phosphorylation which facilitates downstream phosphorylation of the Thr^{308} site (Andjelkovic *et al*, 1997). To overcome

this mLST8 knockout cells were used. mLST8 is vital for the interaction of TORC2 with its substrates, and the result of this knockout is continual dephosphorylation of the Ser^{473} site (Guertin *et al*, 2006). Thus any Thr^{308} phosphorylation would be due to PI3K activity and not a result of Ser^{473} mediated phosphorylation. Using this approach Thoreen *et al*, 2009 found that Torin1 only affected PI3K activity when cells were exposed to concentrations well above that required to completely inhibit TORC2 (> 1 μM and > 0.1 μM respectively). Thus Toirn1 would appear to be specific for mTOR inhibition at low concentration and is therefore a useful compound in which to investigate mTOR activity. Nevertheless, rapamycin should be used to exclude the possibility that effects of Torin1 are not as a result of TORC1 inhibition. As the results from chapter four demonstrate rapamycin does not affect PI3K, SGK1 or ENaC activity, thus any effects of Torin1 arise as a result of TORC2 inhibition. It should also be noted that this study along with another (Feldman *et al*, 2009) who developed a different mTOR inhibitor (PP242) report rapamycin-resistant effects of mTORC1. However this requires the development of a cell line where TORC2 activity has been abolished (for example by mLST8 deletion). Furthermore these rapamycin-resistant effects are yet to be fully explored and rapamycin is still considered to be the most effective means of mTORC1 inhibition.

The lack of a specific inhibitor of SGK1 seems to have been addressed with the development of GSK650394. Sherk *et al* (2008) have shown that this small-molecule inhibitor was able to block the enzyme activity of both SGK1 and SGK2 in scintillation proximity assays and this corresponded with the inhibition of an aldosterone induced amiloride-sensitive current in mouse cortical collecting duct cells (M-1 cell line). Furthermore using an *in vitro* kinase assay they were able to determine that GSK650394

was selective for SGK1 over PKB, PDK1 and other related kinases (Sherk *et al*, 2008). Therefore this compound would appear to offer a method of inactivating SGK1 whilst leaving PI3K, PKB and PDK1 unaffected. Indeed GSK650394 has been used for this purpose; to investigate the role of SGK1 in insulin-dependent Na^+ transport in a mouse derived cortical collecting duct cell line (mpkCCD). This study found that GSK650394 did cause some inhibition of PKB as judged by Ser^{473} phosphorylation (Mansley and Wilson, 2010b). However they argued that this slight inhibition was not sufficient to compromise PKB activity as it has been shown that this must be reduced by less than 10% of the basal level in order for downstream targets to be affected (Logie *et al*, 2007). Taking this into account, GSK650394 was found to fully suppress insulin-induced Na^+ transport, thereby supporting the view that SGK1 is vital to this response. This also demonstrates that the effects of inhibitors can vary even in similar cell lines (i.e. mouse cortical collecting duct cells) and therefore specificity should not be taken for granted and tested for in order to confirm the action of the inhibitor.

Using the approach outlined previously to investigate the effects of PI-103 and rapamycin, we investigated the effects of Torin1 and GSK650394 on endogenous protein phosphorylation and the effect on the dexamethasone-induced amiloride sensitive current in H441 cells to determine whether SGK1 activity is dependent upon TORC2. Furthermore specific inhibition of SGK1 will allow me to test the hypothesis that SGK1 activity is vital to the hormonal control of ENaC activity via glucocorticoids.

Results

The aim of this section is to determine the role of SGK1 in the maintenance of the dexamethasone-induced amiloride sensitive current. This was achieved using the same experimental format as previously described in chapter four. The novel SGK1 inhibitor, GSK650394 was used to establish the role of SGK1 and endogenous protein phosphorylation was used to examine its effects on TORC2 and PDK1 activity, as well as SGK1 activity, in order to confirm a specific action. Using the TOR inhibitor Torin1 and the results from rapamycin treatment (see chapter four), we were able to investigate the role played by mTORC2, which has been identified as the kinase responsible for phosphorylation of the hydrophobic motif of SGK1.

Effects of Torin1 upon TORC2 and PDK1

Low concentrations of Torin1 used over a prolonged period of time can cause hyperactivation of PI3K which can overcome the inhibitory effects of Torin1. Therefore, in order to ensure this was not the case, we tested a range of concentrations over the three hour time period. The secondary aim of this was to test whether or not Torin1 inhibited TORC2 and PDK1 activity equally. Figure 5.2 shows representative western blots of the effects of increasing Torin1 concentration (0-100 nM, 3 h) upon PKB -Ser^{473} (A) and –Thr^{308} (B) phosphorylation. The combined densometric analysis of four such experiments for PKB -Ser^{473} and -Thr^{308} is shown in (B) and (D) respectively. These results clearly demonstrate that Torin1 inhibits PKB-Ser^{473} phosphorylation more effectively than -Thr^{308} phosphorylation. Moreover they confirm that Torin1 is not causing hyperactivation of PI3K under these conditions and therefore can be used as an effective inhibitor against TOR.

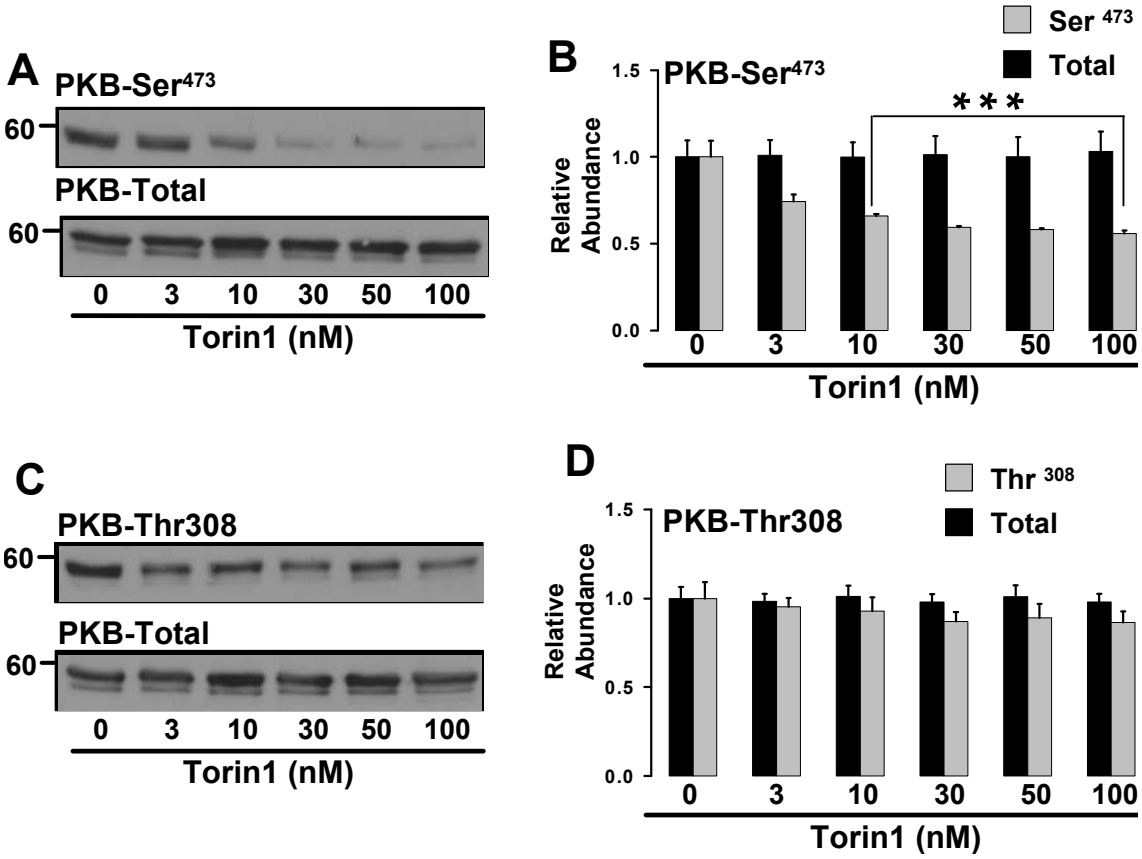


Figure 5.2 Effect of Torin1 upon PKB phosphorylation. Cells were maintained in dexamethasone-free medium and then exposed to dexamethasone for ~24 hours with 3 hours exposure to a range of Torin1 concentrations (0-100 nM) and then subjected to western blot analysis to determine phosphorylation status of –Ser^{473} (A) and –Thr^{308} (B) and total protein abundance of PKB. Shown are typical blots representative of 4 such experiments. Densometric analysis of all four experiments is shown for –Ser^{473} (B) and for –Thr^{308} (D). Asterixes denote a statistically significant effect of Torin1 (*, p = 0.001 one-way ANOVA)

Does Torin1 inhibit mTORC2 activity?

Figure 5.3 shows the effects of dexamethasone-treatment and mTOR inhibition on SGK1 activity as monitored by NDRG1-Thr$^{346/356/366}$ phosphorylation, mTORC2 activity as monitored by PKB-Ser^{473} and PDK1 activity as monitored byPKB-Thr^{308} phosphorylation.

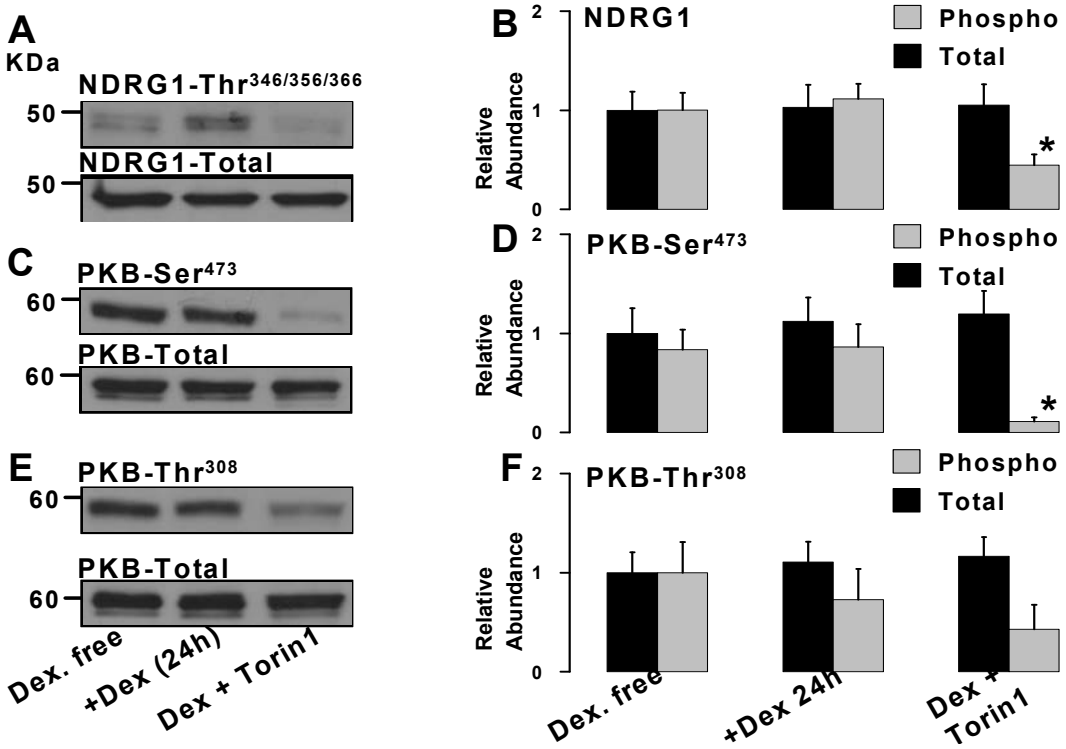


Figure 5.3 Effect of Torin1 upon endogenous protein phosphorylation. Cells were maintained in dexamethasone-free medium and then exposed to dexamethasone for ~24 hours or ~24 hours with 3 hours exposure to Torin1 (0.1μM) and then subjected to western blot analysis to determine phosphorylation and total protein abundance of NDRG1 and PKB. Shown are typical blots representative of 4 such experiments (A) NDRG1-Thr$^{246/256/366}$ / Total-NDRG1 (C) PKB-Ser^{473} / PKB-total (E) PKB-Thr^{308} / PKB-total. Densometric analysis of 4 such experiments are shown in (B), (D) and (F). Asterixes denote a statistically significant effect of Torin1 (*, $p < 0.01$ one-way ANOVA, $n = 4$)

