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SALT-INDUCIBLE KINASE 1: A NOVEL REGULATOR WITHIN A CELL SODIUM-SENSING NETWORK

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Stockholm 2010

Published by Karolinska Institutet and printed by Larserics Digital Print AB, Sundbyberg, Sweden

ISBN 978-91-7409-855-6

The pathways to heaven and hell are activated by the same signaling substance

love

the difference is in how it is received

how it is given

which catalyst that is used to guide the traveler to its destination

the results cannot be measured or calculated

feeling is essential

To Per & Vera

ABSTRACT

Na^+, K^+-ATPase (NK) is expressed in all animal cells and plays a crucial role in cellular ion homeostasis. The NK activity is tightly regulated by specific signaling networks that must be well controlled in time and space (at the plasma membrane or in the intracellular compartments) to guarantee the specificity of the physiological response. The aim of this thesis was to identify a sodium-sensing network that is ubiquitously present in mammalian cells and senses accurately small elevations in intracellular sodium and translates this signal to the NK molecule. Furthermore, it was investigated whether this network could participate or has cell biology relevance during the development of hypertension and complications such as cardiac hypertrophy.

We identified a new protein, salt-inducible kinase 1 (SIK1) that associates with the NK complex and participates in the regulation of its activity during elevations in intracellular sodium. The increases in intracellular sodium in the opossum kidney proximal tubule cell line (OK cells) are paralleled by the elevation in intracellular calcium through the Na^+/Ca^{2+} exchanger, which is leading to the activation of SIK1 (Threonine 322 phosphorylation) via a calcium-calmodulin dependent kinase. The activation of the SIK1 triggers the phosphorylation of the protein phosphatase methylesterase-1, which dissociates from its protein phosphatase 2A (PP2A). PP2A dephosphorylates the NK α-subunit, which leads to its increased NK activity. We also observed that SIK1 activity (pT182 phosphorylation) was significantly elevated in both hypertensive Milan rats and OK cells carrying the hypertension linked mutation in the cytoskeleton protein α-adducin when compared with the α-adducin normotensive control. By blocking the SIK1 network, we prevented the increase in NK activity induced by the mutated variant of α-adducin. Furthermore, we examined in cardiomyocytes whether angiotensin II (AngII) *per se* and independently of the rise in blood pressure may contribute the development of cardiac hypertrophy by influencing the SIK1 network. Our data demonstrated that AngII and the presence of α-adducin carrying the hypertension-linked mutation increase myocyte enhancer factor-2 (MEF2) and nuclear factor of activated T-cells (NFAT) activity, effects that were blocked by inhibition of SIK1. We also demonstrated that AngII increases the hypertrophic-associated cardiac genes: natriuretic peptide precursor type B, skeletal actin, and α-myosin heavy chain.

Together, the results of this thesis reveal novel information about a cell sodium-sensing network, with SIK1 at its core, and the regulation of the active sodium transport in renal epithelial cells. Additionally, it highlights the significance of this network in hypertension and derived complications such as cardiac hypertrophy.

Keywords: Na^+, K^+-ATPase, α-adducin, Milan hypertensive and normotensive strain of rats, hypertension, angiotensin II, cardiac hypertrophy, myocyte enhancer factor-2 and nuclear factor of activated T-cells

POPULÄRVETENSKAPLIG SAMMANFATTNING

Högt blodtryck är en folksjukdom som idag drabbar i genomsnitt var fjärde människa i världen och kan leda till bl a hjärnblödning, hjärtinfarkt samt hjärtsvikt. Högt blodtryck kan uppstå när blodkärlets väggar drar ihop sig och/eller när hjärtats pumpkraft ökar. En bristande jämvikt av vatten och natrium (som finns i vanligt bordssalt) i kroppen kan leda till ökad vätskevolym, vilket också kan bidra till ett ökat blodtryck.
Det har visat sig att ett förhöjt saltintag ökar risken för högt blodtryck. Njurarna är delvis ansvariga för kroppens natrium- och vattenbalans genom att reglera natriumutsöndringen. En stor del av kroppens natrium återupptas till blodet i en del av njuren som kallas *proximala tubuli*, vilket medför att vatten blir kvar i kroppen, med ett högt blodtryck som följd. Cellerna i *proximala tubuli* innehåller en stor mängd natrium-kaliumpumpar, proteiner som reglerar natrium- och kaliumnivån i våra celler. Utan dessa livsviktiga pumpar skulle natriumkoncentrationen inuti cellen öka, vatten skulle strömma in och cellen skulle sprängas. Genom att studera dessa pumpar kan vi få ett mått på hur aktiv cellen är i sitt upptag av natrium.

Syftet med den här avhandlingen var att identifiera en natriumkänslig mekanism som möjliggör regleringen av ett för högt saltintag i njurceller, samt att undersöka om en sådan mekanism kan ha någon relevans för uppkomsten av högt blodtryck samt komplikationer som *hypertrofi* (ökad cellstorlek, i detta fall hjärtmuskelvägg).
I den första studien visar vi att natrium-kaliumpumpen är associerad med proteinet *salt-inducible kinse 1* (SIK1) i njurceller från *proximala tubuli* och att detta protein reglerar natrium-kaliumpumpens aktivitet under saltstress. Forskare har tidigare visat att råttor som intagit saltrik kost har mycket SIK1 i sina binjurar. Vi utförde flera olika experiment för att visa hur SIK1s mekanism hänger samman med regleringen av natrium-kaliumpumpens aktivitet. Vi visar att kalcium samt en rad viktiga proteiner deltar i denna signalväg.
I den andra studien undersökte vi betydelsen av SIK1 i njurceller med eller utan ett defekt cellskelett. Andra forskare har visat att mutationer hos proteinet *α-adducin* förändrar cellskelettet och har betydelse för natrium-kaliumpumpens reglering samt att denna förändring bidrar till högt blodtryck hos både råttor och människor. Vi visar att SIK1s aktivitet är högre i njurceller samt hos råttor med defekt α-adducin, vilket då innebär att SIK1 är involverad i en signalväg som leder till högt blodtryck.
I den tredje studien har vi studerat signalvägar i hjärtmuskelceller från mus. Forskare har tidigare visat att det kroppsegna hormonet *angiotensin II* (AngII) är involverat i högt blodtryck och uppkomsten av *hypertrofi*. Vi använder oss av AngII för att stimulera signalvägar i dessa hjärtmuskelceller. Här undersöker vi om SIK1 kan vara involverad i reglering av två proteiner, *MEF2* och *NFAT*, som har visat sig vara viktiga för regleringen och uppkomsten av *hypertrofi*. Vi visar att SIK1-aktiviteten ökar då hjärtmuskelcellerna utsätts för AngII samt att SIK1 reglerar MEF2 samt NFAT-aktivitet i dess celler.

För att summera arbetet i denna avhandling, så har vi, i och med SIK1s association med natrium-kaliumpumpen, funnit en saltkänslig mekanism som reglera saltbalansen i njurceller från *proximala tubuli*. Vi har även visat att SIK1 har betydelse för reglering av högt blodtryck hos råttor med defekt cellskelett samt bidragit med kunskap om hur SIK1 kan reglera viktiga proteiner som bidrar till förtjockad hjärtmuskelvägg. De nya kunskaperna har bidragit till en ökad förståelse för reglering av natrium-kalimpumpen samt bidragit till nya spännande forskningsprojekt inom detta område. I förlängningen kan denna kunskap även leda till förbättrade behandlingar och mediciner mot högt blodtryck.

LIST OF PUBLICATIONS

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals (I-III):

I. Sjöström M*, **Stenström K***, Eneling K, Zwiller J, Katz AI, Takemori H, Bertorello AM. SIK1 is part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process. *Proc. Natl. Acad. Sci. U S A, 104:16922-16927, 2007*
*authors contributed equally to this work.

II. **Stenström K**, Takemori H, Bianchi G, Katz AI, Bertorello AM. Blocking the salt-inducible kinase 1 network prevents the increase in cell sodium transport caused by a hypertension-linked mutation in human α-adducin. *J Hypertens, 27: 2452-2457, 2009.*

III. Popov S, **Stenström K**, Chedrese PJ, Bertorello AM. Blocking SIK1 in cardiac myocytes prevents the increases in transcription induced by Angiotensin II and a hypertension-linked mutation in α-adducin. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
AMPK	Adenosine monophosphate activated protein kinase
AngII	Angiotensin II
$AT_{1\ and\ 2}$	Angiotensin II subtype 1 and 2
ATP	Adenosine triphosphate
CaMK	Calmodulin-dependent protein kinase
cDNA	Complementary DNA
CNS	Central nervous system
CREB	Cyclic AMP-responsive element binding protein
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ENaC	Epithelial Na^+ channel
GFP	Green fluorescent tag
GST	Glutathione-S-transferase
HA	Hemagglutinin epitope
HDAC	Class II histone deacetylase
HEPG2	Human-hepatoma-derived cell line
HL-1	Cardiac muscle cell line
HT-ADD	Hypertensive variant of α-adducin
MEF2	Myocyte enhance factor-2
MHC	Myosin heavy chain
MHS	Milan hypertensive strain of rats
MNS	Milan normotensive strain of rats
Mon	Monensin
mRNA	Messenger RNA
NCX	Na^+/Ca^{2+} exchanger
NFAT	Nuclear factor of activated T-cells
NHE3	Na^+/H^+ exchanger type 3
NK	Na^+, K^+-ATPase
NPPB	Natriuretic peptide precursor type B
NT-ADD	Normotensive variant of α-adducin
OK cells	Opossum kidney proximal tubule cell line
OLC	Ouabain-like compounds
PCT	Proximal convoluted tubule
PKA	Protein kinase A
PKC	Protein kinase C
PME-1	Protein phosphatase methylesterase-1
PNS	Post-nuclear supernatant
PP2A	Protein phosphatase 2A
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
RPLPO	Large ribosomal protein PO
scRNA	Scrambled RNA
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIK1	Salt-inducible kinase 1
siRNA	Small interfering RNA

SkA	Skeletal actin
SNF1	Sucrose-nonfermenting 1
SNH	Sucrose-nonfermenting homologue
SOS2	Salt overly sensitive 2
TORC2	Transducer of regulated CREB activity coactivator 2
WB	Western blot
WT	Wild type

1. INTRODUCTION

During the evolution of animal cells, the large accumulation of cytosolic proteins resulted in surviving mechanisms that compensated for high intracellular osmolarity and removed sodium (Na^+) and water from the cytosol. The ability to maintain a low and relatively constant concentration of cytosolic Na^+ requires high levels of energy. This transport could consume up to 40 % of the total ATP produced by the cells (Kaplan 2005). The responsible membrane bound transporter for Na^+, present in all mammalian cells, is the Na^+, K^+ ATPase (NK). NK is not only regulating the fluid balance in the cells but is also known to generate a transmembrane chemical and electrical balance which is critical for vital functions such as muscle contraction (Blaustein 1977) and release of neurotransmitters (Gilbert and Wyllie 1980). NK has also been reported to function as a signaling receptor, for exogenous ligands such as ouabain. The endogenous ouabain-like compounds (OLC) detected in mammalian plasma, known to be produced in the adrenal glands, regulate the catalytic activity of the NK (Blaustein, Zhang et al. 2009). The binding of ouabain to NK, results in activation of protein kinase cascades and generation of second messengers in cultured proximal tubule cells (Tian, Cai et al. 2006) and has been reported to participate in the natriuretic response to salt load in mice (Loreaux, Kaul et al. 2008). In humans, a high salt diet elevates the OLC in plasma (Manunta, Hamilton et al. 2006) and an infusion of a low-dose of exogenous ouabain increases vascular resistance and elevates blood pressure (Mason and Braunwald 1964).

The balance of Na^+ (retention or excretion) in the human body is a tightly regulated process by the kidney to maintain fluid volume. Abnormal regulation and failure of the kidney to excrete excessive amounts of sodium are associated with an increased risk for cardiovascular disease such as high blood pressure. The potential failure takes place in the functional part of the kidney, the nephron, which is composed of glomerulus and renal tubules. The renal tubule can be divided into several segments, the proximal convoluted tubule (PCT), loop of Henle, distal tubule and collecting duct. About 70 % of the filtered Na^+, Cl^-, K^+, and bicarbonate are reabsorbed in the PCT cells. The Na^+ reabsorption in these cells is mediated via the apical epithelial Na^+ channel (ENaC), Na^+/H^+ exchanger type 3 (NHE3), Na^+/glucose cotransporter system, Na^+/phosphate cotransporter system and/or the Na^+/amino acid cotransporter system. The uptake of Na^+ at the apical part of the cell is driven by the NK localized in the basolateral, which provides the driving force for vectorial Na^+ transport (Al-Khalili, Kotova et al. 2004). Recent data suggest that an upregulation of molecular expression and function of the NK is paralleled by increases in expression and function of the apical NHE3 (Gomes and Soares-da-Silva 2006).

A defective regulation of the NK within the PCT cells has been shown to be associated with hypertension (Doris 2000). The NK could be regulated both short-and long-term. Short-term regulation (seconds) of NK can be mediated by changing its kinetic activity. The NK could also be regulated by a variety of stimuli (*i.e.,* hormones, hypoxia) that can modify its phosphorylation state. This leads to internalization of the NK units from the plasma membrane into intracellular stores (Ewart and Klip 1995; Therien and Blostein 2000; Teixeira, Katz et al. 2003; Bertorello and Sznajder 2005; Pedemonte, Efendiev et al. 2005). The long-term regulation (hours) is mediated via changes in gene expression and protein synthesis of new pumps, which affect number of functioning units at the plasma membrane (Ewart and Klip 1995).

1.1 CELL HOMEOSTASIS

In order to survive, the signaling mechanisms in all animal cells have to be well synchronized to avoid unnecessary alteration in intracellular equilibrium of fluids and ions (homeostasis). A well regulated cell volume allows the cell to function properly and to keep its structural shape. Cell volume homeostasis requires constant regulation of mechanisms to avoid cell swelling or shrinkage. This is due to the fact that most cells are highly permeable to water which follows the osmotic sodium gradient.

1.1.1 The control of cell volume and sodium concentration

During evolution, an essential necessity for the animal cell survival was the control of alteration in cell volume. The cell is surrounded by a plasma membrane that separates the intracellular compartments from the extracellular milieu. The plasma membrane serves not only as an anchor for the cytoskeleton to keep the cell shape, but also as an important barrier for different molecules to enter and exit the cell. The intracellular compartment contains a large number of molecules that carry a negative charge since their isoelectric point is lower than the physiological pH of the cell. These molecules (proteins, amino acids or carbohydrate metabolites) create intracellular osmotic pressure that requires regulatory mechanisms to maintain the optimum volume of the cell.

