

Department of Woman and Child Health, Paediatric Unit,
Astrid Lindgren Children's Hospital
Karolinska Institutet, Stockholm, Sweden

Na,K-ATPase As a Signaling Transducer

Juan Li



Stockholm 2007

Published articles and figures have been reprinted by permission from:

J Am Soc Nephrol. 17(7):1848-57 (2006) © 2006 *The American Society of Nephrology.*

J Biol Chem. 281(31):21954-62 (2006) © 2006 *The American Society for Biochemistry and Molecular Biology*

© 2007 Juan Li

ISBN 978-91-7357-453-2

*To my family: my son Ruimin, husband
Huisheng and my parents*

III

Abstract

It is now generally agreed that Na,K-ATPase (NKA), in addition to its role in the maintenance of Na⁺ and K⁺ gradients across the cell membrane, is a signal transducer. Our group has identified a novel signaling pathway where NKA interact with IP₃R to form a signaling microdomain. Ouabain, a specific ligand of NKA, activates this pathway, triggers slow Ca²⁺ oscillations and activates NF-κB. In current study, the molecular mechanisms and some important downstream effects of NKA signaling are demonstrated.

The critical binding motifs in NKA/IP₃R signaling microdomain and the role of ankyrin B (Ank-B) were demonstrated. N-terminal tail of the NKA α-subunit (αNT-t) binds directly to N terminus of IP₃R. Three amino acid residues, LKK, conserved in most species and α-isoforms of NKA, are essential for binding. Ank-B expressed in most mammalian cells, plays a pivotal role for NKA/IP₃R signalosome. The N-terminal tails of NKA αsubunit and IP₃R are novel binding sites for Ank-B. In Ank-B silenced cells, the interaction between NKA and IP₃R is decreased.

The role of NKA/IP₃R signaling for activation of NF-κB was elucidated. Cells overexpressing a peptide corresponding to αNT-t, which binds to IP₃R and blocks NKA/IP₃R binding, suppresses ouabain's effect. Knockdown of Ank-B abolishes the ouabain effect on NF-κB.

The downstream effects of this signaling pathway include protection from apoptosis and stimulation of cell proliferation. Ouabain (nM) completely abolishes serum deprivation induced apoptosis. Ouabain protection from apoptosis is not observed in cells overexpressing a mutant NKA α subunit with deletion of the N-terminal tail or a peptide corresponding to αNT-t. Both of them block the interaction between NKA and IP₃R. Inhibition of Ca²⁺ release from intracellular stores via IP₃R or inhibition of NF-κB activity abolishes the anti-apoptotic effect of ouabain. Ouabain stimulates cell proliferation which depends on Ca²⁺ release via IP₃R.

Activation of this signaling pathway rescues nephrogenesis in growth factor deprived embryonic rat kidney. Exposure to ouabain triggers Ca²⁺ oscillations and activates NF-κB in embryonic kidney cells. Growth factor deprivation retards formation of new glomeruli and increases apoptotic index. Ouabain (nM) completely prevents these effects. The protective effects of ouabain are abolished by depletion of intracellular Ca²⁺ stores and by inhibition of NF-κB. The expression of the inductive factors Wt1 and Pax2 activated by NF-κB, are increased in ouabain exposed growth factor deprived kidneys. Thus we have identified a novel mechanism by which kidney development can be protected under adverse intrauterine circumstances.

In conclusion, this thesis demonstrates that NKA directly binds to IP₃R to form a signaling microdomain and Ank-B tethers this binding. Ouabain activates this signaling pathway that results in NF-κB activation, the downstream effects of which are stimulation of cell proliferation, protection from apoptosis and rescue of growth factor deprivation-induced inhibition of embryonic kidney nephrogenesis.

Key words: *Na,K-ATPase, IP₃R, ouabain, Ca²⁺ oscillations, NF-κB, apoptosis, proliferation, nephrogenesis.*

List of Original Papers

This thesis is based on the following publications that will be referred to by their respective Roman numerals:

- I. Zhang SB, Malmersjö S, **Li J**, Ando H, Aizman O, Uhlén P, Mikoshiba K, Aperia A.
Distinct Role of the N-terminal Tail of the Na,K-ATPase Catalytic Subunit as a Signal Transducer
J Biol Chem. 2006 Aug 4;281(31):21954-62.
- II. Liu X, Špicarová Z, Rydholm S, **Li J**, Brismar H, Aperia A.
Ankyrin B modulates the function of Na, K-ATPase/Inositol 1,4,5-trisphosphate receptor signalosome
Submitted to J Biol Chem.
- III. **Li J**, Zelenin S, Aperia A, Aizman A.
Low doses of ouabain protect from serum deprivation-triggered apoptosis and stimulate kidney cell proliferation *via* activation of NF- κ B.
J Am Soc Nephrol. 2006 Jul;17(7):1848-57.
- IV. **Li J**, Perini I, Kruusmägi M, Aizman O, Zelenin S, Aperia A.
Ouabain rescues nephrogenesis in growth-factor deprived embryonic rat kidney
Submitted to J. Clin. Invest.