Effects of mTOR inhibition on the properties of H441 cells

Figure 5.4 shows the I_m-V_{hold} relationship from recordings of H441 cells that had been exposed to control conditions (dex: 0.2 μM, 18-36 h) and after 3-4 hours exposure to Torin1 (0.1 μM). Initial recording was made under ECS control solution and then ECS + amiloride (ECS + Amil., 10 μM). The bathing solutions from Torin1-treated cells contained 0.1 μM Torin1. The C_m for control cells was 39.8 ± 10.1 pF and C_m for Torin1-treated cells was 40.5 ± 5.8 pF.

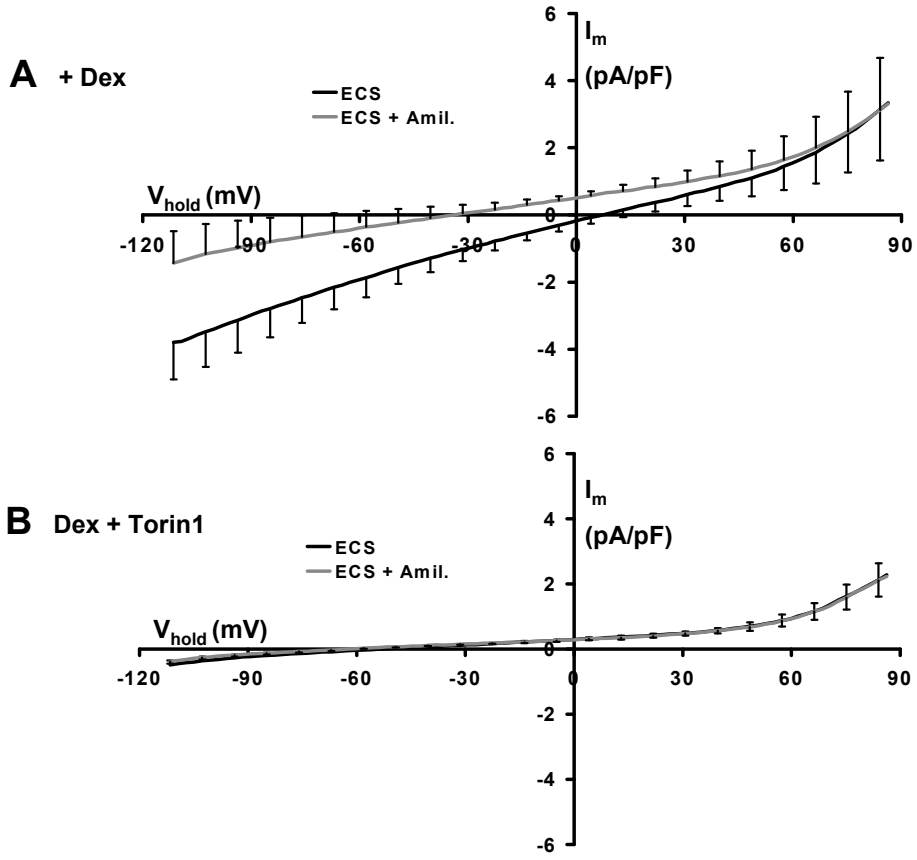
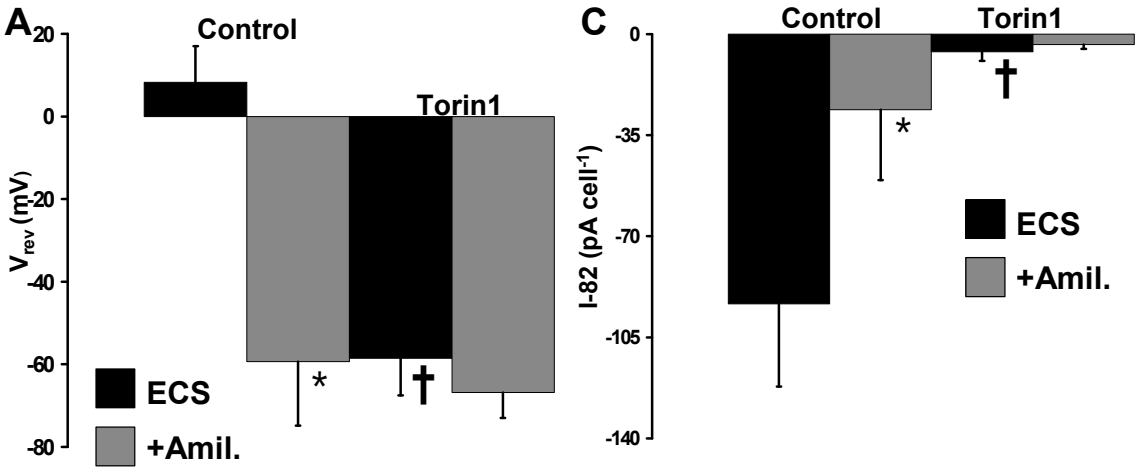


Figure 5.4 Dexamethasone and Torin1 treated cells. (A) Relationship between membrane current (I_m) and holding potential (V_{hold}) quantified in dexamethasone-treated cells (0.2 μM dex, 18-36 h, n = 4) during exposure to ECS and ECS + amiloride (10 μM). (B) Equivalent data from dexamethasone-treated cells that have been exposed to 3-4 hours of Torin1 (0.1 μM, n = 4).

Effect of Torin1, an inhibitor of TORC1 and 2, upon V_{rev} and I_{-82}

Figure 5.5 shows the values of V_{rev} and the I_{-82} derived from data in figure 5.3. Dexamethasone (0.2 μM, 18-36 h) causes depolarisation and exposure to 10 μM amiloride causes a hyperpolarising response. However exposure to Torin1 (0.1μM, 3-4 h) abolishes the dexamethasone induced depolarisation and there is no effect of amiloride on the value of V_{rev}. Torin1 reduces the magnitude of the I_{-82} and is amiloride insensitive, whereas under control conditions there is clear amiloride sensitivity to the I_{-82}.



B **Values of V_{rev}**

	Control	Torin1
ECS	8.3 ± 8.7	-58.5 ± 9.0
+ Amil.	-59.3 ± 15.5	-66.8 ± 6.2

D **Values of I_{-82}**

	Control	Torin1
ECS	-93.4 ± 28.6	-6.1 ± 3.1
+ Amil.	-26.2 ± 24.4	-3.7 ± 1.4

Figure 5.5 V_{rev} and I_{-82} in dex-treated (control) and Torin1-treated cells. (A) and (B) values of V_{rev} derived by analysis of the data in figure 5.3 A and B (control, n = 4; Torin1-treated, n = 4). (C) and (D) current flowing at -82mV, derived from data in fig. 5.3 A and B. Asterixes denote a statistically significant effect of amiloride (*, $P < 0.05$, Student's paired t-test), cross denotes a statistically significant effect of Torin1 (†, $P < 0.05$, one-way ANOVA with Bonferroni post hoc test).

Does GSK650394 inhibit SGK1?

Figure 5.6 shows the effects of dexamethasone-treatment and the effect of GSK650394 on SGK1 activity as monitored by NDRG1-Thr$^{346/356/366}$ phosphorylation, mTORC2 activity as monitored by PKB-Ser^{473} and PDK1 activity as monitored byPKB-Thr^{308} phosphorylation. While there was no statistical significance was found with found upon the effects of GSK upon NDRG1 phosphorylation it does tend towards significance, and furthermore phosphorylation of NDRG1 is clearly abolished when looking at the blots e.g. panel A.

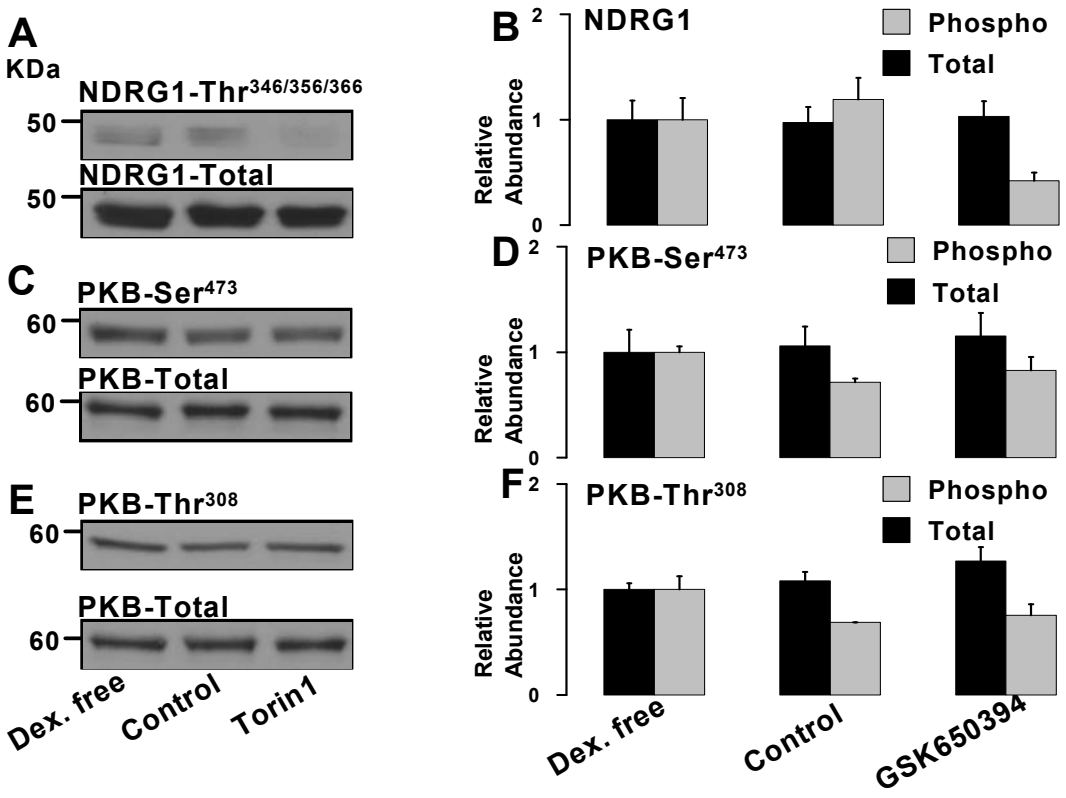


Figure 5.6 Effect of GSK650394 upon endogenous protein phosphorylation. Cells were maintained in dexamethasone-free medium and then exposed to dexamethasone for ~24 hours or ~24 hours with 3 hours exposure to GSK650394 (10μM) and then subjected to Western blot analysis to determine phosphorylation and total protein abundance of NDRG1 and PKB. Shown are typical blots representative of 4 such experiments (A) NDRG1-Thr$^{246/256/366}$ / Total-NDRG1 (C) PKB-Ser^{473} / PKB-total (E) PKB-Thr^{308} / PKB-total. Densometric analysis of 4 such experiments are shown in (B), (D) and (F).