Because all animal cells are highly permeable to water, it is the short-term concentration of ions across the plasma membrane that is of critical importance for maintaining an adequate cell volume (Macknight 1988; Lang, Busch et al. 1998). High intracellular osmolarity, due to cellular accumulation of organic substances, is compensated by lowering cytosolic ion concentrations. Small, charged inorganic ions could easily leak through the plasma membrane whereas large charged molecules cannot move freely through the membrane. This could create

an uneven distribution of charged molecules and an electrical field that influences the movement of ions and water across the plasma membrane into the cell, known as the *Donnan effect* (Figure 1). This uneven distribution is prevented by the exchange of 3 Na^+ (out of the cell) and 2 K^+ (into the cell) via the NK. Additionally, this is prevented indirectly by water that follows sodium towards the extracellular milieu to stop the cell swelling. This also compensates for the distribution of uneven electrical charge created by the accumulation of negatively charged molecules intracellularly. The cell membrane is poorly permeable to Na^+ but highly permeable to K^+, which will create an outside-positive cell membrane potential and drive the Cl^- ions out of the cell compensating for the excess of negative intracellular molecules (Lang, Busch et al. 1998).

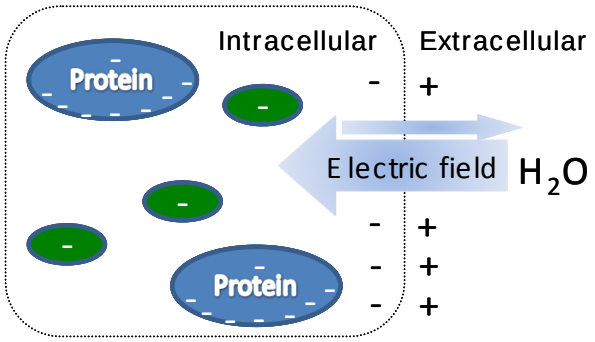


Figure 1. The *Donnan effect of proteins*; charged molecules are unevenly distributed across the plasma membrane, due to large anionic intracellular molecules that are unable to move across the plasma membrane, which contribute to a high intracellular osmolarity. The presence of NK in the plasma membrane will prevent this uneven distribution and consequently the cell to

The NK activity compensates for the osmotic pressure by removing Na^+ ions against its gradient towards the extracellular milieu, which is a necessary survival mechanism, since too high concentration of intracellular sodium is toxic and can create osmotic stress for most living cells (Bohnert, Nelson et al. 1995). The electrochemical gradient for Na^+ into the cell and K^+ out of the cell is maintained by the NK, which is a crucial for several housekeeping functions within the cell, such as the regulation of internal pH via the counter transporter Na^+/H^+ and also for the transport of glucose and/or amino acids into the cell (Glynn 1985; Jorgensen 1986; Shou 1988).

1.2 CELL REGULATION

Cell regulation is a complex system of communication within the cell to coordinate cell actions and to alter cell behavior. The cell regulation and activation in its microenvironment could be initiated by activation of different pathways. A cell signaling cascade could start at the plasma membrane with a receptor-mediated signal. The signaling molecule, "first messenger", binds to the receptor protein and thereby activating an intracellular signaling pathway. The cell signaling cascade could be amplified and/or modulated by both extracellular and intracellular signals. The activation may lead to interaction between proteins and/or second messenger that will regulate the cell activity. A cell signaling event could also affect channels and/or carrier proteins that transport ions across the plasma membrane. A *uniporter* is an integral membrane

protein that only transports one protein or ion in one direction of the plasma membrane. Whereas a *symporter*, transport two or more different molecules or ions across the plasma membrane in the same direction, like the Na^+/Cl^- cotransporter. The *antiport* or counter transporter exchange two or more different molecules or ions across the plasma membrane in the opposite direction, like the NK. Note that these three general classes of transport systems (*uniporter*, *symporter* and *antiporter*) do not reveal whether these systems are energy-requiring or energy-independent.

The NK could be regulated by interaction with various proteins, membrane transporters, channels and cellular receptors (Bertorello, Aperia et al. 1991; Yudowski, Efendiev et al. 2000). A regulation that requires complex intracellular signaling cascades and is organ specific (Bertorello and Sznajder 2005).

1.2.1 The Na^+, K^+-ATPase and its regulation

The Na^+, K^+-ATPase

In 1957 Jens C. Skou discovered the NK as an integral-membrane protein with catalytic ATPase activity in leg nerves of crabs (Skou 1957). This protein is a well known energy transducing ion pump that transports 3 Na^+ out and 2 K^+ into the cell by hydrolysis of one molecule of adenosine triphosphate (ATP) (Figure 2) (Lingrel and Kuntzweiler 1994). The NK belongs to the *P-type ATPases* that form a phosphorylated intermediate during transport, hence the name P-type (Kuhlbrandt 2004). The NK expressed in mammalian cells consists of alpha (α_1,-α_4) and beta (β_1-β_4) subunits. The α_1 and β_1 subunits are expressed in the kidney. The catalytic α subunit, with 10 transmembrane segments, contains a binding site for ATP, Na^+, K^+ and ouabain as well as regulatory phosphorylation sites, whereas the regulatory β subunit participates in the folding, targeting and stabilization of the enzyme to the plasma membrane (Geering 1991; Lingrel and Kuntzweiler 1994). Besides the α and β isoforms, a third subunit, gamma (γ), interacts with the α-β complex in some tissues. This small subunit appears to be

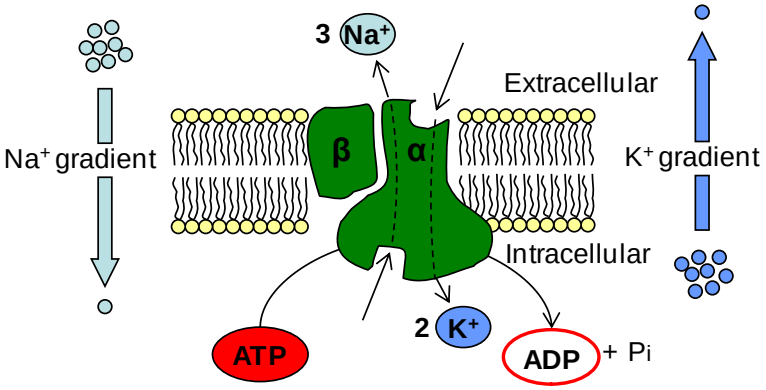


Figure 2. The NK transports 3 Na^+ out and 2 K^+ into the cell by hydrolysis of one molecule of ATP [modified from (Alberts 2002)]. The NK is a heterodimer that consist of a catalytic α-subunit and a regulatory β-subunit. The α-subunit contains the Na^+, K^+, ATP and ouabain binding sites, and is phosphorylated by ATP during each pump cycle. The enzyme actively transports Na^+ out and K^+ into the cell, against their electrochemical gradients.

involved in the modulation of the enzymatic properties of the NK (Beguin, Wang et al. 1997). The NK's are predominantly located at the basolateral membrane of renal epithelial cells. This could be explained by the inability of NK to board the glycosphingolipid-rich rafts that transport proteins to the apical part of the membrane (Rodriguez-Boulan and Zurzolo 1993).

Besides its housekeeping functions, the NK contributes to other regulated and vital functions such as muscle contraction (Blaustein 1977), release of neurotransmitters (Gilbert and Wyllie 1980), renal sodium reabsorption in polar epithelial cells (Rang 1999) and fluid transport in lung alveolar cells (Sznajder, Olivera et al. 1995). Recently it has been suggested that the NK has two functionally separate pools in the plasma membrane; one performing the pumping function and the other a non-pumping function such as signal transduction. It has been suggested that the active NK-pump only work at 1/3 of its capacity and that the non-pumping NK could serve as a reservoir for cells to rapidly adjust the intracellular milieu (Liang, Tian et al. 2007).

Regulation of the Na^+, K^+-ATPase

There are several signaling networks that regulate the NK activity in response to activation of G protein-coupled receptors present at the plasma membrane (Ogimoto, Yudowski et al. 2000; Yudowski, Efendiev et al. 2000). Intracellular signaling pathways, such as cAMP-dependent kinase (PKA) and protein kinase C (PKC) activated upon a stimulation of G protein-coupled receptors, have been shown to phosphorylate the catalytic α-subunit of the NK and thereby modulate its activity (Lowndes, Hokin-Neaverson et al. 1990; Bertorello, Aperia et al. 1991; Chibalin, Vasilets et al. 1992). Chibalin and coworkers determined that the natriuretic hormone dopamine induced NK endocytosis in OK cells, by phosphorylation of PKC at the specific Serine 18 residue within the α_1-subunit (Chibalin, Ogimoto et al. 1999). Furthermore, dopamine favored the association of the protein 14-3-3, which has phosphopeptide binding property and binds to the α-subunit, a critical link in the mechanism to recruit phosphatidylinositol 3-kinase to the site of NK to promote endocytosis (Chibalin, Zierath et al. 1998; Yudowski, Efendiev et al. 2000; Efendiev, Chen et al. 2005). In this complex regulation, the interaction with adaptor protein 2 and clathrin recruitment are also required for the initiation of endocytosis (Ogimoto, Yudowski et al. 2000; Done, Leibiger et al. 2002). The internalization of NK molecules from the basolateral part of renal epithelial cells into the endosomes requires dopamine. Inhibition of the renal NK activity by dopamine during high salt intake leads to an increase in urinary sodium excretion (Bertorello, Hokfelt et al. 1988), and failure in this mechanism has been associated with the development of hypertension (Chen, Beach et al. 1993; Nishi, Eklof et al. 1993).

In contrast, angiotensin II (AngII) functions as antinatriuretic hormone by partly increasing the NK activity in isolated rat proximal straight tubules (Garvin 1991). In the renal epithelia the recruitment of NK from the endosomes to the plasma membrane is initiated by PKC-β dependent phosphorylation within the catalytic α-subunit followed by adaptor protein 1 recruitment (Efendiev, Budu et al. 2003).

The NK is regulated by several circulating hormones. Apart from dopamine and AngII, many others are known to be involved in the control of the NK such as: aldosterone, thyroid hormone, catecholamines, insulin and serotonin (Middleton, Raymond et al. 1990; Ewart and Klip 1995). Furthermore, it has also been demonstrated that the cytoskeleton proteins regulate the NK activity (Bertorello and Katz 1993).

The cytoskeleton is composed of a number of different proteins such as actin, spectrin, ankyrin, fodrin, tubulin and adducin, which all have essential roles in the development and maintenance of structural and functional organization of the cells. The NK is associated and regulated by both ankyrin and α-adducin (Ferrandi, Salardi et al. 1999). Adducin has been shown to play an important role in the determination of cellular morphology and motility and in the regulation of membrane ion transport (Tripodi, Valtorta et al. 1996), as well as indirectly influencing the regulation of the NK activity (Efendiev, Krmar et al. 2004). Adducin promotes association of spectrin with actin (Gardner and Bennett 1987) and caps the fast growing end of actin filaments (Kuhlman, Hughes et al. 1996). Torielli et al., 2008 determined that besides its modulatory effects on actin cytoskeleton dynamics, adducin also plays a direct role in clathrin-dependent endocytosis. A mutation within the α-adducin increases the rate of actin polymerization, which leads to a stiff and rigid cortical cytoskeleton (Tripodi, Valtorta et al. 1996). Efendiev et al., 2004 determined that this defect in the cytoskeleton protein α-adducin lead to defect endocytosis of the NK unit from the PM, and as a consequence increased NK activity (Efendiev, Krmar et al. 2004). Rats expressing the mutated form of α-adducin had an increased NK activity and high blood pressure compared to the normotensive control rats (Melzi, Bertorello et al. 1989).

In summary, the regulation of the NK in the renal tubules and presumably other epithelial cells requires specific interactions, at least transiently, with kinases, phosphatases and scaffolding molecules in time and space (either at the plasma membrane or within intracellular endosomes) to accomplish this complex regulatory machinery.

1.3 HYPERTENSION AND THE KIDNEY

Among other regulatory systems, a defective regulation of the NK has been shown to be associated with the development of hypertension, which is a condition that will cause failure and damage of many organs, including the kidney and the heart. It has been suggested that the control of sodium excretion by the kidney is the dominant mechanism of blood pressure regulation by the renin-angiotensin-aldosterone system (RAAS) (Guyton 1991). Since the NK provides the driving force for vectorial Na^+ transport in renal epithelial cells following reabsorption of Na^+ and water it is likely that the alterations in NK may be involved in the etiology of hypertension.

1.3.1 Hypertension

High blood pressure, a raise in the arterial pressure, is a major contributor to cardiovascular disease (Ezzati, Lopez et al. 2002). It has been recently reported that approximately 25 % of the adult population worldwide suffer from hypertension, and an increased prevalence by 60 % by 2025 will lead to the alarming number of 1.56 billion people that may be affected (Kearney, Whelton et al. 2005). As many as 90-95 % of all cases of hypertension are classified as essential (primary) hypertension, (blood pressure >139/89 mmHg systolic/diastolic), with unknown cause of the disease (Chobanian, Bakris et al. 2003). This severe condition is associated with a significant increase in the risk for progression to heart failure, arrhythmias or sudden death (Levy, Garrison et al. 1990; Lorell and Carabello 2000). A variety of genetic-, lifestyle- and environmental-factors have been associated with the development of hypertension. In the Western population, overweight, physical inactivity, high salt- and low potassium-intake appear to be the major contributors to hypertension (Geleijnse, JM et al., 2004). Today there are many different drugs for reducing a high blood pressure. The β-blockers, diuretics, calcium channel blockers, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers are all available therapies alone or in combinations. In most patients, two or more antihypertensive agents are needed to reach the blood pressure level recommended by national and international guidelines (Hansson, Zanchetti et al. 1998; Chobanian, Bakris et al. 2003; Whitworth 2003). Individual variation in the efficacy and tolerability to antihypertensive therapy could be due to genetic heterogeneity of this multifactorial disease. New strategies with selective treatment in individuals, with fewer side effects and reduced treatment cost, will be of big benefit for the patients.

Different available rat models with genetically derived hypertension, such as the Milan hypertensive strain (MHS) that carries a mutation in the membrane-skeleton protein α-adducin

(Add1; F316Y) (Bianchi 2005), have provided researchers with an excellent tool for studying the mechanisms underlying hypertension. The ubiquitously expressed adducin is composed of either α and β or α and γ subunits (Matsuoka, Li et al. 2000). The α- and β-adducin gene polymorphisms present in these rats account for approximately 50 % of the difference in blood pressure between the MHS and the Milan normotensive strain (MNS) (Bianchi, Tripodi et al. 1994). The α-adducin gene has ~ 94 % homology between rats and humans, which strongly support a role for α-adducin polymorphisms even in humans (Manunta, Barlassina et al. 1998). In fact, the functional mutation in the human α-adducin gene (Gly460Trp) has been demonstrated to be associated with hypertension, and to be linked to a greater sensitivity to sodium, which may link the α-adducin polymorphism to the salt-sensitive form of hypertension (Cusi, Barlassina et al. 1997). A retrospective population-based case control study determined that individuals who carried the mutated α-adducin had a lower risk of combined myocardial infarction and stroke when treated with diuretics rather then other hypertensive drugs (Psaty, Smith et al. 2002).