Effects of GSK650394 upon PKB activity

In order to confirm that GSK650394 does not inhibit PKB activity, a kinase closely related to SGK1, I looked at the phosphorylation of PRAS40 at residue Thr^{246} which is a known substrate of PKB (Kovacina *et al*, 2003). Figure 5.7 shows the effect of dexamethasone and GSK650394 treatment upon the phosphorylation status of PRAS40. GSK650394does not affect the phosphorylation status of PRAS40, thus confirming that GSK650394 does not inhibit PKB activity. Furthermore dexamethasone treatment has no effect upon PRAS40 therefore supporting the observation that dexamethasone does not increase PKB activity.

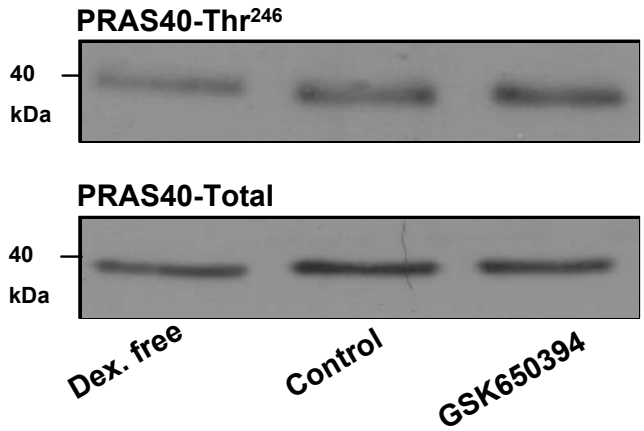


Figure 5.7 Effect of GSK650394 upon PRAS40 phosphorylation. Cells were maintained in dexamethasone-free medium and then exposed to dexamethasone for ~24 hours or ~24 hours with 3 hours exposure to GSK650394 (10μM) and then subjected to Western blot analysis to determine phosphorylation and total protein abundance of PRAS40 (n = 2).

Effects of SGK1 inhibition on the properties of H441 cells

Figure 5.8 shows the I_m-V_{hold} relationship from recordings of H441 cells that had been exposed to control conditions (dex: 0.2 μM, 18-36 h) and after 3-4 hours exposure to GSK650394 (10 μM). Initial recording was made under ECS control solution and then ECS + amiloride (ECS + Amil., 10 μM). The bathing solutions from GSK650394-treated cells contained 10 μM GSK650394. The C_m for control cells was 69.7 ± 11.7 pF and C_m for GSK650394-treated cells was 35.4 ± 2.9 pF.

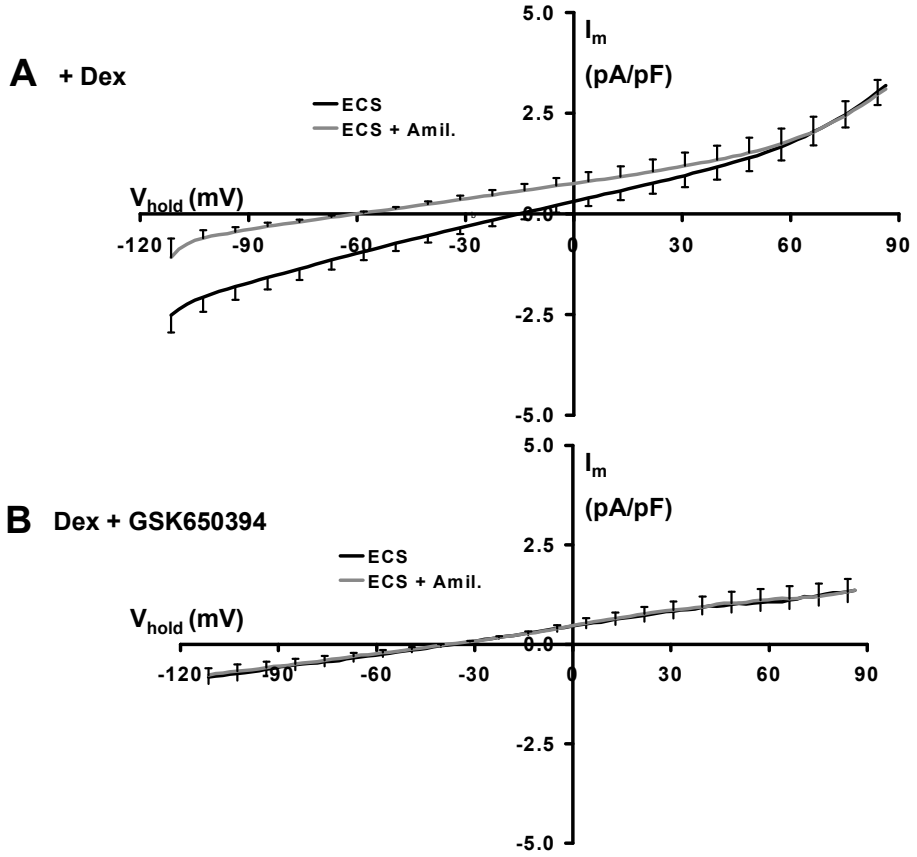
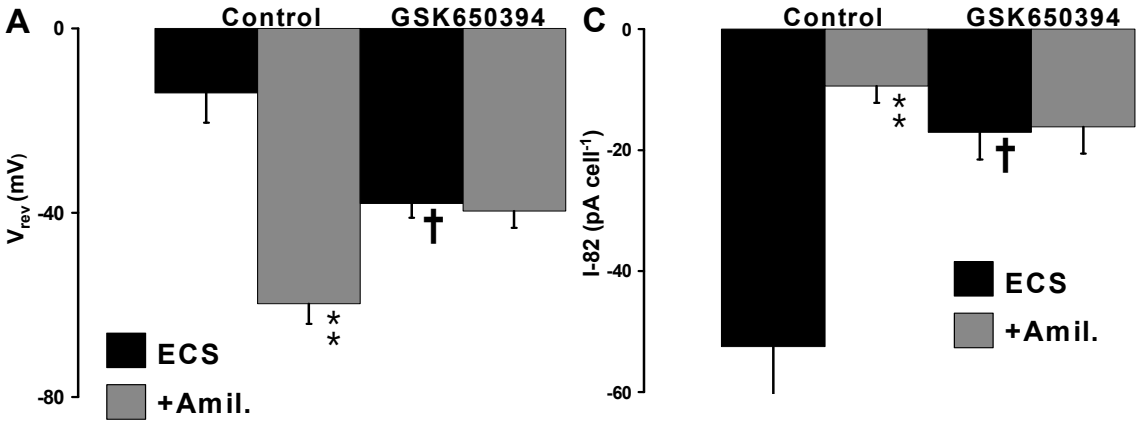


Figure 5.8 Dexamethasone and GSK650394 treated cells. (A) Relationship between membrane current (I_m) and holding potential (V_{hold}) quantified in dexamethasone-treated cells (0.2 μM, 18-36 h, n = 4) during exposure to ECS and ECS + amiloride (10 μM). (B) Equivalent data from dexamethasone-treated cells that have been exposed to 3-4 hours of GSK650394 (10 μM, n = 4).

Effect of SGK1 inhibition on V_{rev} and I_{-82}

Figure 5.9 shows the values of V_{rev} and the I_{-82} derived from data in figure 5.8. Dexamethasone (0.2 μM, 18-36 h) causes depolarisation and exposure to 10 μM amiloride causes a hyperpolarising response. However exposure to GSK650394 (10 μM, 3-4 h) abolished the dexamethasone induced depolarisation and there was no effect of amiloride on the value of V_{rev}. GSK650394 reduces the magnitude of the I_{-82} and is amiloride insensitive, whereas under control conditions there is clear amiloride sensitivity to the I_{-82}.



B

Values of V_{rev}

	Control	GSK650364
ECS	-14.0 ± 6.5	-38.0 ± 3.1
+ Amil.	-59.7 ± 4.4	-39.6 ± 3.7

D

Values of I_{-82}

	Control	GSK650394
ECS	-50.3 ± 8.2	-17.1 ± 4.5
+ Amil.	-6.8 ± 3.4	-16.2 ± 4.4

Figure 5.9 V_{rev} and I_{-82} in dex-treated (control) and GSK650364 treated cells. (A) and (B) values of V_{rev} derived by analysis of the data in figure 5.8 A and B (control, n = 4; GSK650364, n = 4). (C) and (D) current flowing at -82mV, derived from data in fig. 5.8 A and B. Asterixes denote a statistically significant effect of amiloride (**, $P < 0.01$, Student's paired t-test), cross denotes a statistically significant effect of GSK650394 (†, $P < 0.01$, one-way ANOVA with Bonferroni post hoc test).

Effect of TOR and SGK1 inhibition on the amiloride sensitive current

Figure 5.10 shows further analysis of the data in figure 5.4 and 5.8 to obtain the I_{amil} for control cells and cells treated with Torin1 or GSK650394. In control cells there is a clear I_{amil} that reverses close to the predicted value for Na^+ therefore indicating a high degree of Na^+ selectivity. Exposure to Torin 1(0.1 μM, 3-4 h) or GSK650394 (10 μM, 3-4 h) completely abolishes the I_{amil}.

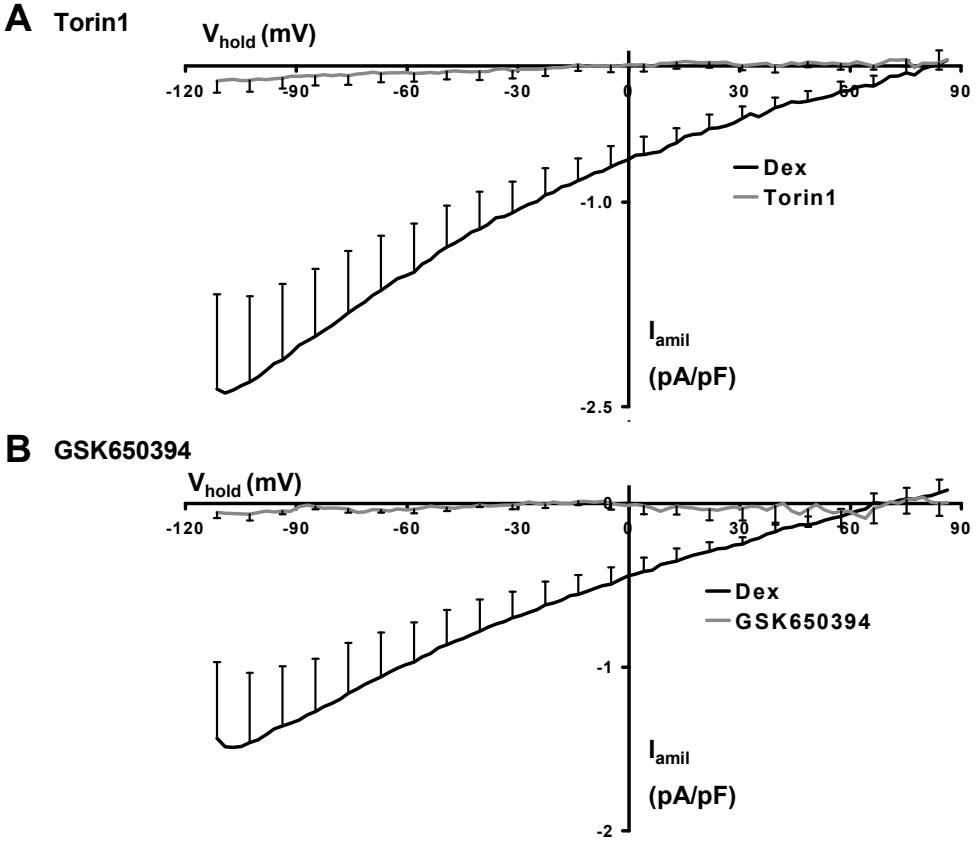


Figure 5.10 I_{amil} of Torin1 and GSK650364 treated cells. (A) Analysis of the amiloride sensitive component of the total membrane current for control cells (n = 4) and Torin1-treated cells (0.1 μM, n = 4) derived from data in figure 5.4 A and B. (B) Comparable data for control (n = 4) and GSK650394-treated cells (10 μM, n = 4) derived from data in figure 5.8 A and B.

Effect of Torin1 and GSK650394 on conductance

To further examine the effects of Torin1 and GSK650394 on the biophysical properties of H441 cells the conductance was determine by further analysis of the data obtained from that shown in figures 5.4 and 5.8. Figure 5.10 clearly shows that dexamethasone-treatment (18-36 h, 0.2 μM) increases the conductance and this is sensitive to 10 μM amiloride. Torin1 and GSK (0.1 μM, 3-4 h; 10 μM, 3-4 h respectively) both completely abolished the effect of dexamethasone.

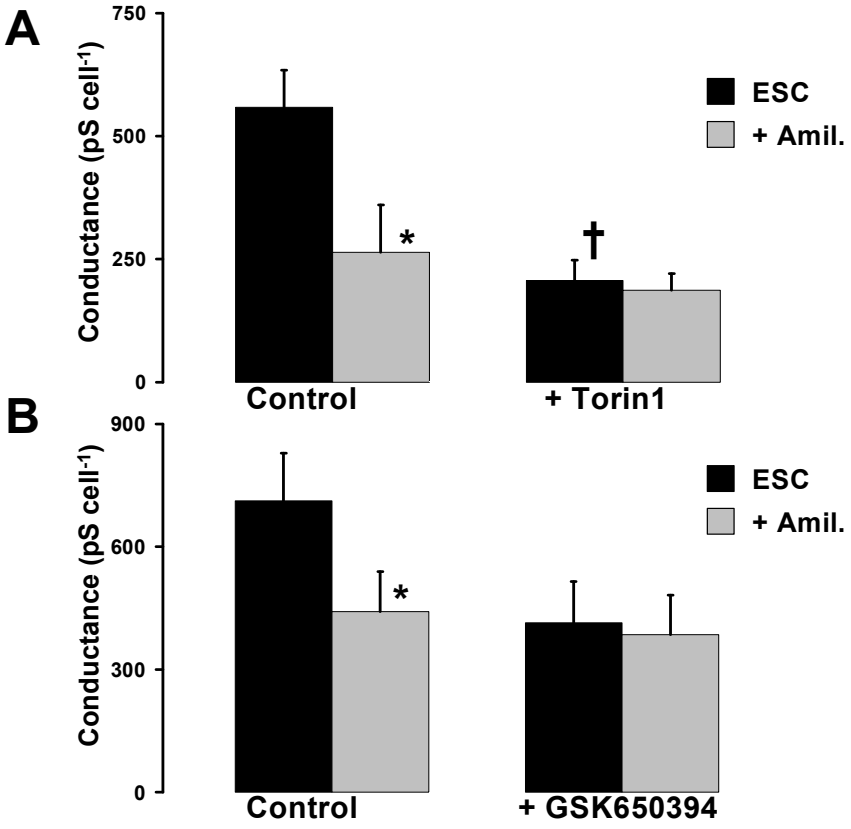


Figure 5.11 Effects of Torin1 and GSK650394 upon conductance. Further analysis of the data obtained from figures 5.4 - panel (A) (control, n = 4; Torin1, n = 4) and 5.8 – panel (B) (control, n = 4; GSK650394, n = 4). Asterixes denote a statistically significant effect of amiloride (* $P < 0.05$, Student's paired t-test), cross denotes a statistically significant effect of Torin1 (†, $P < 0.01$, one-way ANOVA with Bonferroni post hoc test).

Discussion

Torin1 clearly abolishes TORC2 activity as judged by PKB-Ser^{473} phosphorylation, and this effect is unlikely to be a result of inhibition of TORC1 as rapamycin has no effect on TORC2 or PDK1 activity (see chapter four, figure 4.8). However rapamycin-resistant effects of TORC1 have been reported (Thoreen *et al*, 2009) and it cannot be ruled out that the results presented in this thesis do not reflect rapamycin-resistant functions of TORC1. Although in order to examine this possibility an H441 cell line, where TORC2 activity has been abolished, would need to be developed, and until this is accomplished, this possibility cannot be eliminated. However this effect is yet to be fully explored and rapamycin is still considered to be the most effective means of mTORC1 inhibition. At first glance it would appear that Torin1 inhibits PI3K / PDK1 as reduced PKB-Thr^{308} phosphorylation was observed. However this effect is likely to be a result of reduced PKB-Ser^{473} facilitated phosphorylation of PKB-Thr^{308} (Andjelkovic *et al*, 1997). Furthermore Torin1 has been shown to only inhibit PI3K / PDK1 at concentrations above 1 μM (Thoreen *et al*, 2009). Thus Torin1 was predicted to cause some inhibition of PKB-Thr^{308} phosphorylation and the results from the dose response (see figure 5.3) support this as there is little effect on PKB-Thr^{308} phosphorylation despite reduced PKB-Ser^{473} phosphorylation. The observation that Torin1 caused a reduction of SGK1 activity as indicated by reduced NDRG1-Thr$^{246/256/366}$ phosphorylation, is consistent with reports that SGK1 activity is dependent on mTORC2 mediated phosphorylation of the hydrophobic motif of SGK1 and therefore supports the view that mTORC2 is involved in SGK1 activation not only in renal epithelia (Garcia-Martinez and Alessi, 2008; Lu *et al*, 2010) but also in pulmonary epithelia. As exposure to Torin1 abolished the dexamethasone-induced I_{amil} it would seem likely that mTORC2 dependent SGK1 activity is vital to the maintenance of

glucocorticoid-induced ENaC activity and further supports the view that SGK1 activity is critical to this in pulmonary epithelia.

In order to determine whether SGK1 is crucial to glucocorticoid-induced ENaC activity, we used the novel inhibitor GSK650394. The results clearly demonstrate that GSK650394 abolished SGK1 activity without affecting TORC2 or PDK1 activity; furthermore it would appear not to inhibit PKB activity as judged by PRAS40-Thr^{246} phosphorylation. As both TORC2 and PDK1 are involved in the activation of PKB and SGK1, these results demonstrate that GSK650394 acts via a direct mechanism to cause specific inhibition of SGK1 and confirms the results reported by Sherk *et al*, 2008. No evidence of PKB inhibition was observed (see figures 5.6 and 5.7), which differs from that reported in Mansley and Wilson (2010b); however this could be a result of species specific variation as those observations were from a mouse cell line (mpkCCD). Electrophysiological data from GSK650394 treated cells clearly show that SGK1 inhibition abolishes the dexamethasone-induced I_{amil} therefore SGK1 must be an essential component of the pathway that allows glucocorticoids to maintain ENaC activity. By specifically inhibiting SGK1, other parts of the PI3K pathway remain unaffected and this is significant as PKB has been reported to be involved in the control of ENaC activity (Lee *et al*, 2007; Diakov *et al*, 2010), as have the phosphatidylinositide second messengers; PIP_2 and PIP_3 (Blazer-Yost *et al*, 2004; Pochynyuk *et al*, 2007). As PKB-Thr^{308} phosphorylation is evident in GSK650394-treated cells, PIP_2 / PIP_3 must be active as PDK1 is dependent upon these second messengers. While these results do not exclude the possibility that PKB or PIP_2 / PIP_3 can modulate ENaC activity, they clearly demonstrate that they cannot be responsible for SGK1 independent maintenance of ENaC activity. Both Torin1 and GSK650394

abolished the dexamethasone-evoked conductance and this data clearly confirms that TORC2 and SGK1 are vital to the maintenance of dexamethasone-induced ENaC activity.

While the I_{amil} was clearly abolished in GSK650394-treated cells, the values of V_{rev} are different from that of the control cells and, in fact, are close to the predicted reversal potential for Cl^- (-42 mV). Insulin which has been shown to induce a Cl^- conductance in single-dexamethasone treated cells (Brown *et al*, 2008) is present in the culture media of these experiments. It would seem likely that groups of H441 cells do have a Cl^- conductance as in dexamethasone free treatments and, upon application of amiloride, the V_{rev} is ~ 60 mV, a value between that of the predicted reversal potential for K^+ and Cl^- (-82 mV and -42 mV respectively). Thus it is possible that GSK650394 inhibits K^+ conductance as well as abolishing ENaC activity and as a result, Cl^- movement is the predominant ion flux. This effect of GSK650394 is unlikely to be related to SGK1 inhibition as it is not observed with Torin1 / PI-103, both of which inhibit SGK1 activity comparably with GSK650394. This effect has not been described previously and may represent as yet undiscovered non-specific effects of GSK650394 on kinases involved in the regulation of K^+ channels.