1.3.2 Salt sensitivity

Individuals with essential hypertension can be classified as *salt sensitive,* if the blood pressure changes in response to altered sodium intake, whereas those whose changes in sodium intake do not elicit a blood pressure response are considered *salt resistant*. Rises in blood pressure when salt is added is characterized by a failure of the kidney to excrete unnecessary amounts of sodium. The prevalence of salt sensitivity is increased in older individuals, black populations, and patients with a low-renin hypertension such as diabetics (Weinberger, Miller et al. 1986).

There is a large variation in sodium intake among different cultures around the world (Figure 3). Vegetarians like Yanomamo Indians in Brazil consume about 0.46 g sodium/day and show no blood pressure rise with age (Oliver, Cohen et al. 1975; Oliver, Neel et al. 1981), whereas in contrast individuals in north of Japan consume about 13.8 g sodium/day and have a high prevalence of hypertension (Dahl 2005; Franco and Oparil 2006).

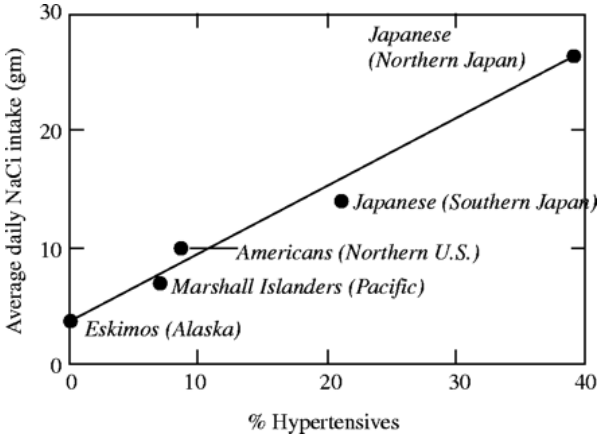


Figure 3. Correlation of average daily salt (NaCl) intake with prevalence of hypertension in different races and among different geographic areas [Reprinted with permission from (Dahl 2005)].

Denton et al., 1982 determined that populations with a high-sodium diet (10-20 g/day) have a high incidence of salt-induced hypertension compared to populations with a low sodium diet (<3g sodium/day). Furthermore, Denton's study on chimpanzees that were fed with high-sodium diet during 20 months demonstrated a significant and progressive elevation of blood pressure (Denton, Weisinger et al. 1995). A similar parallel between the sodium intake and blood pressure was also seen in healthy humans, where a lowered sodium intake significantly reduced the arterial blood pressure (Melander, von Wowern et al. 2007).

Enhanced sympathetic nervous system activity and reduced activity of the renal dopaminergic system are both features that have been proposed to have a role in salt sensitive subjects (Campese, Romoff et al. 1982; Gill, Grossman et al. 1991).

The role of genetic polymorphisms in salt sensitivity of blood pressure has been assessed in many studies (Beeks, Kessels et al. 2004). One of the candidate genes associated with the salt sensitivity of blood pressure is the cell-membrane skeletal protein α-adducin. The α-adducin polymorphism has in rats and *in vitro* transfection studies been shown to be associated with increased NK activity, hence increased tubular sodium reabsorption in the kidney and consequently hypertension (Tripodi, Valtorta et al. 1996; Ferrandi, Salardi et al. 1999). The increased NK activity seen in the MHS of rats could be the result of an increased ATP affinity for the NK (Ferrandi, Salardi et al. 1999) and/or reduced NK endocytosis via the clathrin coated vesicles in response to dopamine (Torielli, Tivodar et al. 2008).

1.3.3 The syndrome of hypertension is intimately related to kidney function

The importance of sodium chloride intake in the pathogenesis of hypertension is still controversial (Simpson 1979; Nicholls 1984; Kesteloot and Joossens 1988; Swales 1988; Swales 1990; Muntzel and Drueke 1992). However, there are several factors involved in the etiology of hypertension that indicates the important role of the kidney as a key mediator of this polygenetic disease. A transplanted kidney, in humans or in animal models, from a hypertensive into a normotensive body has been shown to induce a higher blood pressure, which demonstrate that "hypertension goes with the kidney" (Curtis, Luke et al. 1983; Guidi, Menghetti et al. 1996; Rettig and Grisk 2005). The kidneys receive about 25 % of the cardiac output and filter on average 180 L blood per day, resulting in 1 to 1.5 L excreted urine/day. The functional unit of the kidney, the nephron, is regulated by different hormones such as aldosterone. Crowley and colleagues demonstrated the critical role of the kidney in the pathogenesis of hypertension. They determined that AngII causes hypertension through direct actions on AT_1 receptors expressed in the kidney, a mechanism that did not involve or require AngII-mediated aldosterone responses in the adrenal glands (Crowley, Gurley et al. 2006). The kidney plays a key role as a target for

many of the blood pressure lowering drugs, which suggest its significant role in blood pressure homeostasis.

There are different syndromes caused by genetic variants affecting salt and water reabsorption by the nephron, leading to alteration in blood pressure. *Liddle syndrome* is a rare hereditary disorder, in which the kidneys excrete potassium but retain too much sodium and water, caused by mutations in the ENaC (Lifton, Gharavi et al. 2001). This mutation contribute to increased number of units at the plasma membrane, which results in an increased ENaC activity (Lifton, Gharavi et al. 2001). On the other hand, patients with *Gitelman syndrome* have a defect in the thiazide-sensitive Na^{+}-Cl^{-} co-transporter in the distal convoluted tubule, causes the kidney to excrete sodium, which results in a lower blood pressure than the general population (Lifton, Gharavi et al. 2001).

1.3.4 The kidney-sodium homeostasis

The main function of the kidney is the excretion of waste products such as uric acid, urea, and creatinine, but also the regulation of the body's fluid volume via the sodium excretion. The pressure natriuresis refers to the increased arterial pressure that leads to an increased renal sodium excretion. The opposite, the anti-natriuresis leads to sodium retention, water retention, which could lead to hypertension. In the body there are many different systems for controlling the blood pressure, but in the end it is the long-term kidney-fluid system, that reacts within hours and days, that is the central method of establishing arterial pressure control by increasing (when pressure falls) or decreasing (when the pressure is to high) the body fluid volume (Guyton 1991). Guyton demonstrated that the kidney-fluid mechanism works with infinite feedback gain to find the exact balance between fluid intake and fluid output (Guyton 1990) (Figure 4). The most rapid system, within seconds, for regulating the arterial blood pressure are based on neural receptors [chemoreceptors, baroreceptors and central nervous system (CNS) ischemic response], whereas the hormonal control system, like the RAAS, is normally activated within minutes

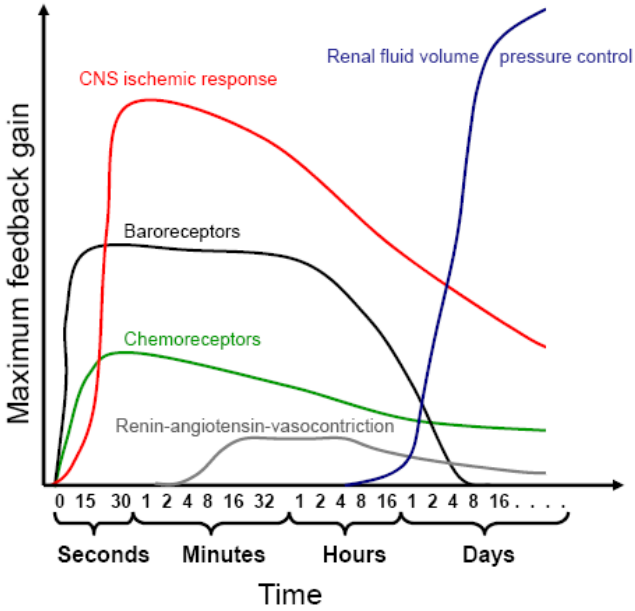


Figure 4. The approximate time and degree of activation of different separate mechanisms for controlling the arterial pressure when it becomes abnormal, expressed in terms of feedback gain [modified from (Guyton 1991)].

(Guyton 1991) (Figure 4). However, worth noting is that salty food activates RAAS, which in combination with the kidney-fluid system then becomes one of the most important controller for the regulation of the blood pressure. Figure 4 summarizes the most important mechanisms that control the arterial pressure in the human body. Neural receptors respond within seconds, hormonal activation within minutes and the kidney-fluid volume system reacts within hours or days. The feedback gain is a quantitative term, which expresses the degree of activation of each of these pressure controlling systems. The feedback gain could be infinite if the control system has corrected the pressure to its exact normal level.

1.3.5 Renin-Angiotensin-Aldosterone system

The RAAS is a major regulator of the blood pressure and plays a central role in the control of sodium excretion and the water balance in the body. The enzyme renin was discovered already in 1898 (Tigerstedt R 1898). It is secreted from the kidney into the blood stream in response to various physiological stimuli, including lowered blood volume or lowered renal perfusion pressure. Sympathetic activation, through the β1 adrenergic receptors and decrease in sodium chloride levels in the ultra-filtrate of the nephron also generates a release of renin. Circulating renin hydrolyzes angiotensinogen, which is produced constitutively and released into the circulation mainly by the liver. The product angiotensin I is converted into AngII by ACE, which is found predominantly in the capillaries of the lung (Figure 5).

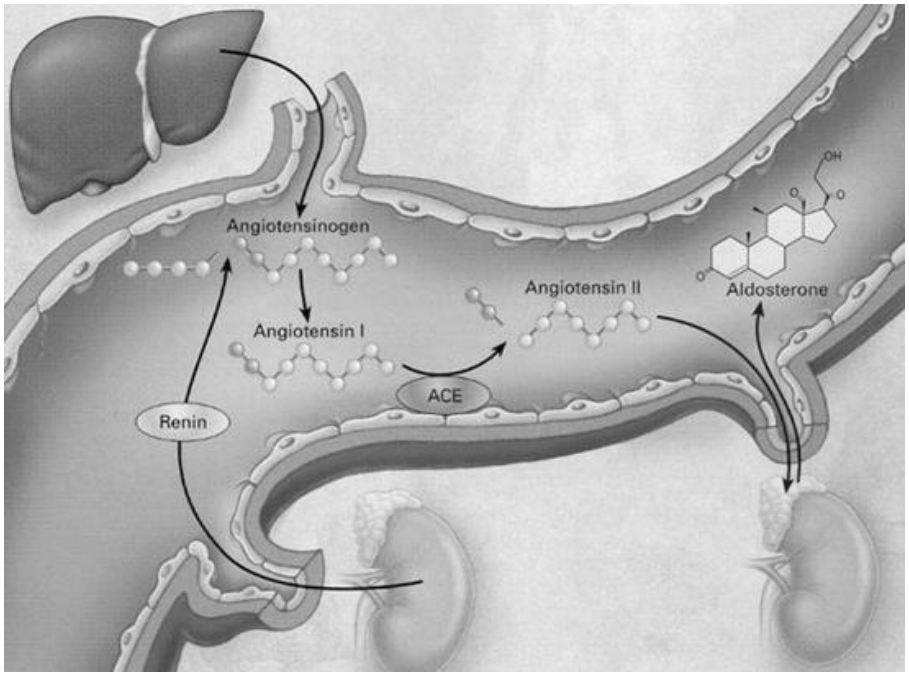


Figure 5. The renin-angiotensin-aldosterone system [Reprinted with permission from (Weber 2001)].

Since renin catalyzes the rate-limiting step in the RAAS cascade it is usually used as an index of the endogenous formation of AngII. The effects of the octapeptide AngII is mediated via specific membrane-bound G-protein-couple receptors called the AngII subtype 1 (AT_1) and 2 (AT_2).

The mechanisms of AngII to increase the blood pressure are mediated via the AT_1 receptors (Crowley, Tharaux et al. 2004). These receptors are expressed in variety of organ systems, thought to play key role in blood pressure homeostasis, including the kidney, heart, blood vessels, adrenal glands and cardiovascular control centers in the brain (Shanmugam and Sandberg 1996). AngII has via the AT_1 receptors a great cardiovascular influence. Some of the established biological effects of AngII are stimulation of proximal tubular reabsorption of sodium ions, increased release of noradrenaline from sympathetic nerve terminals, generalized vasoconstriction and cell growth in the cardiac of left ventricle and in the arterial wall (Rang 1999).

The AT_2 receptor, which has a lower expression than the AT_1 receptor, has been shown to mediate the opposite effects to those mediated by AT_1. The AT_2 receptor stimulates vasodilation and natriuresis, which have a role in blood pressure regulation (Carey, Wang et al. 2000).

Overall, to control high blood pressure there are different ways to interrupt different steps in the RAAS system. For example there are agents that inhibit the ACE and the AT_1 receptors.

1.3.6 Cardiac hypertrophy

One of the major complications of sustained elevations in blood pressure is cardiac hypertrophy, which at the cellular level is characterized by enhanced protein synthesis, higher organization of the contractile unit (sarcomere) and an increase in cardiomyocytes size (Chien, Zhu et al. 1993; Komuro and Yazaki 1993; Sadoshima and Izumo 1997). Cardiomyocytes have been known to grow in cell size without cell division to adapt to a demand for an increased workload, since they lose their ability to proliferate soon after birth (MacLellan and Schneider 2000). However, recent data from a research group at Karolinska Institutet indicate that there is renewal of cardiomyocytes in humans (Bergmann, Bhardwaj et al. 2009).

Cardiac hypertrophy is a resultant of the heart response to a sustained mechanical stress, either as a consequence of pathological hypertrophy, due to stimuli such as hypertension, ischemic heart disease, valvular insufficiency, infectious agents, or mutations in sarcomeric genes (Lorell and Carabello 2000) or a physiological hypertrophy, in response to exercise (Oakley 2001).

The development of cardiac hypertrophy involves, at the molecular level, activation of numerous relevant genes and transcription factors. These transcription factors bind to specific

deoxyribonucleic acid (DNA) sequences and thereby control the transcription of genetic information from DNA to messenger ribonucleic acid (mRNA) (Molkentin, Lu et al. 1998; Frey and Olson 2003), which influence cardiac growth. Several, more or less known, molecular pathways are involved in cardiomyocytes hypertrophy, such as the calcineurin- nuclear factor of activated T-cells (NFAT) and myocyte enhance factor (MEF2)/ histone deacetylase (HDAC) pathways (Frey and Olson 2003).

The transcription factor NFAT was initially described in T-cells, hence the name, nuclear factor of activated T-cells (Molkentin, Lu et al. 1998). NFATc1-c4 represents a family of Ca^{2+}/calcineurin-dependent transcription factors (Hogan, Chen et al. 2003). Molkentin et al., 1998 were the first to describe the calcineurin and NFAT as hypertrophic transducers, which promotes a dramatic hypertrophy response in transgenic mouse hearts (Molkentin, Lu et al. 1998). In T-cells as well as in cardiomyocytes increased intracellular calcium activates the calmodulin-dependent phosphatase calcineurin. The NFAT protein is dephosphorylated by calcineurin, which results in translocation of NFAT into the nucleus and induction of NFAT-mediated gene transcription (Frey and Olson 2003).