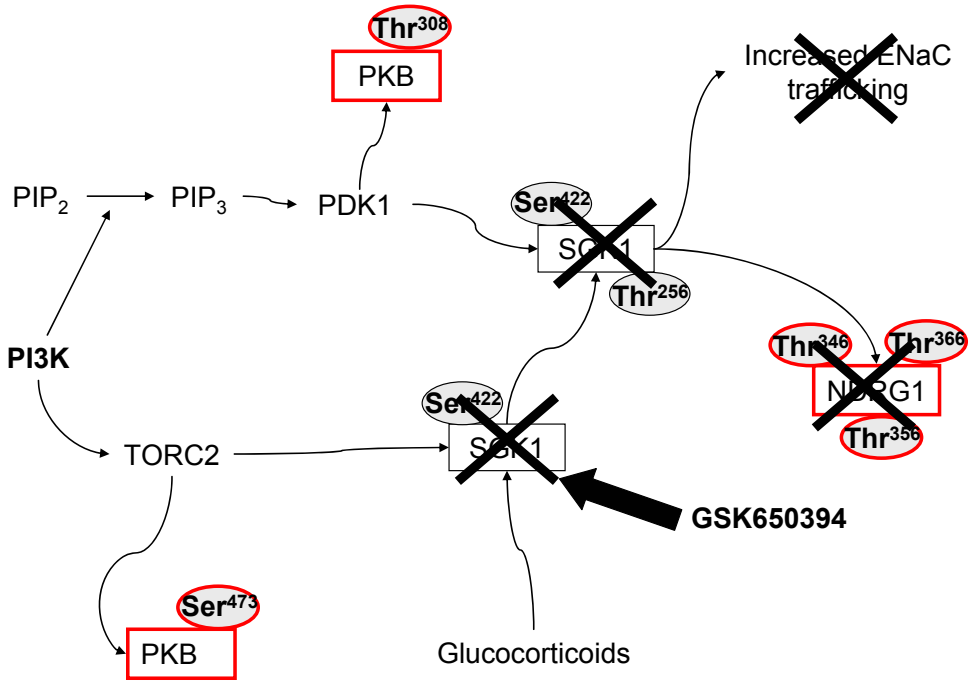


Figure 5.12 Diagram showing effect of SGK1 inhibition. SGK1 inhibition resulted in the abolition of glucocorticoid-induced ENaC activity and abolished the phosphorylation of NDRG1 without affecting the phosphorylation of PKB. Thus indicated that SGK1 was specifically inhibited and that SGK1 is required for the maintanence of glucocorticoid-induced ENaC activity.

Conclusions

This data clearly demonstrates that Torin1 and GSK650394 are specific inhibitors of their respective targets, for the first time in H441 cells and, as such, confirm reports (Garcia-Martinez and Alessi, 2008; Lu *et al*, 2010) that TORC2 is required for conferring catalytic activity to SGK1. This demonstrates that both TORC2 and SGK1 are vital components of the dexamethasone-induced ENaC signalling pathway in a human derived pulmonary epithelium which has previously only been speculated. These results also demonstrate for the first time that PBK and / or $PIP_{2/3}$ cannot account

for the maintenance of ENaC activity in the absence of SGK1 activity. The results of this chapter and the previous results chapters will be discussed further in the general discussion.

Chapter 6 – General Discussion

Introduction

Since the initial observations by Aherne and Dawkins (1964) on rabbit lung wet weight, it has been assumed that the fluid secreted during gestation and development of the lung is absorbed at birth in order for efficient gas exchange to take place. The trigger for this switch is thought to be a result of increased levels of circulating adrenaline, thyroid hormone and GC before birth (Lagercrantz and Bistoletti, 1977; Baines *et al*, 2000) and that Na^+ absorption was the likely candidate driving fluid absorption (Olver *et al*, 1986). However it was not until 1993 that the ion channel responsible for this was identified as ENaC (Canessa *et al*, 1993) which required expression of three ENaC subunits, α-, β- and γ-ENaC, in order for selective Na^+ transport to take place (Canessa *et al* 1994). The observations that GCs can cause an increase in α-ENaC transcription (Sayegh *et al*, 1999; Otulakowski *et al*, 1999) led to further investigation into the molecular mechanisms that underlie GC-mediatated Na^+ transport. SGK1 appeared to be important as it was shown to have PY motifs that were able to interact with Nedd4-2 (Bhalla *et al*, 2005; Ichimura *et al*, 2005). This taken with the observation that Nedd4-2 was able to interact with all three ENaC subunits and target them for degradation (Staub 1996; Snyder *et al*, 2002) led to the theory that SGK1 was the major kinase responsible for GC-mediated Na^+ transport. However the development of *sgk1* gene knockout mice that did not display any lung problems associated with improper fluid clearance seriously questions the role that SGK1 plays. Thus the role of SGK1 is not fully understood in the induction and maintenance of GC-induced Na^+ transport in the lung. However the results of this thesis would suggest that SGK1 is in fact critical to the maintenance of GC-induced ENaC activity.

The results in this thesis further confirm that glucocorticoids activate a Na^+ current in H441 cells that is essentially identical to that associated with the expression of α-ENaC, β- ENaC and γ- ENaC co-expression (Canessa *et al*, 1993). Despite this, the molecular mechanisms that underlie this control are poorly understood, even though GCs are used clinically to treat lung problems such as oedema and IRDS. Therefore we designed experiments to investigate the effects of kinase inhibitors upon the GC-induced Na^+ currents in order to examine the mechanisms underlying this control. In order to verify that full inhibition was achieved and that other closely related kinases were not inhibited, we used Western blot analysis to monitor the phosphorylation status of physiological substrates. However it is important to consider the limitations of this work.

The H441 Cell model

It is important to remember that a model by definition does not represent *in vivo* mechanisms. For example H441 cells are derived from a lung adenocarcinoma (Gazdar *et al*, 1990) and therefore a transformed cell line. Furthermore H441 cells also display characteristics that are similar to that of Clara cells (Gazdar *et al*, 1990), which are not thought to be important to fluid clearance or airway hydration. Furthermore the fact that GCs cause Na^+ transport in H441 cells does not fit with the theory that GC only prepare the foetal lung for fluid clearance and that adrenaline acts as the "switch" to initiate the change to a Na^+ absorbing phenotype (Barker *et al*, 1988; Collett *et al*, 2002). Indeed it is likely that thyroid hormone, as well as GCs, is required in order to prepare the developing lung for fluid clearance at birth (Collett *et al*, 2002). However despite this, it is clear from a number of studies, and the work in this thesis that GCs do induce an ENaC like, amiloride-sensitive Na^+ current in H441 cells (see chapter 3). Therefore while not ideal these cells do provide a model by which to investigate GC-

induced ENaC activation in human derived cells. However it is also important to further develop this work, for example, in a human derived primary cell culture, due to these limitation of the H441 cell model.

Cell-cell contact determines electrophysiological properties of H441 cells

It has been suggested that cell coupling is a determining factor in the ability of H441 cells to express ENaC activity in response to glucocorticoid stimulation (Brown *et al*, 2008). This observation is supported by the finding that dome formation in confluent H441 cells display ENaC activity but that the surrounding cells do not (shlyonsky *et al*, 2005). However the results presented in this thesis would suggest that it is cell contact and not the electrical coupling that is important to the development of amiloride sensitivity in H441 cells. The degree of cell-cell coupling was associated with amiloride sensitivity in glucocorticoid-treated cells in a previous study (Brown *et al*, 2008). However this was not found to be the case in this thesis the results presented here as amiloride sensitivity was seen in "groups" of cells with a C_m of 20 pF, suggesting that the cells were not electrically coupled, which is much lower than the reported average of 75 pF reported by Brown *et al* (2008). However Gallacher *et al* (2009) reported amiloride sensitivity from groups of H441 cells that were not electrically coupled, C_m of 22 pF. As a result this led to the conclusion that cell-cell contact and not necessarily cell coupling was the important determining fact in the ability of H441 cells to express ENaC activity which could be examined future experiments by introducing a dye into the pipette solution.

Na^+ / K^+ ATPase

It could be argued that the increase in Na^+ transport could be driven by increased activity of the Na^+ / K^+-ATPase, however this is unlikely in the case of H441 cells. Na^+ / K^+-ATPase activity was inhibited using ouabain and dexamethasone-treatment had no significant effects on this compared to apically permeabilized cells (i.e. cells with apical Na^+ transport). However dexamethasone has been reported to increase Na^+ / K^+-ATPase in epithelial cells isolated from rat lung (Barquin *et al*, 1997; Dagenais *et al*, 2001), although this difference was suggested to be due to species differences as H441 cells are human derived. It would therefore seem likely that dexamethasone would have no effect upon Na^+ / K^+-ATPase in the cells used in this thesis due to the similarity in cell culture.

Aldosterone

The role of aldosterone was not explored in this thesis, however it is important to note that there is a degree of overlap between the mechanisms involved in aldosterone mediated ENaC regulation in the kidney and how GCs are thought to control ENaC in the lung. Aldosterone binds to the mineralocorticoid receptor and upregulates the gene encoding for SGK1 (Chen *et al*, 1999; Robert-Nicoud *et al*, 2001), and has been proposed to mediate effects upon Na^+ transport by inducing expression of SGK1, which then phosphorylates and inhibits Nedd4-2 (Flores *et al*, 2005), which is the same mechanism proposed for GC regulation of ENaC in pulmonary epithelium. As GC can bind to the mineralocorticoid receptor, and this receptor is known to be expressed in the lung (Keller-Wood *et al*, 2005) an alternative interpretation of the results in this thesis could be that dexamethasone is binding to the mineralocorticoid receptor to bring about the increase in Na^+ transport. As GSK650394 abolished SGK1 activity and the

maintenance of the dexamethasone induced Na^+ transport it is likely that if dexamethasone was acting via mineralocorticoid receptor activation instead of glucocorticoid activation, it is clear that SGK1 is required for the hormonal activation and maintenance of Na^+ transport in H441 cells. Whether dexamethasone is acting via the glucocorticoid or mineralocorticoid receptor could be could be investigated by the use of mineralocorticoid receptor antagonists such as eplerenone, which would determine whether dexamethasone is indeed acting via the glucocorticoid receptor. Furthermore as mentioned in the introduction aldosterone may be able to compensate for a lack of SGK1 via its ability to upregulate GILZ. The possible role of this will be discussed later in this chapter.

Insulin

The role of insulin was not addressed by the experiments in this thesis and dialysed medium was supplemented with this hormone in order to aid growth in this media. The major ionic flux in hormone deprived H441 cells is reported to be K^+ as the membrane potential of these cells is ~-80 mV (Brown *et al*, 2008), however GC-deprived cells in this thesis typically had a membrane potential of ~60 mV, therefore it is likely another ion flux aside from K^+ is contributing to this. The likely candidate for this is an increase in Cl^- transport as mentioned and described in chapter three. It would seem highly likely then that there is an insulin-induced Cl^- current in the cells used in this thesis. This would help explain the amiloride insensitive conductance seen in dexamethasone-treated cells and would also explain why GSK653094-treated cells display a V_{rev} of ~-40 mV which is almost identical to that of E_{Cl} (-42 mV) under these conditions. However, if this was the case, then it might be expected that PI-103-treatment would have abolished amiloride-insensitive and -sensitive conductance. Therefore this is a PI3K independent conductance although, as the nature of this was not investigated, it

cannot be confirmed if this represents a Cl^- conductance. Indeed there are other possibilities as to the nature of this conductance such as cyclic nucleotide gated channels or acid sensing ion channels. There is some evidence for a role of cyclic nucleotide gated channels as pimozide, which has been used to block cyclic nucleotide gated channels (Johnson *et al*, 2006), has been shown to reduce the non-selective cation conductance in single H441 cells (Brown *et al*, 2008) although Albert *et al* (2008) found that pimozide and a cyclic nucleotide gated channel activator (8-bromo-cGMP) had no effect on non-selective conductances in H441 cells. Therefore further investigation would need to be carried out in order to ascertain the true nature of the amiloride-insensitive conductance.

Despite these limitations, the fact that H441 cells do not display an endogenous basal Na^+ current and that this can be activated by GC-treatment, confirms that they provide a model system in which to investigate the molecular mechanisms underlying GC-mediated Na^+ transport. Investigation into the mechanisms that underlie this control will provide a basis in order to direct future work in either animal models or primary cultures of human lung epithelium.

PI3K is essential to glucocorticoid-induced ENaC activity

The results of this thesis also clearly demonstrate that the action of $PIP_{2/3}$ upon channel gating properties such as those reported by Pochynyuk *et al* (2007), cannot account for GC-induced ENaC activity (see chapter five). Furthermore PIP_3 mediated ENaC insertion into the apical membrane can also not account for the action of GCs. Blazer-Yost *et al* (2004) used real-time confocal fluorescence microscopy with a biosensor for PIP_3 that allowed visualisation of the generation and movement of PIP_3 following basolateral insulin stimulation (PIP_3 biosensor was general receptor for 3-phosphoinositides (GRP1) which had been fused to GFP which had been used

previously to visualise PIP_3 distribution (Oatey *et al*, 1999; Yang *et al*, 2000). Using this method they came up with an alternative pathway that would allow for ENaC insertion into the membrane. This model predicted that PIP_3 was very rapidly generated in the lateral membrane and crossed the tight junctions to the apical membrane resulting in changes to lipid composition of the membrane which may favour channel insertion. However this response is very rapid (Na^+ transport plateaus after ~10 minutes) and, as the response to GCs is not active after three hours, it would seem unlikely that this action of PIP_3 could account for GC-induced ENaC activity seen in H441 cells. On the other hand they cannot exclude a role for $PIP_{2/3}$, although they do demonstrate that GC-induced ENaC activity cannot be maintained by these second messengers independently of SGK1.

While the experiments in this thesis confirm that the activation of GC-induced ENaC activity involves the PI3K – TORC2 – SGK1 pathway, they also demonstrate the importance of PI3K. While SGK1 may appear to be a vital component downstream of PI3K in maintaining ENaC activity, the fact that PI-103 abolished this effect demonstrates that, while PI3K activity is not increased via GC-stimulation, without its activity, the response to GC cannot be maintained. Thus PI3K exerts overall control over GC-induced ENaC activity even though its activity is not affected by GCs. Thus PI3K has a permissive role in the GC-induced activation of ENaC. This may represent a level of control that helps protect against hyperactivation of ENaC, whereby further increases in ENaC activity may be possible with additional stimulation of PI3K. This would help prevent hyperactivation of ENaC as the level of ENaC activity induced by GC is limited by PI3K.

TORC1 does not play a role in glucocorticoid-induced ENaC activity

The results of this thesis are in agreement with earlier reports that suggest PI3K is critical to the GC-dependent control of ENaC and also provide evidence against a role for TORC1, but support a role for TORC2, in the underlying mechanism which accords with recently published studies (Garcia-Martinez and Alessi, 2008; Lu *et al*, 2010). However there is evidence for the involvement of TORC1 in SGK1 activation (Hong *et al*, 2008). Hong and colleagues report that TORC1 is responsible for hydrophobic motif phosphorylation of SGK1, as rapamycin inhibited phosphorylation of SGK1 at the Ser^{422} site. Garcia-Martinez and Alessi were unable to replicate this and found no evidence of SGK1-Ser^{422} inhibition by rapamycin, although was found to inhibit S6K1 and / or S6K2, both TORC1 substrates (Terada *et al*, 1992; Lee-Fruman *et al*, 1999). Furthermore previous studies have not reported SGK1 inactivation with the use of rapamycin (Kobayashi and Cohen, 1999; Park *et al*, 1999). As a result of this they suggested that the differences observed from Hong *et al*, 2008 arose because of confusion between non-specific binding of the SGK1 antibody to S6K. Interestingly, further evidence against a role for TORC1 in the activation of SGK1 comes from a study that identified a role for TORC1 in GC-induced Na^+ transport in the mpkCCD cell line (Mansley and Wilson, 2010a). In support of Garcia-Martinez and Alessi (2008) this study found that rapamycin had no effect upon the activity of SGK1 as monitored by NDRG1 phosphorylation; nevertheless they also found that it caused inhibition of GC-induced Na^+ transport. Thus, it would appear that TORC1 is not involved in the activation of SGK1, although it may play a role in regulating GC-induced Na^+ transport. To further complicate matters another study using the same cell line as Mansley and Wilson found that rapamycin did not inhibit hormone sensitive currents (Lu *et al*, 2010). This disparity could be a result of high concentrations of aldosterone in combination

with insulin being used which may lead to the specific effect of rapamycin not being detected (Mansley and Wilson, 2010a). Otulakowski *et al*, (2007) have shown in rat FDLE cells that rapamycin treatment reduces the amiloride sensitive current. While this would appear to be evidence of TORC1 mediated control of ENaC it may actually represent TORC2 inhibition. The FDLE cells used to investigate the bioelectric properties were incubated with 3nM rapamycin for 48 hours before the experiment was carried out. It is therefore important that prolonged exposure to rapamycin has been reported to cause TORC2 inhibition by preventing the formation of the TORC2 complex (Sarbassov *et al*, 2006) and therefore this experiment may in fact represent inhibition of both TORC1 and 2. It would be possible to test this possibility by utilising the approach I used in this thesis to assay for TORC2 activity. It would appear that the role of TORC1 in the regulation of Na^+ transport is still not fully understood and differences in its involvement may exist between the kidney and the lung; however, despite this, it is clear that in the H441 cell line, TORC1 does not play a role in GC-induced ENaC activity.

TORC2 is vital for SGK1 activation and glucocorticoid-induced ENaC activity

In contrast to TORC1, TORC2 seems to play a prominent role in the activation of SGK1. This enzyme requires phosphorylation of its hydrophobic motif and its activation loop at Ser^{422} and Thr^{256} for full activation. The identity of the kinase responsible for activation loop phosphorylation was identified as PDK1. This was shown to increase the phosphorylation of SGK1 and was enhanced by an SGK1 phospho-mimic of the hydrophobic motif (S422D) which resulted in increased SGK1 activity (Kobayashi and Cohen, 1999). This was further supported by the observation

that SGK1 activity and Thr^{256} phosphorylation was reduced by an expression of an inactive PDK1 mutant (Biondi *et al,* 2001). However these studies were not able to identify the kinase responsible for hydrophobic motif phosphorylation and it was not until the development of TORC2 subunit knockouts that TORC2 was identified as the kinase responsible (Garcia-Martinez and Alessi, 2008). Although this suggested that TORC2 may be involved in the control of Na^+ transport, this study did not investigate the ENaC conductance. The first evidence directly linking TORC2 with a role in Na^+ transport was Lu *et al*, 2010. They found that in mpkCCD cells PP242, an ATP-competitive inhibitor of both TORC1 and 2 resulted in reduced SGK1 phosphorylation and crucially this was not mimicked by rapamycin. PP424 also blocked the hormone-induced current whereas rapamycin did not. Furthermore inhibition of rictor by shRNA, a critical TORC2 component, also inhibited the hormone-stimulated current. These results taken together therefore provided the first evidence that TORC2 dependent SGK1 activity is crucial to Na^+ transport (Lu *et al*, 2010). It is important to note that in the mpkCCD cell line GC-mediated increases in Na^+ transport have been observed to be inhibited by rapamycin and is therefore dependent on TORC1 activity (Mansley and Wilson, 2010a). This appears to reflect differences in the mechanism of control between hormones and indicates that GC-induced Na^+ transport is not dependent on SGK1 activity. While this may be the case in the mpkCCD cell line, or for that matter, the kidney, it is clearly not the case in the H441 cell line as GC-induced ENaC activity was abolished by TORC1/2 inhibition but not abolished by TORC1 inhibition. Thus it is clear that GC-induced ENaC activity is dependent upon TORC2 in lung derived epithelia. This may represent specific differences in the mechanisms controlling Na^+ transport in different cell lines and, while investigation into the processes that control Na^+ transport in other tissues may provide valuable insight, it is

important that detailed investigation is carried out in lung derived cell lines or tissue in order to enhance understanding of the molecular mechanisms involved in hormonal control of ENaC in the lung.

SGK1 is essential to maintain glucocorticoid-induced ENaC activity

While it seems clear that SGK1 activity is dependent upon hydrophobic motif phosphorylation by TORC2, and that inhibition of TORC2 results in reduced ENaC activity, it could be argued that this does not represent evidence of SGK1 mediated ENaC activity. Indeed as the results of Lu *et al* (2010) and Mansley and Wilson (2010a) do not provide evidence of independent SGK1 inhibition, the results may be interpreted as suggesting that TORC2 is the major regulatory kinase and that, while SGK1 activity may be dependent on TORC2, SGK1 plays no role in the regulation of ENaC activity. This alternative interpretation may explain why in *sgk1* knockout mice there is no overt lung phenotype (Wulff *et al*, 2002; Rexhepaj *et al*, 2006) as it could be that TORC2 acts independently of SGK1 to regulate ENaC activity. In order to address this discrepancy we made use of a novel SGK1 inhibitor that does not inhibit other closely related kinases such as mTOR or PKB (Sherk et al, 2008; Mansley and Wilson, 2010b) and see chapter five. As SGK1 activity was abolished along with the GC-induced current this, for the first time using pharmacological inhibition in an epithelial cell line derived from lung epithelium, proves that GC-induced ENaC activity is strictly dependent upon SGK1. The significance of this finding is two fold as it also rules out the possibility that other factors are responsible for the maintenance of ENaC activity following GC-stimulation. For example in the α-ENaC subunit there is a consensus motif for SGK1 and its mutation at Ser-^{621} prevents the increase in ENaC activity that is induced by recombinant constitutively active SGK1 (Diakov and Korbmacher, 2004). This consensus motif has also been shown to be involved in PKB mediated ENaC

stimulation as constitutively active PKB was shown to increase amiloride-sensitive currents in *Xenopus laevis* oocytes expressing α, β and γ-ENaC (Diakov *et al*, 2010). As these studies were carried out using the outside-out patch technique it is unlikely that the effect of SGK1 and PKB could be the result of increased channel trafficking. Furthermore okadaic acid, a non-specific phosphatase inhibitor mimicked the effects of SGK1 and PKB which was abolished by mutation of Ser^{621} (Diakov and Korbmacher, 2004; Diakov *et al*, 2010). Thus it would appear that both SGK1 and PKB can cause increases in ENaC activity, possibly through phosphorylation of the α-ENaC subunit at Ser^{621}. In Fisher rat thyroid cells siRNA directed against PKB and SGK1 was found to decrease ENaC activity and that both kinases were required to maintain ENaC activity (Lee *et al*, 2007). Despite this, they suggest that the reason for the requirement of both kinases could be to provide a safeguard in the event one kinase becomes ineffective. Given these findings it would seem that this may represent a possible alternative mechanism for activating ENaC that is independent of SGK1 and could explain why *sgk1* knockout does not result in lethality. However the results of this thesis clearly show that SGK1 inhibition abolishes ENaC activity while PKB activity is unaffected, therefore it would seem unlikely that PKB mediated ENaC activity could account for the lack of lethality in *sgk1* knockout mice. This does not exclude a role for PKB in the modulation of ENaC activity, for example it may be involved in acute regulation via channel phosphorylation, but it does show that PKB itself cannot maintain ENaC activity independent of SGK1 in H441 cells. Furthermore the results of this thesis cannot determine the exact mechanism by which SGK1 acts and further investigation would be required to discover if SGK1 was acting via an effect on channel gating or by increasing ENaC trafficking and insertion into the membrane.