The transcription factor MEF2 is expressed at high levels in the adult heart and regulates numerous cardiac genes. Calcium-dependent protein kinase (CaMK) is via HDACs a potent inducer of MEF2 activity in cardiomyocytes in vivo (Passier, Zeng et al. 2000). HDACs control the MEF2 activity and gene expression, by influencing the acetylation status of nucleosomal histones. Deacetylation of nucleosomal histones promotes chromatin condensation and transcriptional repression (Zhang, McKinsey et al. 2001) Histone acetyltransferases, on the other hand, relaxes the chromatin and thereby makes it possible for transcription factors to bind DNA and activate target genes (McKinsey, Zhang et al. 2001). Of the three different classes of HDACs, especially the class II HDACs (HDAC-4, HDAC-5, HDAC-7, and HDAC-9) associate with MEF2 to repress MEF2-induced target gene expression (Miska, Karlsson et al. 1999; Sparrow, Miska et al. 1999). Phosphorylation of specific sites within the class II HDACs is required to regulate the associations between these two proteins (McKinsey, Zhang et al. 2001). In skeletal myocytes, salt-inducible kinase 1(SIK1) is required for phosphorylation of HDAC-5 and for expression of the MEF2 target genes (Berdeaux, Goebel et al. 2007).

Overall, a better understanding of the cellular signaling pathways in cardiac myocytes will lead to the development of new therapeutic agents to treat cardiac hypertrophy.

2. AIM

The overall aim of this thesis was to identify a network of proteins that may sense the changes in intracellular sodium and regulates its active transport and, if present, to determine its role during disease development.

Specific aims that will be addressed:

1. Identify a signaling target that senses accurately small elevations in intracellular sodium and translates this signal into the Na^+, K^+-ATPase complex (Figure 6A).

2. To elucidate whether a sodium-sensing network mediates the elevations in Na^+, K^+-ATPase activity in renal epithelial cells induced by a hypertension-linked mutation in the α-adducin molecule (Figure 6B).

3. To examine whether the effects of angiotensin II influence transcriptional activation and gene expression in cardiomyocytes and whether a sodium-sensing network mediates this effect (Figure 6C).

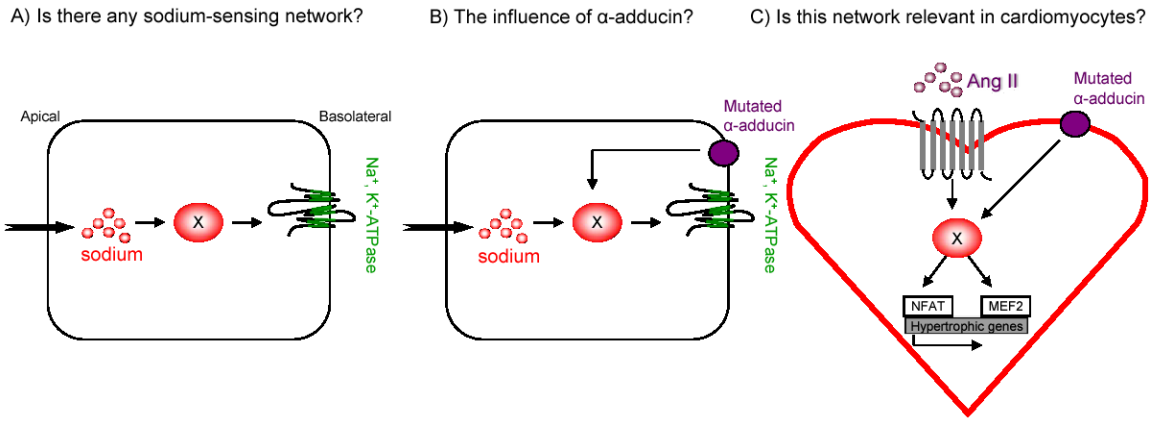


Figure 6. Schematic figures depicting the specific questions in each signaling pathway (A), (B) and (C) that will help in the development of the overall aim of this thesis.

3. METHODOLOGY

Cell lines (article I, II and III)

The OK cell line was used as a model for proximal convoluted tubule cells. OK cells were derived from the kidney of a North American opossum, and has many characteristics of kidney proximal convoluted tubules (Malstrom, Stange et al. 1987). Since this cell line express the NK and response to hormones it has been an important *in vitro* model for proximal tubular physiological studies (Efendiev, Bertorello et al. 2002). The native OK cells were maintained at 37 ºC (5 % CO_2) in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (GIBCO, Invitrogen) and 100 units/ml Penicillin– 100 μg/ml Streptomycin and L-Glutamine. OK cells stably expressing the NK α_1-subunit carrying a green fluorescent tag (GFP) as previously described (Efendiev, Bertorello et al. 2000), were maintained in the same media as mentioned above with the addition of 13 μM ouabain to select for the stable clones.

The human-hepatoma-derived cell line (HEPG2, American Type Culture Collection, Manassas, CA) was used as a human liver model and these cells were maintained at 37 ºC (5 % CO_2) and grown in GlutaMAX-DMEM (GIBCO, Invitrogen) with 10 % fetal bovine serum (GIBCO, Invitrogen) and 100 units/ml Penicillin– 100 μg/ml Streptomycin.

The cardiac muscle cell line (HL-1) was used as an experimental model to elucidate transcriptional activation and gene expression in cardiomyocytes. HL-1 is derived from AT-1 mouse atrial cardiomyocytes tumor lineage. The cells can be serially passaged and retain differentiated cardiac morphological, biochemical, and electrophysiological properties (White, Constantin et al. 2004). The HL-1 exhibit an adult cardiomyocyte-like gene expression profile and express the genes for α-cardiac myosin heavy chain (α-MHC), α-cardiac actin and atrial natriuretic factor (Claycomb, Lanson et al. 1998). The HL-1 cardiac cell line were maintained at 37 ºC in an atmosphere of 5 % CO_2 and 95 % air and cultured in Claycomb media (Claycomb, Lanson et al. 1998) supplemented with 10 % fetal bovine serum, 100 units/ml Penicillin– 100 μg/ml Streptomycin, 1 % L-Glutamine, and 10 μM Norephinephrine. The cells were cultured in flasks pre-coated with fibronectin [25 μg fibronectin (Sigma) in 2 ml of 0.02 % gelatin (Sigma)].

Plasmids and expression vectors (article I, II and II)

The complementary DNA (cDNA) SIK1-GST, SIK1-HA, SIK1-K56M, and SIK1-T322A was kindly provided by Dr. H. Takemori (Laboratory of Cell Signaling and Metabolism, National Institute of Biomedical Innovation, Osaka, Japan). Site-directed mutagenesis of SIK1

(K56M) was preformed (Lin, Takemori et al. 2001) by using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The SIK1 mutant lacking a CaMK regulatory residue was generated by exchanging nucleotides as follows: Thr322Ala (ACA-GCA). The hemagglutinin epitop (HA) and glutathione-S-transferase (GST) tags were inserted at the N-terminal region of the SIK1 molecule (Katoh, Takemori et al. 2004).

pNFAT-TA-Luciferase (NFAT-Luc) vector TATATAATGGAAGCTCGAATTCAGCG; Clontech, Laboratories Inc., Palo Alto, CA) was used to measure NFAT activity. Three tandem copies of NFAT consensus sequence acting as NFAT enhancer element were inserted upstream of the TATA box promoter in the plasmid. Luciferase reporter was inserted downstream of the TATA box. pTA-Luc vector (Clontech, Laboratories Inc., Palo Alto, CA) lacking the NFAT consensus sequence was used as negative control.

The MEF2-Luciferase (MEF2-Luc) vectors were provided by Dr. Zixu Mao (Departments of Pharmacology and Neurology, Emory University School of Medicine, Atlanta, GA 30322, USA). The reporter plasmid pMEF2X2Fluc was generated by cloning two double-stranded oligonucleotide (MEF2- wild type (WT); TCGAGGGCTATTTTTAGGGCC) spanning the c-jun MEF2 site into the Xhol site of pOFLuc. A mutant sequence in the MEF2 binding site was constructed and used as negative control (MEF2 Mut-Luc) (Shin, Seoh et al. 1999).

The cDNAs for protein phosphatase 2A (PP2A) WT and L199P were kindly provided by Dr. B. A. Hemmings (Friedrich Miescher Institute, Basel, Switzerland).

The α-adducin mutant plasmids (G460/S586, W460/C586) were provided from Giuseppe Bianchi (Division of Nephrology, Dialysis, and Hypertension, S Raffaele Hospital, Milan, Italy) and previously described elsewhere (Efendiev, Krmar et al. 2004).

The SIK1- small interfering RNA (siRNA) or scrambled RNA (scRNA) as control were kindly provided by Sznajder Laboratory, Division of Pulmonary & Critical Care Medicine, Northwestern University, Chicago, IL.

All constructs were verified by DNA sequence analysis.

Cell transfection (article I, II and III)

The plasmids were introduced into the cells by liposome-mediated transfection (LipofectAMINE 2000, Invitrogen, Carlsbad, CA). The expression time of the plasmid was 12-48 hours. Reporter tags (HA-tag or GST-tag) were linked to the plasmid to check the expression efficiency by Western blotting, using antibodies against either the HA or the GST-tag.

Cell surface biotinylation (article I)

The abundance of NK α-subunits at the plasma membrane were determined with the cell surface biotinylation method. OK cells were incubated with or without 5 μM of Monensin (Mon) for different period of times. The incubation was stopped by placing the cells on ice and washed with ice-cold PBS (containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+}) followed by addition of biotin (1.5 mg/ml) during 30 min at 4 °C on a shaking platform protected from light. Thereafter the cells were washed with ice-cold PBS (containing 100 mM glycine, 1 mM Ca^{2+} and 0.5 mM Mg^{2+}) and with ice-cold PBS without glycine. Subsequently, the PBS was replaced with RIPA buffer containing: 50 mM Tris-HCl, 150 mM NaCl, 1% IGEPAL CA-630 (NP-40), 1 % sodium deoxycholate and protease inhibitors [(1mM) PMSF, (5μg/ml) leupeptin, antipain, pepstatin A and (10 μg/ml) aprotinin], and cells were scraped, vortex and centrifuged at 14000 × g at 4 °C for 1 min. Streptavidin was added to the supernatant (250-350 μg of protein) and incubated at 4 °C with end-over-end rotation overnight. The pellet was washed once with a buffer containing 150 mM NaCl, 50 mM Tris-HCl, 5 mM Ethylenediamine tetraacetic acid (EDTA), there after washed two times with a buffer containing 500 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA and finally the beads were washed three times with a buffer containing, 500 mM NaCl, 20 mM Tris-HCl and 0.2 % BSA. Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB) with a NK α-subunit (1:1000).

Confocal microscopy (article I)

To support our findings that the NK associates with the SIK1 we performed confocal microscopy. The expression of GFP tagged NK α_1-subunit was detected by confocal laser scanning microscope (Leica TCS SP2, Leica Lasertechnik GmbH, Heidelberg, Germany). SIK1 was visualized after incubation with a specific antibody against the SIK1.

Determination of Na^+, K^+-ATPase activity in OK cells (article I and II)

Active transport of sodium ions mediated by the NK was determined as described previously (Efendiev, Bertorello et al. 2002). OK cells were re-plated to a 24 well plates (~80 000 cells/well) the day before the experiment. Cells were incubated in 2 mM of ethylene glycol tetraacetic acid (EGTA) for 2h at 37 ºC, and thereafter with or without 5 mM of ouabain at 23 ºC for additional 20 min. Rubidium ($^{86}Rb^+$, PerkinElmer) was added to the media, a trace amount (1μCi/μl), and further incubation at 23 ºC for 20 min. The reaction was stopped by washing three times with ice cold saline. Cells were dissolved in 3 % SDS and aliquots taken for $^{86}Rb^+$ quantitation by counting the β-radiation. NK-mediated $^{86}Rb^+$ transport was calculated

from the differences in tracer uptake between samples incubated with and without the Ouabain. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). NK activity was expressed as nmol of Rb^+/mg protein/min from triplicate determinations. To asses the effect of 5 μM of Mon, the cells were incubated at room temperature for different period of time.

Immunoprecipitation (article I and II)

Cells were grown in 10 cm Petri-dishes and treated according to experimental procedure. The reaction was stopped by putting the cells on ice. The cells were washed in cold PBS and after addition of immunoprecipitation buffer (IP) buffer: 50 mM Tris-base, 100 mM NaCl, 30 mM NaF, 2mM EDTA, 2 mM EGTA, 1 % Triton X-100 and protease inhibitors (PMSF (1mM), leupeptin, antipain, pepstatin A (5 μg/ml of each) and aprotinin (10 μg/ml) they were scraped and transferred to Eppendorf tubes. They were further ruptured by passing through 27.5-G needle and further homogenized using a motor pestle. The homogenate was centrifuged at 1000 x g for 5 min at 4 °C and the post-nuclear supernatant (PNS) pre-cleared with 40 μl protein A/G agarose (Santa Cruz Biotechnology) during 1 h at 4 °C. Equal amounts of protein (500 μg) in 1 ml of IP buffer were incubated with appropriate antiserum for 16 h at 4 °C with end-over-end rotation. After addition of protein A/G agarose (2h at 4 °C) the complexes were spun down and the pellet washed three times with IP buffer. Samples were analyzed by SDS-PAGE and Western blot with desired antibodies using enhanced chemiluminescence (ECLPlus, GE Healthcare) and quantitated by scanning following by ImageJ analysis. Scans were performed using a Scan Jet IIc scanner (Hewlett Packard, Palo Alto, CA).

Back phosphorylation (article I)

For estimation of the amount of in vivo phosphorylation, the back phosphorylation method was used, where the signal is inversely proportional to the degree of phosphorylation. OK cells over expressing the WT phosphatase methylesterase 1 (PME-1) were incubated with or without Mon 5 μM at 23 ºC for 2.5 min. The incubation was stopped by transferring the samples to ice and addition of IP buffer as described above with the addition of 30 mM $Na_4P_2O_7$ and 2 mM of activated Na_3VO_4. PME-1 was immunoprecipitated and phosphorylated (Nestler and Greengard 1980; Chibalin, Ogimoto et al. 1999) by purified GST-SIK1 (~10 ng) in the presence of 50 mM Tris-HCl (pH 7.4); 10 mM $MgCl_2$; 1 mM DTT; 250 μM Na_2ATP; 0.5 μCi/μl [γ-^{32}P] ATP (PerkimElmer) for 30 min at 30 ºC. The reaction was stopped by

addition sample buffer and heated for 5 min at 90 ºC. The proteins were separated on a 15 % SDS-PAGE and transferred to PVDF membranes. The radioactivity incorporated in the PME-1 was analyzed by autoradiography and the amount of PME-1 by WB.