Alternative pathways

GCs appear to activate ENaC through a type of biological "switch" mechanism whereby they cause an initial increase in SGK1 activity which subsequently reduces over time back to the level of activity seen in un-stimulated cells; however continued ENaC activity is reliant on basal SGK1 activity, as inhibition of SGK1 abolishes ENaC activity. This situation is different from that reported in kidney cells, as GC-induced Na^+ transport is reported to require TORC1 activity (Mansley and Wilson, 2010a) which is clearly not the case for H441 cells. Furthermore inhibition of PI3K and SGK1 does not abolish basal Na^+ transport although, it does prevent increased Na^+ transport that is induced by insulin, therefore PI3K and SGK1 are not required to maintain basal Na^+ transport but are required for insulin-dependent increase in Na^+ transport in the kidney (Mansley and Wilson, 2010b). As stated earlier this may be a result of tissue specific differences and while these findings may help explain why *sgk1* gene deletion does not have a significant effect upon renal Na^+ handling in animals fed a normal diet (Wulff *et al*, 2002), the opposite is true for the lungs as, from the results in this thesis, it would be expected that *sgk1* gene deletion would be fatal as it would result in the inability of animals to clear the lungs of fluid at birth. However this is clearly not the case in mice lacking the *sgk1* gene (Wulff *et al*, 2002; Grahammer *et al*, 2006; Rexhepaj *et al*, 2006; Fejes-Toth *et al*, 2008) and part of the explanation may represent a limitation of the H441 cell line as an appropriate model or represent species specific differences. However an alternative explanation may lie with another GC regulated protein kinase. Indeed there is evidence to support an alternative mechanism that would enable GCs control over ENaC without the involvement of the PI3K – TORC2 – SGK1 pathway, this will be discussed in the next section. It would seem likely that an event

such as fluid clearance which is critical to survival would not be reliant upon the activity of just one kinase in order to take place.

Possible role of GILZ

Soundararajan *et al*, (2005) used western blot and electrophysiological analysis to show that aldosterone treatment resulted in decreased ERK phosphorylation which was paralleled by an increase in amiloride-sensitive Na^+ transport. This effect was mimicked by GILZ expression and the mitogen-activated protein kinase inhibitor U0126, thus suggesting that the effect of GILZ was due to ERK inhibition (Soundararajan *et al*, 2005). Building on these observations, Soundararajan and colleagues proposed the idea of an ENaC regulatory complex which would contain Nedd4-2 and Raf-1 and would be able to interact with ENaC, resulting in ubiquitination and degradation. They were able to show, using co-IP experiments, that there was an interaction between Nedd4-2 and Raf-1 with ENaC and with each other. Furthermore GILZ and SGK1 were shown to interact with Nedd4-2 and Raf-1 and that GILZ expression enhanced SGK1 interaction with Nedd4-2 and Raf-1. Expression of GILZ and / or SGK1 also resulted in increased surface expression of ENaC (Soundararajan *et al*, 2009). This provides compelling evidence that in the kidney there is a mechanism that could regulate ENaC activity / expression independently of SGK1 via GCs. While GILZ and SGK1 appeared to have a synergistic action the possibility that GILZ could compensate for SGK1 cannot be ruled out and may provide an answer as to why *sgk1* gene deletion does not result in failure to clear the lungs of fluid in *sgk1* knockout mice. However this work was carried out in kidney cell lines and there is little investigation into the effects of GILZ on Na^+ transport in lung epithelium, however some preliminary evidence has shown that GILZ1 transfection in H441 cells can mimic the effect of dexamethasone-stimulation on the electrophysiological properties of H441 cells

(unpublished observation, Michael Gallacher). However due to the nature of the transfection procedure this could only be observed in single H441 cells and, as the evidence in this thesis as well as numerous studies have confirmed, there are differences in the way that single H441 cells respond when compared to groups / confluent monolayers (Clunes *et al*, 2004; Ramminger *et al*, 2004; Brown *et al*, 2008; Gallacher *et al*, 2009). Nevertheless this still provides compelling evidence that GILZ may be able to compensate for *sgk1* gene deletion in human pulmonary epithelium and warrants further investigation. Aldosterone release may be able to compensate for the lack of SGK1 as it can also upregulate the genes encoding for GILZ (Robert-Nicoud *et al*, 2001), and this would seem likely as there is increased levels of aldosterone in *sgk1* gene knockout mice (Wulff *et al*, 2002).

If GILZ can compensate for a lack of SGK1 then the question, why does inhibition of SGK1 abolish ENaC activity, is pertinent. It could be that the compensatory role only takes effect under exceptional circumstances, such as that of gene knockout, while under "normal" conditions GILZ plays a supporting role whereby it acts to enhance the activity of SGK1. The observation that GILZ was able to enhance SGK1 interaction with Nedd4-2 may support this. Furthermore transfection of higher levels of SGK1 was required to bring about a similar effect on ENaC surface expression than when SGK1 and GILZ were co-transfected (Soundararajan *et al*, 2009). Electrophysiological and Western blot analysis from cells in which the *sgk1* gene has been knocked out, may shed light on this and an increase in GILZ expression over control cells would likely be observed if this mechanism was compensating for a lack of SGK1. As Nedd4-2 is the likely convergence point of both GILZ and SGK1 then it might be expected that Nedd4-2 knockout would result in rapid or even premature fluid clearance. In agreement with this, a recent study using Nedd4-2 knockout mice has shown that these mice are not

viable and that very few manage to survive even a few days after birth. The chief cause of death is respiratory distress. However, unlike α-ENaC knockout mice and consistent with a role for Nedd4-2 in ENaC ubiquitination and degradation, this is due to premature fluid clearance rather than a failure to clear fluid which results in lung collapse (Boase *et al*, 2011). Furthermore mice that survived after birth developed fatal inflammation of the lung which can be associated with the over-expression of ENaC (Mall *et al*, 2004). Interestingly, another group who developed an independent Nedd4-2 knockout mouse known as the Yang mouse, found that there was no lung phenotype (Shi *et al*, 2008). However, this was argued to be the result of a mixed genetic background and hypomorphic nature leading to a milder phenotype, as Boase *et al*, 2011 found Ned4-2 mRNA and protein bands when they analysed the Yang mice. While this study did not look at SGK1 activity, its results would seem to fit well with the findings in this thesis, as they indicate that the major regulatory force controlling ENaC activity in lung epithelium involves the SGK1/Nedd4-2 pathway.

The model most often used to explain the role of SGK1 in the hormonal control of ENaC is based upon these theories:

- That GC-induced Na^+ transport is dependent upon SGK1 activity.
- That the magnitude of the Na^+ current correlates with SGK1 activity.
- That the increase in Na^+ transport is the result of increased ENaC trafficking and insertion into the apical membrane.

The results presented within this thesis confirm that GC-induced Na^+ transport is dependent upon SGK1 activity. However, this does not correlate with sustained activity of SGK1, and the effects of GCs on SGK1 activity appear to be transient. As with PI3K and TORC2, the role of SGK1 would appear to be permissive. The increase in SGK1 activity is likely to reflect an increase in protein synthesis which has been demonstrated

in renal epithelia (Gonzalez-Rodriguez *et al*, 2006), and it could be that the initial increase in SGK1 activity is required to "switch on" ENaC activity; however continued maintenance of this may be reliant upon continued SGK1 synthesis, at basal levels, which requires activity of PI3K – TORC2 to confer catalytic activity of the newly synthesised protein. As SGK1is reported to have a short half-life (Arteaga *et al*, 2006) continued synthesis could be required in order to maintain ENaC activity. The third predication that SGK1 results in increased trafficking and membrane insertion of ENaC cannot be verified by the techniques used, as, although it may seem logical to suggest that increased ENaC activity is a result of increased surface expression, the results obtained here could also represent a change in gating kinetics and / or activation of silent ENaC already present in the membrane, therefore allowing for increased current density without affecting the number of channels. In order to further understand the role that SGK1 plays it would be reasonable that future experiments were carried out that were designed to investigate surface expression of ENaC in response to GC-stimulation.

Do glucocorticoids affect surface expression of ENaC?

A number of studies have employed different techniques such as immunocytochemistry and surface biotinyalation (for examples see: Debonneville *et al*, 2001; Soundaraajan *et al*, 2005; Frindt *et al*, 2008) in order in investigate surface expression of ENaC. However, problems exist with all these methods, for example immunocytochemistry, while useful, is unable to provide sufficient resolution, whereby pools of ENaC near the membrane surface cannot be differentiated between ENaC that has been inserted into the membrane. Surface biotinyalation assays would appear to be a very effective way to investigate the surface expression of ENaC and have been used to show aldosterone induced increases in ENaC in the apical membrane of the amphibian renal cell line A6

(Alvarez de la Rosa *et al*, 2002) and increases in ENaC density in whole rat kidney in response to aldosterone (Frindt *et al*, 2008). This method relies on the use of antibodies in order to detect ENaC protein and there is a lack of efficient antibodies available which has hindered progress in this area. ENaC antibodies often detect multiple bands and this is likely because of proteolytic cleavage of channel subunits. Protease and protease inhibitors can modify Na^+ transport (Myerburg *et al*, 2006; Planes *et al*, 2009; Lazrak *et al*, 2009) and it is likely that post translational processing of the α- and γ-subunits but not the β- subunit are required for maximal activation of the channel (Hughey *et al*, 2004; Myerburg *et al*, 2006; Passero *et al*, 2008; Diakov *et al*, 2008). However it is poorly understood how the cleaved products of ENaC subunits relate to ENaC activity and it has even been suggested that proteolytically cleaved forms of ENaC may not be detectable by current methods (Myerburg *et al*, 2010). Therefore significant advances will need to be made in order to better understand how this relates to ENaC activity. The end result of this is that ENaC antibodies often detect multiple bands and it is unclear which best represent ENaC. Despite this surface biotinylation represents one of the most effective ways to investigate surface expression. In rat kidney a two to five-fold increase in ENaC surface expression was observed, yet channel activity was increased over fifty-fold. Thus it was argued that changes in surface expression cannot fully account for the increase in channel activity and it would appear that increased Na^+ transport is a result of both increased trafficking and increased channel activity (Frindt *et al*, 2008). These were also the findings of Myerburg *et al* (2010) who used trafficking inhibitors to demonstrate that ~60% of the Na^+ current in human bronchial epithelial cells (HBEs) was due to trafficking and that further increase was due to increased channel activity by proteolytic cleavage. It is therefore interesting that SGK1 inhibition completely blocks amiloride-sensitive Na^+

transport in H441 cells, as it might be expected to reduce this only if it was involved in trafficking. A likely explanation for this is the strict conditions in which H441 cells were maintained. Insulin and GCs were the only hormones present in the media of H441 cells that were used for experiments, for example the media that HBEs were maintained in contained hydrocortisone, insulin and epinephrine. As the release of epinephrine is critical to the activation of the Na^+ absorbing phenotype, it is possible that if it were introduced into the experiments in this thesis, it would result in augmenting the response to GCs, possibility by activating ENaC channels already present in the membrane or by increasing the activity of newly inserted channels.