In vitro phosphorylation of SIK1 by CaMKI (article I)

To prepare a SIK1- sucrose-nonfermenting homologue (SNH) peptide, a cDNA fragment of the SIK1 SNH domain (301aa-354aa) was amplified by PCR with primers linked with a BamHI site in the forward primer and a NotI site in the reverse primer. To express the SNH domain as a GST fusion protein in E. coli, the amplified product was ligated into the BamHI-NotI site of pGEX-6P3. An active CaMK was prepared using an expression plasmid (pSport6-CaMK1; IMAGE: 4483612, Invitrogen). To convert a native CaMK1 into a constitutive active form, a stop codon was inserted at the amino acid 296 position by site directed mutagenesis. The resultant cDNA was ligated into the BamHI-NotI site of pEBG vector (mammalian GST fusion vector), and the active GST-CaMK1 was expressed in COS-7 cells. The purified GST-CaMK1 was mixed with GST, GST-SNH WT or GST-SNH (T322A mutant) and incubated with or without ATP (0.1 mM) for 30 min at 30 ºC in a CaMK1 reaction buffer (50 mM Tris pH 7.4, 10 mM MgCl2). The reaction was terminated by addition of Laemmli sample buffer and an aliquot subjected to SDS-PAGE followed by Western blot analysis using an anti-pT322 antibody.

Determination of protein phosphatase activity (article I)

Phosphatase activity was assayed using the EnzoLyte MFP Protein Phosphatase Assay system (AnaSpec, San Jose, CA). After treatment, cells were homogenate, centrifuged (100,000 x g, 4 °C, 20 min), and enzymatic activity was assayed in the pellet. Dephosphorylation of MFP (3-O-methylfluorescein phosphate) was monitored by measuring the fluorescence of MF product every 15 min for 1 h in a 96-well fluorescence plate reader (MicroLumat Plus LB; excitation, 485 nm; emission, 535 nm). Phosphatase activity was calculated as the slope of fluorescence recordings and expressed as arbitrary fluorescence units/μg protein.

Mass Spectrometry (article I)

The NK was immunoprecipitated from OK cells expressing stably the NK tagged with an enhancer GFP reporter in its α-subunit (Done, Leibiger et al. 2002) using an antibody against GFP. The proteins were resolved in 12 % SDS-PAGE using the Laemmli buffer system (Laemmli 1970) and visualized with a Silver-plus kit according to the procedure recommended by the manufacturer (Invitrogen) or by Coomassie Blue staining. The bands were excised from

the Coomassie Blue stained gel and digested with trypsin. The tryptic peptides analysis was performed by MALDI-TOF mass spectrometer (Voyager-De PRO) at the Karolinska Institutet core facility (http://www.mbb.ki.se/pac/). The peptide mass spectrum was acquired over a range of 500-3500 Da. Peptide mass mapping was carried out against the NCBI database (http://prospector.ucsf.edu) using the MS-FIT program.

Determination of SIK1 activity (article I, II and III)

SIK1 activity was determined in isolated proximal tubule cells, HL-1 cells and OK cells expressing the GST-SIK1 isoform as described (Lin, Takemori et al. 2001). Isolated GST-SIK1 or immunoprecipitated SIK1 from PCT homogenates were separated on SDS-PAGE and phosphorylated (activated) GST-SIK1 or endogenous SIK1 (from PCTs) was examined by Western blot using a specific SIK1 antibody raised against the phosphorylated (activated) Thr-182 residue (Katoh, Takemori et al. 2006) by using chemiluminescence (ECL Plus, GE Healthcare). WB signals were quantitated using the ImageJ software (http://rsb.info.nih.gov/ij/). The state of SIK1 phosphorylation was determined as the ratio between the phosphorylated signal and the amount of SIK1 immunoprecipitated and changes expressed as percentage of control.

The determination of the SIK1 activity in OK cells were also determined by incubating the isolated SIK1-GST with a SIK1 phosphorylation buffer [50mM Tris (pH 7.4), 10 mM MnCl2, 0.3-0.5 μCi/μl [γ-32P] ATP (PerkinElmer, Norwalk, CT)] and TORC2 substrate at 30 ºC for 30 min with constant shaking. The reaction was stopped by adding sample buffer. Proteins were separated on SDS-PAGE, and phosphorylated GST-SIK1 and TORC2 were examined by autoradiography.

Proximal convoluted tubules from Milan normotensive and hypertensive strain of rats (article II)

The PCT from Milan normotensive strain (MNS) and Milan hypertensive strain (MHS) of rats were kindly provided from Giuseppe Bianchi (Division of Nephrology, Dialysis, and Hypertension, S Raffaele Hospital, Milan, Italy). The PCTs were re-suspended into a lysis buffer: 50 mM Tris-base, 100 mM NaCl, 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 1 % Triton X-100, 50 mM β-Glycerophosphate, 2 mM DTT, 2 mM Na_3VO_4, 30 mM Na_4P2O_7 and protease inhibitors (1 mM PMSF, (5 μg/ml of each) leupeptin, antipain, pepstatin A and (10 μg/ml) aprotinin. The tubules were ruptured by passing through 27.5-G needle and further homogenized using a motor pestle. The homogenates were centrifuged at 1000 x g for 3 min at

4° C and the PNS pre-cleared with 40 µl protein A/G agarose (Santa Cruz Biotechnology) during 1 h at 4 °C. Equal amounts of protein (1000 µg) were used in each assay.

Luciferase assay (article III)

Luciferase activity was determined in HL-1 cells transfected with the MEF2-Luc vector (Shin, Seoh et al. 1999) or the NFAT-Luc vector (Clontech, Laboratories Inc., Palo Alto, CA) in combination with either the SIK1 WT, K56M, T322A or the α-adducin (G460/S586, W460/C586).

HL-1 cells were re-plated to a 24 well plates (40 000-70 000 cells/well) the day before the experiment. Cells were starved in basal media 15h-17 h before the experiment, incubated with or without 1-10 pM of AngII at 37 ºC for different periods of time. The reaction was stopped by washing two times with ice-cold PBS, and dissolved in 100 µl of Luciferase Assay Buffer (Promega, Madison, WI, USA), vortexed for 30 sec and incubated at -20 ºC for 5 min before it was placed on a shaker for 30 min at 4 ºC to disrupt the cells. The cell lysate (20 µl) was mixed with 100 µl of Luciferase Assay Reagent (Promega, Madison, WI, USA), and the light produced was measured in a Luminometer (Turner Designs, Sunnyvale, CA, USA). Protein concentrations of cell lysates were determined by the Bradford technique (Bradford 1976). The luciferase activity was expressed as percentage change of the ratio between the light produced (Figure 7) and the protein concentration from triplicate determinations.

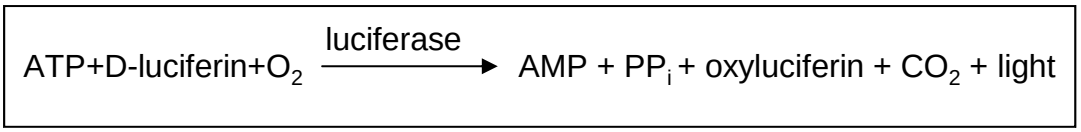


Figure 7. The reaction of the firefly luciferase, with stable light emission.

Real-time PCR (article III)

Total RNA was extracted from HL-1 cells using E.Z.N.A.TM Total RNA Kit I (Omega Bio-Tek, Norcross, GA, USA) according to manufacturer's instructions and quantified using a nanodrop 1000 Spectrophotometer (Thermo Scientific, Walthman, MA, USA). Total RNA (100 ng) was reverse transcribed using RevertAidTM H Minus M-MuLV reverse transcriptase (Fermentas, Life Science, Vilnius, Lithuania) following manufacturer's instructions. Predesigned Taqman gene expression assays for the natriuretic peptide precursor type B (NPPB; Mm01255770_g1), α-MHC (α-myosin heavy chain; Mm01313844_mH), skeletal actin (SkA; Mm00808218_g1), salt-inducible kinase 1 (SIK1; Mm00440317_m1) and large ribosomal

protein PO (RPLPO; Hs99999902_m1) genes were obtained from (Applied Biosystems, Foster City, CA, USA.). Real-time polymerase chain reaction (RT-PCR) was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Reactions were performed in 96-well plates. The RT-PCR was performed during following conditions: 50 ºC, 2 minutes; 95 ºC, 10 minutes; 95 ºC, 15 s; 50 cycles. Results were analyzed using the 7000 System Software Version 1.2.3 (Applied Biosystems). For each sample the software calculated the number of the cycles in which the reader detects a significant increase in fluorescence with respect to the baseline signal, referred to as the threshold cycle (Ct). In each sample the mRNA levels of NPPB, SkA, α-MHC and SIK1 were normalized to the mRNA levels of RPLPO.

Statistic analyses (article I, II and III)

The values are mean ± SEM. Statistical analysis was performed using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA, USA). All statistical tests were performed with the unpaired Student t test or ANOVA when appropriated. Differences with a value of $P < 0.05$ were considered statistically significant. Significance is denoted as *<0.05, **<0.01 and ***<0.001.

4. RESULTS AND DISCUSSION

The physiological regulation of the NK requires the interaction and binding of signaling molecules within the NK complex. Our hypothesis that an unidentified target is associated with the NK was examined and the results revealed the identity of a novel protein, the salt-inducible kinase 1 (SIK1) (Okamoto, Takemori et al. 2004). This kinase was found to be associated with the renal NK complex and to participate in the regulation of its activity following small elevations in intracellular sodium concentrations. SIK1 has been found in several transporting epithelia (Feldman, Vician et al. 2000) and is regulated by an increase of salt intake (Okamoto, Takemori et al. 2004). In addition, the NK activity and intracellular trafficking are regulated by a phosphorylation-dephosphorylation process (Carranza, Rousselot et al. 1998; Chibalin, Ogimoto et al. 1999), which made SIK1 a crucial component to study within this regulatory network.

SIK1 is part of a cell sodium-sensing network that regulates active sodium transport through a calcium-dependent process (article I)

Wang et al., 1999 were the first to describe the SIK1, in adrenal glands of high salt diet-fed rats (Wang, Takemori et al. 1999). SIK1 is a member of the sucrose-nonfermenting 1 protein kinase/AMP- activated protein kinase (SNF1/AMPK) family of the serine/threonine protein kinases (Jaleel, McBride et al. 2005). The SNF1 protein kinase is a yeast homologue of mammalian AMPK family and plays a role in transcriptional activation and transcriptional repression of genes (Sanz 2003).There are three isoforms of the SIK protein; SIK1 (molecular mass ~ 86 kDa), SIK2 (molecular mass ~ 120 kDa) and SIK3 (molecular mass ~ 170 kDa). Our hypothesis that an unidentified target was associated with the NK was evaluated by using mass spectrometry. By using this method we identified in renal epithelial cells (OK cells) SIK1 which associates with the renal NK complex. Western blot analysis and confocal microscopy with antibodies against the SIK1 and the α-subunit of the NK also confirmed the association between theses two proteins. We did not further analyze whether the interaction between NK and SIK1 is direct or indirect (via an adaptor protein). This is still to be elucidated. However, one could speculate that the adaptor protein ankyrin, that is known to be associated with the NK (Nelson and Veshnock 1987) and also shown to be brought down together with the NK and SIK1 in our mass spectrometry study, may serve as a linker between these two proteins.

It has been previously shown that the intracellular sodium concentration can be increased by using the ionophore Mon in OK cells without changing the architecture or the morphology of

these cells (Efendiev, Bertorello et al. 2002). We demonstrated that NK activity increased in a time dependent fashion when cells were treated with Mon, without changing the NK α-subunit abundance at the plasma membrane. This result was interpreted to suggest that the elevations in intracellular sodium concentration has a direct effect on the catalytic activity of NK, since the number of NK molecules within the plasma membrane remains the same regardless of the change in intracellular sodium concentration.

Next we examined whether the increased intracellular sodium associated with an increase in SIK1 activity. We found that there was a time dependent increase in SIK1 activity induced by Mon. This was confirmed both by studying the autophosphorylation of SIK1 and by determining the phosphorylation of SIK1's substrate the transducer of regulated cyclic AMP-responsive element binding protein (CREB) activity coactivator 2 (TORC2).

To find out the functional relevance of SIK1 during the sodium-dependent regulation of NK we introduced the SIK1 negative mutant, K56M [lysine (K) is changed to methionine (M) at the position 56] that abolish the kinase activity, into OK cells. The results demonstrated that the NK activity was significantly increased in the mock and WT transfected cells but not in the cells expressing the SIK1 K56M mutant, which indicates that SIK1 is required during stimulation of NK activity in the presence of elevated intracellular sodium. The fact that the SIK1 mutant (K56M) does not affect the basal (nonstimulated) NK activity may indicate that SIK1 associates with a limited pool of NK molecules that can be subject to short-term regulation.

To further elucidate the biological significance of SIK1, we introduced the SIK1-siRNA to silence the expression of the SIK1 protein. The negative control for the siRNA contains the same nucleotide composition, but scrambled RNA, which lack significant sequence homology to the genome. The human-hepatoma-derived cell line, HEPG2, was transiently expressing either the negative control SIK1-scRNA or the SIK1-siRNA and treated with or without Mon. The result demonstrated a significant reduction in the ability of Mon to increase the NK activity in the cells expressing the SIK1-siRNA compared to the control cells expressing the SIK1-scRNA.

It is possible that Mon or sodium directly increases the SIK1 activity. However, it was observed that in a cell-free system that neither sodium nor Mon generated an increase in the SIK1 activity, suggesting that there is an intracellular signaling factor that is a possible mediator in this regulation.

Increases in intracellular sodium is paralleled by increases in intracellular calcium (Efendiev, Bertorello et al. 2002) and because calcium is a well known intracellular messenger, we examined its role during this sodium-dependent regulation of NK activity. By quenching

calcium with both EGTA (outside the cell) and the selective intracellular calcium chelator [1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)] BAPTA-AM it was found that the increase in NK activity was absent in response to Mon. Most likely the calcium could be increased intracellularly by its releases from intracellular stores, endoplasmic reticulum via the inositol trisphosphate receptor or extracellular via the Na^+/Ca^{2+} exchanger (NCX). We found that the NCX was expressed in the OK cells and by blocking the exchanger, with two different selective inhibitors (KB-R7943 and SEA0400) the increase in NK activity induced by Mon was prevented (Figure 8). We examined the effect of rising intracellular calcium by using a calcium ionophore (A23187) on SIK1 activity. We found that SIK1 activity was not increased, indicating its specificity for the sodium signal, and suggesting that increases in intracellular calcium alone were not sufficient to increase the activity of SIK1.