Logical experiments that would follow on from the experiments presented in this thesis would be to assay for surface expression in response to GC-treatment. It might be expected that, if following the general conceptual model, this would result in increase in surface expression of all three ENaC subunits in H441 cells treated with dexamethasone. Such experiments using surface biotinylation have been carried out (in our lab) with unexpected results. All three channel subunits were found to be expressed in the surface of GC-deprived cells, therefore the absence of ENaC in the membrane does not account for the lack of an amiloride sensitive current in these cells. GC-stimulated cells on the other hand, increased the surface expression of α-ENaC, although had no effect on either β- and γ-ENaC. Therefore despite all subunits containing PY motifs that are able to interact with Nedd4-2 (Staub 1996; Snyder *et al*, 2002), only α-ENaC surface expression is increased; therefore this data in combination with that presented in this thesis, does not fit with the hypothesis that GC-induced ENaC activity is a result of coordinated increase in trafficking of all three channel subunits to the membrane. However, the increase in α-ENaC may be required in order for the appropriate channel stoichiometry to form and these results may reflect the

formation of a 2α, 1β and 1γ stoichiometry. Furthermore the use of kinase inhibitors (PI-103, Torin1 and GSK650394) only reduced α-ENaC expression. This could be seen to be consistent with the theory that α-ENaC insertion results in channel activity. This would also accord well with the data suggesting that the α-subunit is the major pore forming subunit (Canessa *et al* 1994; Hummler *et al*, 1996). Indeed it could be that an α-ENaC subunit, phosphorylated via SGK1 (Diakov and Korbmacher, 2004) could be recruited to an ENaC complex that contains only one subunit each, therefore allowing channel activation when this second α-subunit is recruited. A surprising result of this thesis was that three hours of GC-treatment did not result in an amiloride sensitive current, although it does correspond with an increase in SGK1 activity. It was therefore interesting that this acute GC-stimulation appeared to increase the surface expression of all three subunits. If the previously mentioned theory is to be believed, then this result may not be too surprising, as the increase in subunits was even, meaning the ratio of subunits will be the same and not favour the formation of a 2α, 1β and 1γ stoichiometry. However the results of these surface expression experiments, taken together with the results of this thesis, do not support the theory that coordinated increases in ENaC membrane expression are a result of SGK1-dependent trafficking of ENaC to the membrane. Despite this they are consistent with the view that SGK1 is vital to the hormonal regulation of ENaC by GCs and therefore the role of SGK1 would still appear to be unclear.

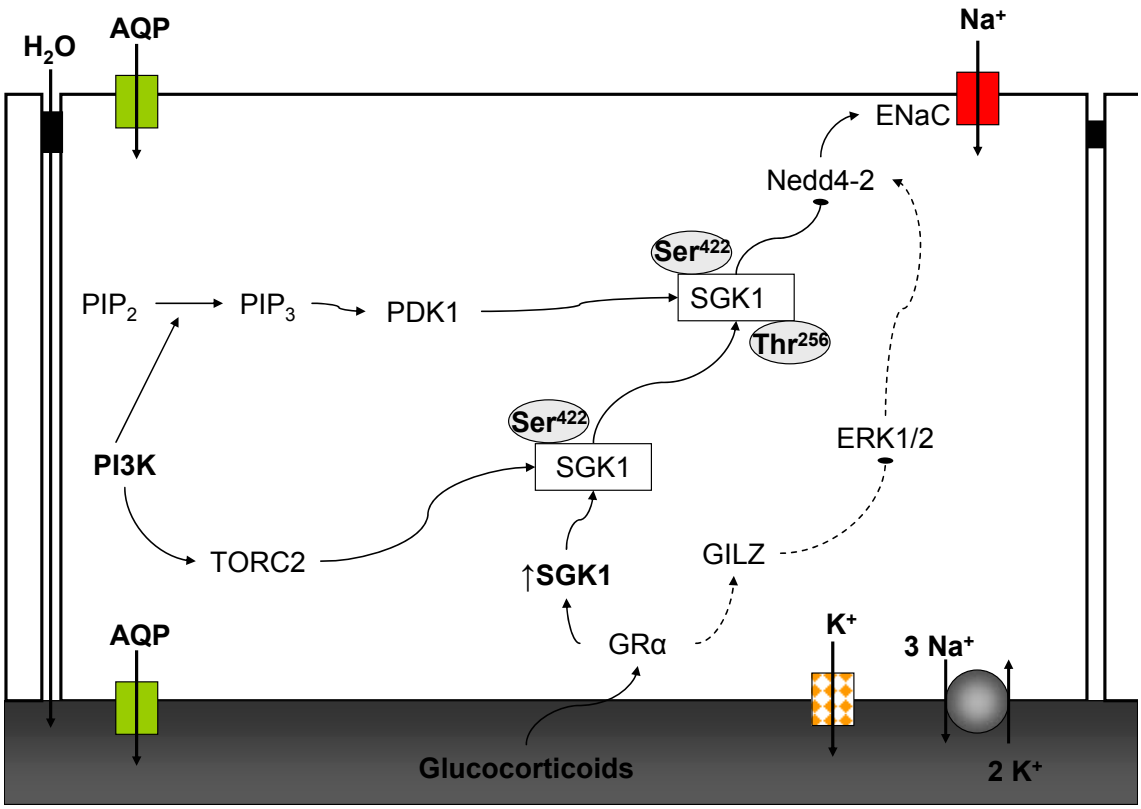


Figure 6.1 Diagram of glucocorticoid induced ENaC activity. Glucocorticoid induced ENaC activity is dependent upon SGK1, however SGK1 activity is dependent upon phosphorylation of two PI3K dependent kinases, TORC2 and PDK1, which phosphorylate Ser^{422} and Thr^{256} residues on SGK1 respectively. However another glucocorticoid induced kinase, GILZ, may be able to compensate for a lack of SGK1 activity, for example in SGK1 gene deletion, in order to ensure that fluid clearance take place at birth.

Main Conclusions

- Glucocorticoids induce an amiloride-sensitive ENaC like conductance in groups of H441 cells.
- Cell-cell contact is crucial for the expression of glucocorticoid-induced ENaC activity in H441 cells and not electrical coupling.
- Glucocorticoids do not increase the activity of PI3K, PDK1 or TORC2. However they do induce a temporal increase in SGK1 activity.
- ENaC conductance does not mimic the temporal increase in SGK1 activity.
- Despite this ENaC activity cannot be maintained in the absence of SGK1 activity.
- PI3K plays a critical, but permissive, role in glucocorticoid-induced Na^+ transport in H441 cells.
- $PIP_{2/3}$ and / or PKB alone cannot maintain glucocorticoid induced ENaC activity
- The mechanism of glucocorticoid regulated ENaC activity in H441 cells involves the PI3K – TORC2 – SGK1 pathway.

Thus the main hypothesis stated in the introduction which was: SGK1 activity is vital to the hormonal control of ENaC activity via glucocorticoids, cannot be rejected in the H441 cell line.

Future work

While this work confirms that SGK1 is vital to GC regulated control of Na^+ transport in the H441 cell line, this work would need to be expanded upon in primary cell culture and lung slices in order to determine whether this is the case *in vivo*. This could be achieved simply be repeating the experiments in this thesis for example using human

nasal epithelia, obtained from nasal scrapings. This study only address the role of glucocorticoids in the regulation of ENaC and as there is many more regulators of ENaC then it is important to take these into consideration for future experiments. For example it could be that *in vivo* other regulators of ENaC could cover of the lack of a lung phenotype seen in *sgk1* gene knockout mice, such as GILZ, cAMP and proteolytic cleavage, but that any compensatory effects of these are only seen *in vivo*. For example patch clamp experiments on lung slices from the *sgk1* gene knockout mice in combination with Western blot analysis could determine whether GILZ is active and if inhibition of GILZ affects Na^+ transport.

While it would seem likely that SGK1 would cause an increase in surface expression, it would appear this is not the case (unpublished observation, Noor Isamal), therefore the role of SGK1 is still unknown. It would perhaps be pertinent to look at alternative mechanisms by which SGK1 may control ENaC activity, such as channel phosphorylation. However, as this work was carried out in a model cell line, it may not represent the true *in vivo* mechanisms behind GC regulation of Na^+ transport, although it does provide an excellent basis from which to investigate further. Therefore greater understanding would be gained by investigating the effects of the inhibitors used in this thesis in primary cell cultures such as human bronchiolar epithelial cells, cultures of isolated ATI and ATII cells or lung slice preparations. This would help clarify the role of the PI3K – TORC2 – SGK1 pathway in systems that better represent an *in vivo* situation and by furthering our understanding of the molecular mechanisms involved in glucocorticoid control of Na^+ transport, new and improved treatments could be developed. For example a drug targeted towards SGK1 inhibition could be developed that would aid the treatment of Liddle's syndrome, a condition underpinned by hyperactivation of ENaC.

The experiments in this thesis could be further expanded upon by investigating whether removal of dexamethasone would abolish ENaC activity. It could be that while SGK1 activity is required at basal levels to maintain ENaC activity, the requirement for GC is only necessary for the initial activation. However as the *sgk1* gene also contains a GRE (Itani et al, 2002) it could be that *de novo* synthesis of SGK1 could be sufficient to maintain ENaC activity. This would still rely on activation by PI3K – TORC2 in order to confer catalytic activity. By replacing media with dexamethasone free media for example after 24 hours stimulation, it would be possible to investigate whether the presence of GC is required for maintenance of ENaC activity or if the initial increase in SGK1 is important. In addition it would be interesting to investigate whether exposing cells to dexamethasone that rises and falls in a cyclical fashion would maintain ENaC activity and / or keep SGK1 activity elevated as this would mimic the pattern of GC release in the human body more closely.

Glucocorticoids are important to the switch between net fluid clearance to net fluid absorption, and this is maintained throughout life in order to maintain a physiological depth of the ASL. The results in this thesis suggest that SGK1 is critical to maintain the Na^+ absorption, via ENaC that drives fluid absorption in order to maintain ASL depth. However, there are many other regulators of ASL depth, such as ATP, flow rate, PO_2, adrenaline and arginie vasopressin, which may be able to compensate for the lack one or more inputs, (for example, *sgk1* gene knockout in mice) in order to achieve normal fluid homeostasis. How all these factors come together to regulate ASL depth is the ultimate goal, while the results in this thesis add to this knowledge much work is still required to fulfil this goal.

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