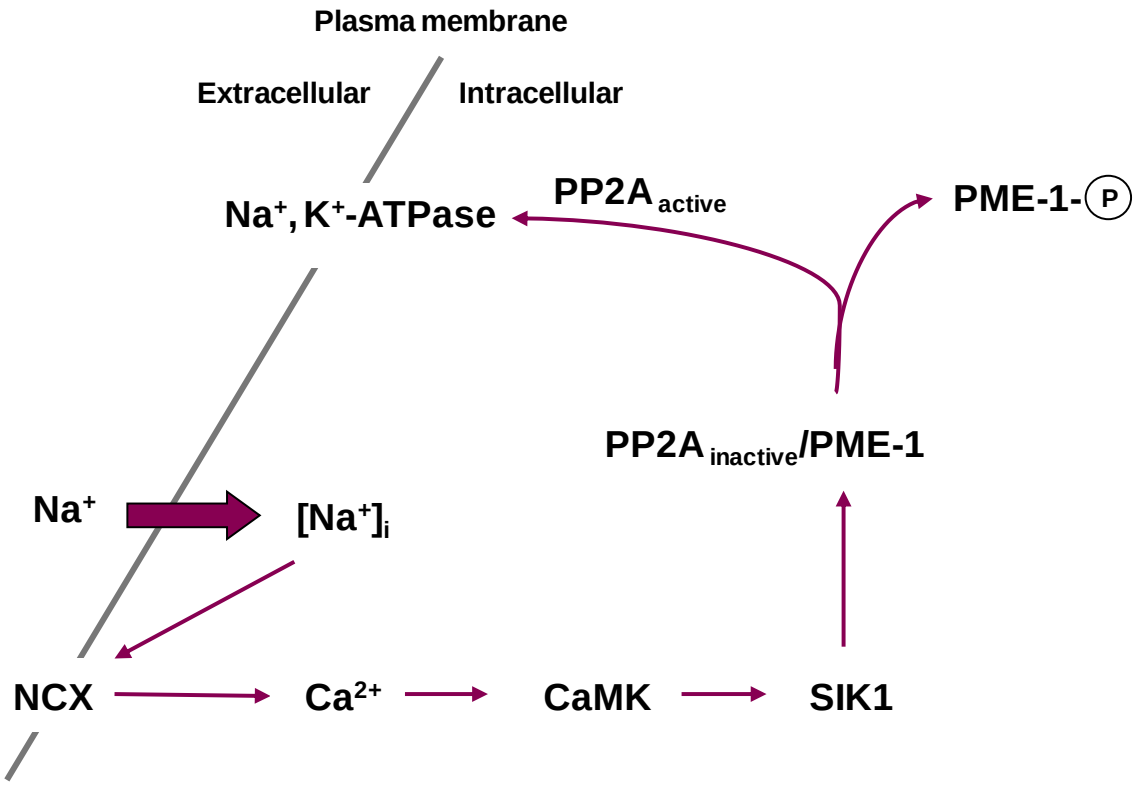


Figure 8. Proposed cellular mechanisms responsible for increases in NK activity in response to elevated intracellular sodium. Increases in intracellular sodium, within a short period of time (5 min), which does not result in changes in cell architecture, is associated with rapid increase in intracellular calcium by activating the NCX. This increase in calcium activates CaMK leading to phosphorylation (T322) and activation of SIK1. Phosphorylation of PME-1 by SIK1 promotes its dissociation from PP2A's catalytic subunit, which leads to an active PP2A that dephosphorylates the NK α-subunit and increase its catalytic activity.

Therefore, we hypothesized that a putative Ca^{2+}-dependent target could provide the link between the sodium induced increase in intracellular calcium and activation of SIK1. By blocking the CaMK with the inhibitor KN-93, we prevented both the increase in SIK1 and NK activity induced by high intracellular sodium.

A consensus site for CaMK phosphorylation was found within the SIK1 protein (IDRQRT322V) (White, Kwon et al. 1998), which strongly suggests that SIK1 could be under the control of CaMK by direct phosphorylation. CaMK1 phosphorylated in vitro the sucrose non-fermenting homologue of the SIK1 (SIK1-SNH) type peptide, but not the SIK1-SNH carrying the T322A mutation, which lacks the CaMK phosphorylation site within the SIK1 protein (Figure 8). The functional significant of this finding was also highlighted in OK cells transiently expressing the SIK1 WT and SIK1 T322A mutant, which also demonstrated that the increased NK activity was prevented in the cells expressing the mutant form compared to the WT.

A summary of several studies done on the NK regulation, stated in the review by Ewart and Klip, established that activation of kinases correlated with inhibition of the NK activity, whereas pathways that activated phosphatases correlated with stimulation of the NK activity (Ewart and Klip 1995). We found that the increase in intracellular sodium resulted in a dephosphorylation of the NK α-subunit and that this dephosphorylation occurred in a time dependent manner. Furthermore, it was determined whether SIK1 was necessary for promoting the dephosphorylation of the NK α-subunit. We observed that OK cells transiently expressing the SIK1 mutant (K56M) were unable to get dephosphorylated, which suggests that SIK1 plays a role in the dephosphorylation of the NK α-subunit in cells treated with Mon. Since we did not find any consensus phosphorylation site for SIK1 within neither the α- nor the β-subunit of the NK molecule, we concluded that it must be another regulatory protein that provides the link between the SIK1 and the NK α-subunit. Previously it has been reported that PP2A dephosphorylates the NK α-subunit (Lecuona, Dada et al. 2006) and that the NK associates with the PP2A and regulates its cellular mechanisms responsible for traffic to and from the plasma membrane (Efendiev, Bertorello et al. 2002).

We hypothesized that the PP2A protein is a crucial component in this sodium-sensing network. By introduced a negative mutant of the PP2A [L199P: leucine (L) is changed to proline (P) at the position 199] in OK cells treated with or without Mon we tried to answer the given hypothesis. The results revealed that the sodium-induced increase in NK activity required activation of the PP2A. Katoh Y., et al 2006 determined that the classically used PKC inhibitor staurosporine, at low concentration was sufficient to inhibit the SIK1, without interfering with other kinases. By using a low concentration of staurosporine (5 nM) it was found that the PP2A activity was inhibited. Interestingly, OK cells with transiently over expressed SIK1 WT showed an increased PP2A activity, whereas over expression the SIK1 mutant (K56M) was not associated with an increase in PP2A activity over the basal. Taken together, these data suggest that SIK1 regulates the PP2A activity. The PP2A is one of the major serine/threonine

phosphates known to be involved in many cellular processes (Janssens, Jordens et al. 2003). PP2A is a heterotrimer containing a 65 kDa regulatory subunit (PR65 or A subunit), a 36 kDa catalytic subunit (PP2Ac), which can act as a dimeric structure independently or can be associated with three major classes of the third subunit: PR55/B, PR61/B and PR72/B (Longin, Jordens et al. 2004). Since direct phosphorylation of the phosphatase has generally been described as a process resulting in inhibition of its activity (Janssens and Goris 2001), we focused on methylation of the phosphates subunits as a potential regulatory mechanism, since that mechanism are known to activate the PP2A. A unique type of reversible methylation occurs at the carboxy group of the C-terminal Leu309 of PP2Ac, which is catalysed by a specific, 38 kDa, AdoMet (S-adenosylmethionine)-dependentthyltransferase, namely leucine carboxyl methyltransferase (Longin, Jordens et al. 2004). This methylation is reversible and demethylation is catalysed by a specific protein called PME-1, which has also been shown to associate with the PP2A (Ogris, Du et al. 1999) (Figure 8). Interestingly, we found that the PME-1 protein co- precipitated with the NK α-subunit. Similarly, PP2A was also found to be co-immunoprecipitated together with the NK/PME-1 complex. From the mass spectrometry study we also recognized a protein with Mr of $\approx$ 45, which could be the PME-1, but we had too little material to confirm this finding in that step. We found that increases in the intracellular sodium concentration by Mon induced the dissociation of PME-1 from the NK/PP2A complex in OK cells, whereas the association of $PP2A_{PR65}$ subunit remained unchanged (Figure 8). This finding was also confirmed when the association between the PME-1 protein and the $PP2A_{PR65}$ subunit was studied under the influence of elevated intracellular sodium concentration. The relevance of SIK1 in this dissociation process between the NK α-subunit and the PME-1 was further elucidated in OK cells transiently expressing the SIK1 WT and SIK1 mutant K56M. The result demonstrated that the SIK1-negative mutant failed to promote the dissociation of PME-1 from the NK α-subunit complex, compared with the control cells expressing the SIK1 WT. Could PME-1 be under the control of SIK1 by direct phosphorylation? We found a consensus site for a potential phosphorylation site within the PME-1 protein and by using the backphosphorylation method, where the signal is inversely proportional to the degree of phosphorylation, we found that the PME-1 protein was phosphorylated in the presence of SIK1. This experiment also determined that Mon treatment decreased the amount of dephosphorylated PME-1.

In summary, SIK1 is a critical mediator within a cell sodium-sensing signaling network present in mammalian cells, that senses changes in $[Na^+]_i$ and transforms this event into a signaling cascade that links sodium transport out of the cells by increasing NK activity.

The analogous to this sophisticated signaling network for controlling intracellular sodium has been shown to exist even in plant cells (Bertorello and Zhu 2009). SIK1's corresponding protein in plant cells is the salt overly sensitive 2 (SOS2) kinase with a catalytic region similar to the yeast SNF1 and the mammalian AMPK. This regulatory model controls the ion balance within plant cells via the SOS3-SOS2-SOS1 network. SOS3 is the calcium sensor that activates SOS2, which convert inactive SOS1 into its active form by phosphorylation. The plasma membrane Na^+/H^+ antiporter, SOS1, exports Na^+ from the cytoplasm (Bertorello and Zhu 2009). This conserved mechanisms in animal and plant cells, CaMK (SOS3)-SIK1 (SOS2)-NK (SOS1), appears to be relevant for cell survival.

Blocking the salt-inducible kinase 1 network prevents the increases in cell sodium transport caused by a hypertension-linked mutation in human α-adducin (article II)

Based on our previous findings (article I), this second study was performed to elucidate whether SIK1 mediates the increase in NK activity in cells expressing the hypertension-linked mutation of the α-adducin. It has been shown that the Milan hypertensive strain of rats carrying the mutation in the α-adducin has a higher NK activity compared to the control rats (Melzi, Bertorello et al. 1989). This phenomenon has also been seen in cell lines transiently expressing the α-adducin mutation as a result of reduced NK endocytosis via clathrin vesicles in response to dopamine (Efendiev, Krmar et al. 2004). Furthermore, we also demonstrated in article I that SIK1 mediates the increase in NK activity in response to moderate elevation in intracellular sodium. Is the SIK1 network involved in the increase in NK activity in renal epithelial cells expressing the human variant of α-adducin carrying following mutation (G460W/S586C)? To answer this question we used OK cells that transiently expressed the normotensive variant of α-adducin (NT-ADD) or the hypertensive variant (HT-ADD) with either the SIK1 WT or SIK1 bearing a mutation (K56M) that renders the kinase inactive (Lin, Takemori et al. 2001). The results suggest that the NK activity was significantly increased in cells co-transfected with the HT-ADD and the SIK1 WT, which is the same effect seen in single transfected OK cells without expressing the SIK1 WT (Efendiev, Krmar et al. 2004). However, when the SIK1 mutant (K56M) was co-expressed it abrogated the increases in NK activity induced by HT-ADD. Overall, the results of this group of experiments suggest that SIK1 is a mediator in the cell signaling network that involves increases in sodium transport caused by a hypertension-linked mutation in human α-adducin.

In article I, we observed that increased intracellular sodium requires elevations in intracellular calcium, via the activation of a reverse NCX, followed by activation of CaMK

leading to SIK1 phosphorylation (T322 residue) and increased NK activity. In the following experiments, OK cells were transiently transfected with either the NT-ADD or the HT-ADD variant and treated with different agents that are known to affect special stages in the SIK1 signaling pathway. Three different approaches were used to investigate whether the presence of HT-ADD might affect the SIK1 network and thereby increasing the NK activity; A) Blocking the NCX, B) Blocking the CaMK and C) Introducing the SIK1 T322A mutant.

The NK activity was examined by treating the cells with or without the specific NCX inhibitor (KB-R7943) or in the presence or absence of the CaMK blocker (KN-93). Because of the results we proposed that both inhibitors blocked the increases in NK activity that were seen in the cells expressing the HT-ADD, which strongly indicate that the hypertension-linked mutation within the α-adducin molecule mediates the increases in NK activity by activation via the cell sodium-sensing network that was described in article I. Accordingly, the introduction of the SIK1 T322A mutant also prevent the increases in NK activity, which strongly support the concept that the α-adducin mutation is not mediating its cellular actions by targeting directly SIK1, but rather through its network, including the calmodulin-CaMK system.

Changes in NK activity could either be the result of changes in its catalytic activity or changes in the number of active molecules at the plasma membrane. The presence of the mutant variant of α-adducin affects the number of units at the plasma membrane in both transient (Efendiev, Krmar et al. 2004) and stable (Torielli, Tivodar et al. 2008) transfected cell lines. We examined whether the association between NK and SIK1 was affected by the mutant variant of α-adducin. The co-immunoprecipitation experiments done indicated no possibility that elevations in NK activity induced by HT-ADD may have been the result of increased association between the NK complex and the SIK1 protein. On the other hand, the isolated renal proximal tubule cells from Milan hypertensive rats showed a significant increased SIK1 activity (phosphorylated Thr-182) compared to the Milan normotensive rats, which suggests that the SIK1 network is important for enhancing the activity of the NK molecules once they have reached the plasma membrane. Similarly, when normal OK cells were transfected with HT-ADD, SIK1 activity was significantly elevated compared to basal (nonstimulated) cells or cells expressing the NT-ADD. This elevation in SIK1 activity could be responsible for sustained increases in renal tubule NK activity and thereby increases in sodium reabsorption, leading to plasma volume expansion and high blood pressure. Interestingly, the magnitude of the increases in SIK1 activity in response to Mon did not change proportionally. The cells expressing the vector or the NT-ADD determined a proportional increase in SIK1 activity in response to Mon, whereas the cells expressing the HT-ADD did not respond to Mon in the same proportion, which could be due to the existence of a limited pool of SIK1 molecules that only respond to

short-term change in intracellular sodium and that has a limited capacity after it reaches its maximal phosphorylation under sodium stimulation. The precise nature of how the mutant α-adducin is interacting with the SIK1 network is not clear and remains to be elucidated. However, one could speculate that the presence of the α-adducin mutant leads to increased influx of extracellular calcium, since inhibition of the NCX blocked the increase in NK activity that was seen in the cells expressing the HT-ADD.

In summary, the increases in NK activity elicited by the hypertensive variant of α-adducin was abolished by inhibiting the SIK1 network.

Understanding SIK1's regulation could not only improve our knowledge in kidney sodium regulation but also improve the treatment for salt sensitive patients and patients with cardiac hypertrophy. Individuals with a mutation in the α-adducin gene are likely to have renal sodium retention and salt-sensitive hypertension (Cusi, Barlassina et al. 1997) as well as cardiac hypertrophy (Winnicki, Somers et al. 2002). It has been shown that blood pressure response to diuretics has been more pronounced in patients with the α-adducin 460Trp allele compared to those homozygous for the Gly allele (Cusi, Barlassina et al. 1997; Sciarrone, Stella et al. 2003). With this knowledge, it would be interesting to specifically block SIK1 in the renal epithelial cells of MHS of rats, to evaluate if there is any blood pressure lowering effect.

Blocking SIK1 in cardiac myocytes prevents the increases in transcription induced by Angiotensin II and a hypertension-linked mutation in α-adducin (article III)

AngII is a well known hormone that plays a role in the blood pressure homeostasis by acting on organs such as the kidney and the heart (Shanmugam and Sandberg 1996). One complication of sustained elevation in arterial blood pressure is cardiac hypertrophy. Therefore, the aim of this third study was to determine whether the effects of AngII could influence transcription activation of MEF2 and NFAT and gene expression in cardiomyocytes and whether the Ca^{2+}-dependent SIK1 network has a mediatory role. Furthermore, in article II, we determined that that SIK1 activity was significantly elevated in renal proximal tubule cells from Milan hypertensive rats, carrying a α-adducin mutation, when compared with normotensive controls. Since the MHS rats are associated with cardiac hypertrophy (Ferrandi, Molinari et al. 2004), this study was also designed to examine whether the presence of a hypertension linked mutation within the human α-adducin influences transcription activation and whether SIK1 has any mediatory role.

The results of several studies suggest that an alteration in intracellular Ca^{2+} signaling is a primary stimulus for the hypertrophic response. In vivo experiments in primary cardiomyocytes

showed that Ca^{2+} agonists (Sei, Irons et al. 1991), treatment with Ca^{2+} ionophores (Sonnenberg 1986) or elevation of extracellular Ca^{2+} (LaPointe, Deschepper et al. 1990) result in hypertrophic responses. Furthermore, overexpressed calmodulin (the Ca^{2+}-binding protein) in the heart of transgenic mice induces hypertrophy (Gruver, DeMayo et al. 1993). Taken together, these studies reveal that the Ca^{2+}-calmodulin signaling is involved in the development of cardiac hypertrophy, which strongly suggest a possible role for the Ca^{2+}-calmodulin-activated SIK1 network in the hypertrophic growth response.

The molecular mechanism of cardiac hypertrophy is still unsolved, but it has previously been reported that intracellular Ca^{2+} alteration leads to activation of crucial transcription factors, such as MEF2 and NFAT, which signaling has been implicated in the regulation of hypertrophic growth response (Molkentin, Lu et al. 1998; Suzuki, Nishimatsu et al. 2002; Frey and Olson 2003; Xu, Ha et al. 2007). It is known that AngII is a hypertrophic agent that activates the Ca^{2+}-dependent intracellular singling system (Sadoshima, Xu et al. 1993), therefore AngII was used in this study to trigger the downstream activation of NFAT and MEF2.

The luciferase reporter gene system was used to detect the MEF2 and NFAT activity, since it is a sensitive system, ease to quantify, when compared with other available systems (de Wet, Wood et al. 1987). By using HL-1 cells, a cell line derived from mouse cardiac myocytes, that transiently expressed either the MEF2-Luc or the NFAT-Luc vector we determined whether AngII could mediate the increase in MEF2 and NFAT activity. The results determined a peak activation of MEF2-luciferase reporter activity in response to moderate increases in AngII, 10 pM, after 5 minutes. However, AngII did not affect MEF2 activity when a mutant sequence in the MEF2 binding site of the Luc-vector was used. Incubation with equal concentration of AngII promotes similar increase in NFAT activity after 15 minutes and with a peak activation that occur after 3h. The NFAT control reporter construct which lacks the NFAT binding site in the promoter showed no NFAT activity. Taken together, these findings illustrate that both MEF2 and NFAT are activated by AngII in cardiac myocytes, which suggest that AngII *per se*, independently of other humoral factors associated with high blood pressure, may trigger cardiac hypertrophy. Similarly, a study done in vascular smooth muscle cells determined that AngII stimulates both MEF2 and NFAT activity (Suzuki, Nishimatsu et al. 2002).

Based on the previous finding we tried to elucidate whether SIK1 could be a mediator for the MEF2 and NFAT activity in cardiac myocytes. We started to reveal whether AngII could increase the SIK1 activity in these cells. HL-1 cells were incubated with AngII for different period of times. The changes in SIK1 activity were assessed by the state of Thr-182 phosphorylation and the result demonstrated that AngII increased the SIK1 activity in a time dependent manner.

Therefore, we hypothesized that increased SIK1 activity mediates the activation of MEF2 and NFAT induced by AngII. In order to prove this hypothesis, we used HL-1 cells that transiently expressed the MEF2-Luc vector or NFAT-Luc vector with either the SIK1 WT or SIK1 bearing a mutation (K56M) that renders the kinase inactive (Lin, Takemori et al. 2001). Also, the SIK1 bearing the T322A mutation (lacking the CaMKI phosphorylation site with SIK1) was used. Interestingly, the results determined that basal (non-stimulated) HL-1 cells significantly decrease MEF2 and NFAT activity in cells expressing the SIK1 mutants (K56M and T322A) when compared to cells expressing the SIK1 WT (data not shown in article III). MEF2 activity was significantly increased in the presence of AngII in cells transiently expressing the SIK1 WT, whereas this effect was abrogated in cells expressing the SIK1 mutants. Similarly, increased NFAT activity induced by AngII were absent in cells transiently expressing the SIK1 mutants. These findings suggest that SIK1 is a mediator for both MEF2 and NFAT signaling in cardiac myocytes.

Because the effects are blocked when cells expressed a SIK1 mutant lacking the phosphorylation site (Thr322) that is Ca^{2+}-calmodulin kinase dependent, these results also suggest that the cell sodium-sensing network described in article I could have a possible role in transcription activation in cardiac myocytes. The decrease (below zero) seen in both MEF2 and NFAT activity in cells expressing the mutants could perhaps be explained by the fact that the sodium-sensing mechanism is critical for these signaling cascades, that when blocked create an imbalance that influence both the MEF2 or NFAT activity. We could even speculate that the calcium/calmodulin-CaMK cascade is more critical for the MEF2 pathway than the NFAT, since there is a more pronounced reduction in the MEF2 activity than the NFAT activity in the presence of the T322A mutant. This observation support previously findings that CaMK is an important regulator for the MEF2 activity, whereas the NFAT pathway seams to be regulated by the calcium/calmodulin- calcineurin cascade (Frey and Olson 2003).

The effect of AngII in these cells is likely to be mediated via the AT_1 receptor, present in HL-1 cells (Filipeanu, Zhou et al. 2004), which is known to mediate increases in intracellular calcium (Touyz, Sventek et al. 1996; Fuller, Hauschild et al. 2005), since its effects are blocked when cells expressed the SIK1 mutant lacking the phosphorylation site (Thr322) that is Ca^{2+}-calmodulin kinase dependent.

The SIK1 protein kinase is encoded by the snf1lk gene and has been shown to be involved in early cardiogenesis in mice (Ruiz, Conlon et al. 1994; Stephenson, Huang et al. 2004). Gene that codes for SIK1 has been found in mouse on chromosome 17. In rat the SIK1 gene has been localized to chromosome 20p12. In spontaneously hypertensive and Lewis rats this locus contributes to the increase in blood pressure in these animals, when exposed to a high-salt diet

(Rapp 2000). In human the snf1lk gene that codes for SIK1 is located within chromosome 21q22.3, which is a region that has been implicated in congenital heart defect in patients with Down syndrome (Barlow, Chen et al. 2001; Stephenson, Huang et al. 2004). Interestingly, the malfunctioning NFAT signaling has been reported to be responsible for the cardiac defects in Down syndrome (Arron, Winslow et al. 2006), which suggests that SIK1 may control other still unknown signaling molecules that participate in the regulation of NFAT. Van Oort, RJ *et al.*, reported that calcineurin-induced MEF2 activation is mediated by NFAT, possibly by direct protein-protein interaction (van Oort, van Rooij et al. 2006), which suggests that SIK1 could be an upstream regulator of both MEF2 and NFAT (Figure 9). Alternatively, and because activation of NFAT is associated with elevated intracellular calcium, we could speculate that SIK1 may promote increase in intracellular calcium by regulating either plasma membrane calcium channels or receptors involved in the release of calcium from intracellular stores (Figure 9).

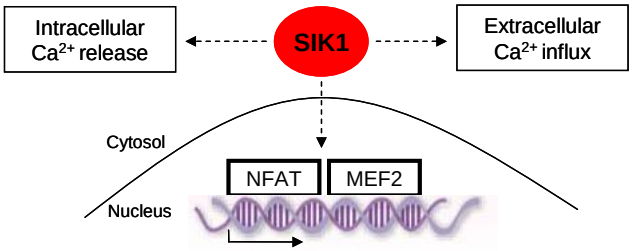


Figure 9. Proposed model for the role of SIK1 in the NFAT and MEF2 signaling pathways in cardiac myocytes. SIK1 is an upstream regulator of both MEF2 and NFAT and promotes increases in intracellular calcium by regulating either plasma membrane calcium channels or receptors involved in the release of calcium from intracellular stores.

We observed that AngII increased the mRNA expression of the SIK1 gene in a time-dependent fashion. Similarly, a time-dependent increase in gene expression induced by AngII was seen in the hypertrophic-associated cardiac genes NPPB, SkA and α-MHC (Chien, Knowlton et al. 1991). Among those genes, α-MHC activation is under the control of MEF2 (Lee, Nadal-Ginard et al. 1997). Similarly, analysis of cardiac gene expression in mice has shown that α-MHC required MEF2C for optimum transcription (Lin, Schwarz et al. 1997). The results suggest that AngII increases expression of structural genes that are associated with the development of cardiac hypertrophy, independently of the rise in arterial blood pressure. This response bears some similarity to the in vivo experiment done in rats that got infusions with AngII and significantly developed left ventricle hypertrophy, even when the blood pressure increases in response to AngII were blocked (Khairallah 1972), suggesting that AngII directly stimulate protein synthesis and cell growth in cardiac tissue.

Because AngII controls SIK1 activity, and SIK1 expression is increased upon incubation with AngII, it is likely that the effect of AngII could be mediated via the SIK1-MEF2 pathway. Alternatively, SIK1 could affect gene expression via CREB activation as previously reported (Takemori, Katoh et al. 2002). It has been speculated, although at present it is not clear, that

activation of SkA and α-MHC could be affected by activation of NFAT (Wilkins and Molkentin 2002; Kuwahara, Wang et al. 2006).

In article II, we showed that the presence of the hypertension-linked mutation within the α-adducin gene that associates with increases of left ventricle hypertrophy (Winnicki, Somers et al. 2002), significantly contributes to an increased SIK1 activity. In this study we examined the MEF2 and NFAT activity in cardiac myocytes expressing the normotensive and hypertensive α-adducin (G460W/S586C). The result demonstrated that in basal (non-stimulated) cells MEF2 and NFAT activity was significantly increased in cells expressing the HT-ADD compared to the cells expressing the NT-ADD variant. AngII induced a dose dependent increase in MEF2 and NFAT activity. The activation of MEF2 and NFAT in cells expressing the HT-ADD variant in response to 1 pM of AngII appears to be significantly different compared to the cells expressing the NT-ADD. In contrast, there are no significantly differences between cells treated with higher doses (5 and 10 pM) of AngII. Interestingly, the increase in basal MEF2 activity was blocked by inhibition of SIK1 activity. This was done by transiently expressing the SIK1-K56M that renders the kinase activity. The dependency on NFAT activation also appears to be dependent on SIK1 activity. Furthermore, SIK1 controlled basal activity of the transcription factors are highlighted by the observation that MEF2 and NFAT are elevated in cells over expressing SIK1 WT when compared to non-transfected cells. The increases observed in MEF2 and NFAT activity in the presence of HT-ADD might be explained by the increased intracellular calcium. It is also possible that accumulation of AT_1 receptors at the plasma membrane might be greater in cells expressing HT-ADD compared to the NT-ADD variant.

Thus, these results may suggest that despite the hypertensive background, the presence of the α-adducin mutation could directly affect transcription of hypertrophic genes, and consequently cardiac hypertrophy, independently of chronic elevation in arterial pressure.

The observations seen in the HL-1 cells have all the limitations of a cell line study, but our results determined that the Ca^{2+}-calmodulin-activated SIK1 may be a critical transducer of the hypertrophic response that uniquely link alterations in intracellular calcium handling in a cardiac myocytes to the hypertrophic growth response.

In summary, a cell sodium-sensing network within SIK1 at its core appears to be responsible for transcription activation induced by AngII in cardiac myocytes. Furthermore, these results suggest that hypertension-linked mutation within α-adducin as well as AngII can *per se* activate transcription factors associated with cardiac hypertrophy independently of the rise in arterial blood pressure (Figure 10).

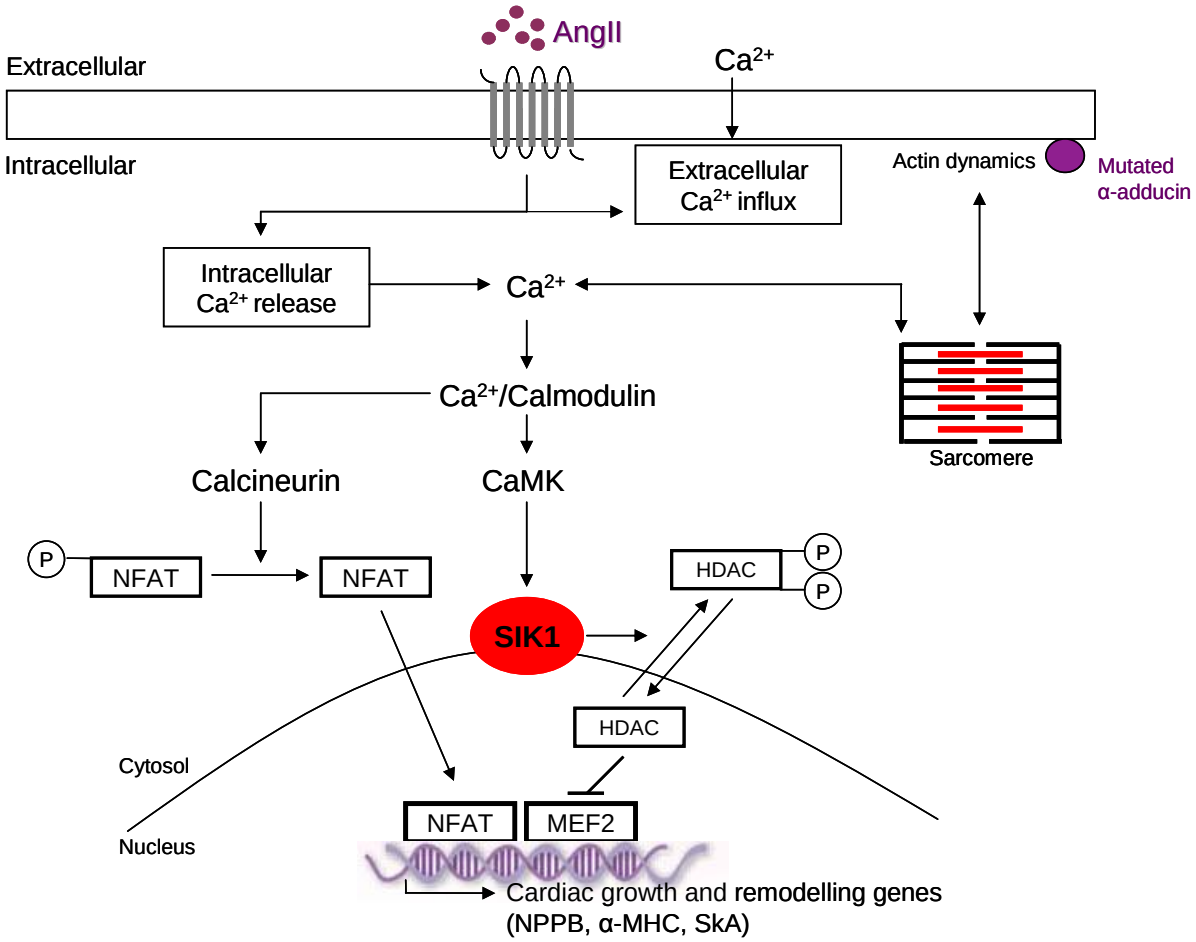


Figure 10. Proposed AngII-mediated signaling pathway involved in the regulation of MEF2 and NFAT in cardiac myocytes. AngII activates the AT_1 receptors followed by increases in intracellular calcium. Calcium/calmodulin activates CaMK that phosphorylates and activates SIK1. SIK1 phosphorylates HDAC that controls MEF2 activity and gene expression. Activation of NFAT is controlled by interaction with the phosphatase calcineurin, which dephosphorylates NFAT followed by its nuclear translocation. Thus, a mutation in the α-adducin gene might increase intracellular calcium levels that influence the activity of both MEF2 and NFAT.

5. SUMMARY AND CONCLUSIONS

The work presented in this thesis intended to investigate if there is any sodium-sensitive network of proteins present in mammalian cells, that may sense the changes in intracellular sodium and regulates its active transport, and if that is the case, if that network could play a role in disease development.

The studies were performed in different cell lines except study II that also includes studies on rat kidney. In summary, we found in OK cells that the signaling target SIK1 is associated with the NK complex. We determined, in these cells, that the increased intracellular sodium is paralleled by elevation in intracellular calcium through NCX exchanger, leading to the activation of SIK1 (Threonine 322 phosphorylation) via a Ca^{2+}-calmodulin dependent kinase. The activation of the SIK1 triggers the phosphorylation of the PME-1 which dissociates from its PP2A. The active PP2A dephosphorylates the NK α-subunit, which leads to its increased NK activity. Furthermore, the SIK1 activity was significantly elevated in renal proximal tubule cells from Milan hypertensive rats when compared to the normotensive controls. Similarly, SIK1 activity was significantly elevated in a normal renal proximal tubule cell line when transfected with α-adducin variant carrying the human hypertensive mutation. Blocking SIK1 activity using negative mutants as well as its activation pathway prevented the effects induced by the hypertensive α-adducin. This cell sodium-sensing network has also been demonstrated to be involved in cardiomyocytes, where SIK1 is responsible for transcription activation elicited by AngII and by the hypertensive variant of α-adducin that associates with cardiac hypertrophy. AngII increases the mRNA levels for the hypertrophic-associated cardiac genes NPPB, SkA, and α-MHC independently of the rise in arterial blood pressure. Taken together, all these data provide a new piece of the puzzle in the understanding of the intracellular sodium-sensing network with SIK1 at its core.

In conclusion, this thesis reveals the identity of the SIK1 intracellular signaling network, present in the mammalian cell. The SIK1 network senses changes in intracellular sodium concentration and transforms this event into a signaling cascade that links sodium transport out of the cells by increasing the NK activity. The SIK1 network mediates the elevation in NK activity in renal epithelial cells induced by a hypertension-linked mutation in α-adducin. Therefore, this network represents an alternative target by which agents can modulate active sodium transport in renal epithelia and avoid the increase in systemic blood pressure that are associated with genetic mutations in the human α-adducin molecule. Furthermore, the wide biological significance of the SIK1 network is indicated by its mediation of the increases in

transcriptional activity of MEF2 and NFAT in cardiomyocytes induced by AngII and by the hypertensive α-adducin.

6. ACKNOWLEDGEMENTS

This thesis was performed at the Membrane Signaling Networks within the Atherosclerosis Research Unit in the Department of Medicine, Karolinska Institutet. I would like to express my sincere gratitude to all of you who have supported, questioned, inspired and helped me during my work to accomplish this thesis, you are all important to me. In particular, I would like to express my deep appreciation to a number of people who made this possible:

My principle supervisor, **Alejandro M. Bertorello**, founder of "Membrane Signaling Networks": Thank you for sharing your knowledge and ideas, for providing guidance and constructive advice and for all your patience with me during these years. Thanks for teaching me a lot of English. Good luck with all future ideas and take care of the lovely orchids.

My co-supervisor, **Per Eriksson**, thanks for reading my thesis and for your true source of inspiration and for sharing your knowledge. Thank you for always believing in me.

My mentor, **Ola Winqvist**, thanks for all support, generosity (all lunches that you have paid for!), discussions and all the knowledge that you have given me.

P. Jorge Chedrese, thanks for always taking your time and helped me during you stay in our lab. You are an amazing personality and a very good listener. You arrived here right in time to help me finish my thesis, I am grateful to you. You have not only taught me the differences between soap and soup and flower and flour, but also a lot about science.

Anders Hamsten, for your generosity and support, for being knowledgeable and strategically managing the unit, for leading the group of Cardiovascular Genetics to the frontier of medicine.

Group members at the Membrane Signaling Networks (MSN): **Kristina "Stina" Eneling**, for being a nice friend and for all the support you have given me. You are an amazing person that I am glad I know. Esther has a lovely mother. Hang in there, you will soon finish this as well; **Sergej "Sigge" Popov**, the 24 hours working man, thanks for all the collaborations on my last manuscript, I couldn't have finished it without you. I wish you a nice life in Japan and maybe I come and visit you one day.
I also would like to thank the former members of MSN: **Zongpei Chen** thanks for all the discussions and knowledge you have shared with me; **Mattias Sjöström**, thanks for all the collaborations on my first publication and for teaching me self-defense.

Angela Silveira, for being helpful and organizing the Wednesday meetings; **Eva Ehrenborg**, for many nice conversations; **Ferdinand van't Hoof**, for interesting talks; **Rachel Fisher**, thanks for all the English words you taught me during my first years at GV. I think the most

useful word has been: syllabus. I hope you will have a nice summer with the family. Big hug to Harry; **Mai-Lis Hellenius**, for showing how to be a successful scientist. You are a role model for all PhD students; **Josefin Skogsberg**, for being a really sincere friend that always has time to help and listen. I hope you will use M. Montazami's home furnishing tip one day; **Maria Nastase Mannila**, for being a nice friend and hard working mother of three; **Anna Aminoff**, for being a ambitious PhD student that also care about other PhD students situation, **Dick Wågsäter** for your knowledge and pedagogy, **Katja Chernogubova**, for all chats; **Maria Jesús Iglesias**, for being an ambitious and positive person, **Malin Andersen**, for all friendly chats, **Sarah-Jayne Reilly**, good luck with all Marathons that you will run, I admire you!, **Petra Thulin**, for being a nice friend that I always can count on. I am looking forward to your coming dissertation and to a nice New Year's Eve at your place☺; **Valentina Paloschi,** for being a energetic person with a happy smile; **Joanna Chmielewska,** for all friendly chats; **Sanela Kurtovic,** for bringing your knowledge and expertise to our unit**, Rona Strawbrige**, for helping me with the English; **Barbro Burt**, you have been a big support for me in the lab, especially during my first years at GV. Thanks for all nice chats and for letting me listen to P3 even if "Lugna favoriter" is more easy listening; **Karin Husman,** for always being helpful; **Fariba Foroogh,** for being helpful and caring, **Olivera Werngren,** for occasional chats in the lab, **Therese Olsson** for your positive and enthusiastic attitude**, Birgitta Söderholm,** for all chats in the lab, **Karin Danell-Toverud**, for taking care of placing the orders; **Ami Björkholm**, for always being helpful with administrative matters; **Magnus Mossfeldt**, for sharing your knee problem and all IT support; **John Öhrvik**, for giving statistical advice; **Karolina Anner**, for many friendly talks**; Johan Björkegren,** for contribution to scientific discussions; **Jesper Tegnér**, for nice conversations. Good luck with the Computational Medicine research group at CMM, **Roland Nilsson**, for sharing the L5 corridor, for occasional chats and for your willingness to helping me with the Swedish abstract part of the thesis, **Jesper Lundström,** for being a happy person and a loving father of three; **Sara Hägg,** for being a dear friend and for all nice lunch breaks; **Shori Maleki** and **Peri Noori**, for always asking how life is; **Alexandra Bäcklund, Louisa Cheung** and **Hovsep Mahdessian,** for asking interesting and valid questions during the Wednesday meetings; **Jacob Odeberg, Maria Sabater, Lasse Folkersen, Bengt Sennblad, Erika Gunnar** and all other for contributing to a pleasant working atmosphere.

Former colleagues: **Alexander Kovacs,** for being a nice friend, good listener and for all nice advice**, Anders Mälarstig**, for sharing your expertise in genetics; **Ann Samnegård,** the sailing doctor who knows how to organize a nice dissertation party; **Helena Ledmyr,** for inviting me to my first dissertation party ever at Hard Rock café; **Justo Sierra Johnson**, for all the fun times when we shared the same office, thanks for providing me with useful articles about hypertension; **Karl Gertow,** for your smile; **Maria Kolak** for sharing your knowledge and nice lunches with me; **Massimilliano "Max" Ria** for all the good conversations we had during the years at GV. I hope you and your family have a nice time in Oxford; **Per "Pelle" Sjögren**, for have a nice sense of humor. I still remember your half time exam. and your dissertation party!; **Sergey Krapivner,** for all helpful advice in the lab; **Vincent Fontaine,** for bringing a piece of France into the lab; **Camilla Berg,** for all the help and advice and for listening**; Anita Larsson**, for being such a nice person; **Kerstin Lundell**, for all funny talks during the years at GV; **Jacob Lagercrantz, Karin Björklund-Jonsson**, for many friendly talks; **Marie Björnstedt-Bennermo, Monsur Kazi, Hanna Björk** and **Fei Chen**, for being very nice former colleagues.

Saikiran Sedimbi, for all friendly chats**, Katrin Brandt**, my friend, good luck with all projects at work and at home. I hope you will have a nice summer with your family, **Markus Dagnell**, I always enjoy your company. **Charlotta Dagnell**, for being a hard working mother that knows what you want.

Thanks to everybody in the **L8:02** and **L5:01** corridor, I hope you will miss me a little!? ☺

The Linköping University people that introduced me to the world of science; **Magnus Grenegård**, I am grateful to you, who introduced me to the world of science and brought me into the professional research world. You provide a good working atmosphere wherein the student can develop in the world of science, **Anna K. Asplund Persson**, for being a nice person and taught me everything important to know about Western blotting. **Peter Gunnarsson**, thanks for sending me your nice thesis, good luck with your future and I hope you found somebody to help you to move your stuff ☺, **Stéphanie Désilets**, we had a great time in the lab in Linköping, I miss you "Lilla trombocyten", please let me know if you visit Stockholm, so we can meet again.

My friends, that are not doing science that have been of big support during my time as a PhD student, without you I will not survive; **Anna L, Anna O**, **Anna T**, **Carlos, Dijana**, **Fredrik ÅV, Ida A**, **Ida J**, **Linda**, **Marie, Marika, Markus D, Marcus O**, **Patrik**, **Sara** and **Victoria** *"Stjärnorna i förorten"= "The stars in the suburbs";* **Johan**, the founder of this group, a very close friend that enjoys fishing with me ☺. **Fredrik,** thanks for letting me borrow your Pharmacology book for this period of time, maybe I give it back now. **Charlotta**, I am looking forward to your dissertation in the near future, **Pär** and **Malin** for both having a nice sense of humor, **Joel ♥ Noémie**, your wedding will be the best ever.

My fiends from my study time in Lund: **Christopher**, **Ingrid**, **Malin, Henrik W, Anna** and **Ulrika.**

Mari Appelstam at Vera's daycare, for being a happy and energetic person with a lot of patience.

Family **Freyling**, for being so nice and lovely, I am already looking forward to next "Christmas walk" with you.

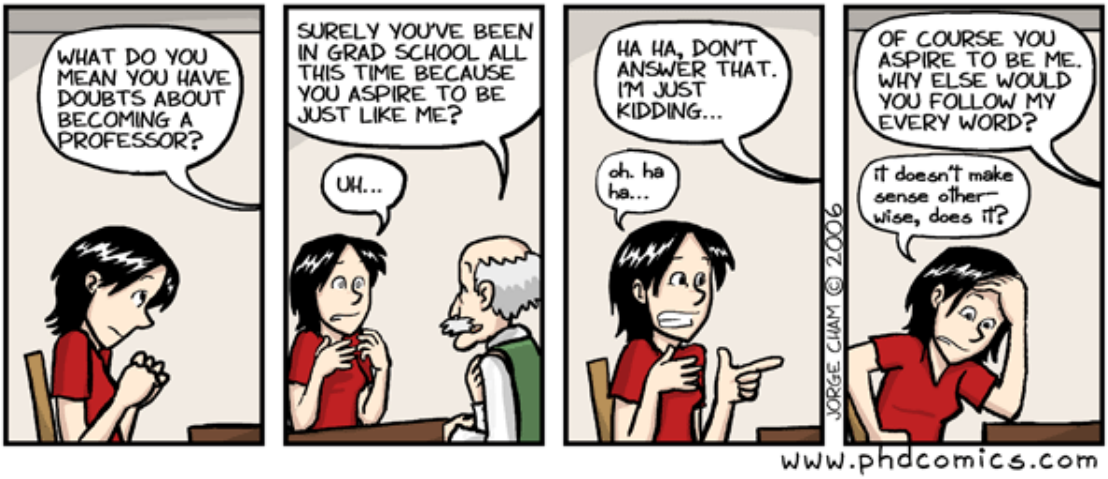
My brother, **Oscar and his family**, for being a nice family that care about the world's politics.

My extended family, **Kersti**, **Lasse**, **Åsa**, **Fredrik**, **Emma** and **Elin**, thanks for all the nice dinners and I am sorry if I had to leave in the middle of the dessert because I had to take care of my precious cells in the lab. Thanks for all support during these years. Thanks for baby-sitting while I was busy writing. You are all very important to me.

My dear parents, thanks **mom** and **dad**, for always being there for me no matter what and for all support during these 32 years. I love you so much.

To my lovely daughter **Vera**, for being so happy and giving me so much energy to distracting my attention away from the thesis work and to more important things in life. You are my sunshine, my tomorrow!

Finally, I would like to thank my husband **Per**, for always being so understanding and supportive. For believing in me and for all the nice dinners you have cooked during these years. I am so grateful to have you in my life. I will never ever erase you from my memory. I love you ♥



Stockholm, 13 April 2010

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