Contents

Abstract	V
List of Original Papers	VII
Contents	IX
Abbreviations	XI
Introduction	1
Na,K-ATPase	1
Ouabain-a specific ligand of Na,K-ATPase	1
Historical notes	
Ouabain chemistry and structure	
Endogeneous ouabain	
Na,K-ATPase-a signaling transducer	8
Ouabain activates Na,K-ATPase/IP ₃ R complex	
Calcium-a second messenger	10
NF-κB	11
Role of ouabain/Na,K-ATPase/IP ₃ R for kidney development	13
Aims of the study	17
Materials and methods	19
Materials	19
Methods	20
Detection and quantification of apoptotic cells	
Measurements of cell proliferation	
NF-κB activity	
Ratiometric imaging	
Whole-mount immunostaining	
Confocal microscope and glomerular and ureter tip counts	
RNA extraction	
Real time RT-PCR	
Results and Comments	29
Na,K-ATPase directly binds to IP ₃ R to form a signaling microdomain and ankyrin B tethers Na,K-ATPase/IP ₃ R complex	29
Ouabain activates NF-κB through Na,K-ATPase and IP ₃ R binding	33
Anti-apoptotic effect of ouabain is the downstream effect of ouabain/Na,K-ATPase/IP ₃ R signaling pathway	36
	IX

Ouabain rescues nephrogenesis in growth factor deprived embryonic rat kidney	37
Discussion and Future Perspectives	39
Acknowledgements	49
References	53

Abbreviations

ATP	Adenosine Triphosphate
Ank-B	Ankyrin-B
Ca ²⁺	Calcium
CPA	Cyclopiazonic acid
ER	Endoplasmatic Reticulum
FRET	Fluorescence Resonance Energy Transfer
IP ₃	Inositol 1,4,5-trisphosphate
IP ₃ R	Inositol 1,4,5-trisphosphate Receptor
K ⁺	Potassium
Na ⁺	Sodium
Na,K-ATPase	Sodium pump
PM	Plasma membrane
RPT	Renal Proximal Tubule
SR	Sarcoplasmatic Reticulum
2-APB	2-aminoethoxydiphenyl borate

Introduction

Na,K-ATPase

Na,K-ATPase, or sodium pump, was discovered in 1957 by J.S. Skou. Sodium pump is a ubiquitous transmembrane protein presents in all mammalian cells. It uses the energy of ATP hydrolysis to elicit a cation-dependent E1 to E2 conformational change that results in the transport of 3 Na⁺ out of the cell and 2 K⁺ into the cell against their electrochemical gradients (Skou and Esmann 1992). This active pumping maintains a high intracellular K⁺ and low intracellular Na⁺ concentration which produces both a chemical and an electrical gradient across the cell membrane. The electrical gradient is essential for maintaining the resting membrane potential of cells. The sodium gradient is necessary for Na⁺-coupled transport of nutrients and cell volume regulation. It is estimated that around 30% of total body ATP consumption goes to powering the sodium pump and in some organs such as kidney and brain, this value can reach up to 80% (Clausen, Van Hardeveld et al. 1991). Sodium pump (Fig. 1) is a member of the P-type ATPases and is closely related to the Ca²⁺-ATPase family and the H⁺-K⁺ ATPase. It is composed of two essential subunits, α and β , and in some tissues, such as heart, kidney, and brain, the enzyme is associated with other proteins, such as members of the FXYD family of proteins. The association with these proteins modulates cation binding affinity of the Na,K-ATPase. The α subunit is the catalytic subunit of the enzyme and it spans the plasma membrane ten times, with both N- and C- termini located in the cytosol. The α subunit is responsible for the majority of transport activity. It contains the ATP binding site, binding sites for Na⁺ and K⁺, regulatory kinase phosphorylation sites and the binding site for the cardiac glycosides. The β subunit crosses the membrane only once; a small N-terminal segment is located in the cytoplasm, whereas the C-terminus and most of the subunit is located outside the cell. The β subunit is an accessory subunit which is involved in the enzyme's

maturation and localization to the plasma membrane (Lingrel and Kuntzweiler 1994). Four isoforms of the α subunit occur in mammals, α_1 , α_2 , α_3 , and α_4 , and three isoforms of the β subunit have been identified, β_1 , β_2 , and β_3 . The α isoforms are expressed in a tissue-specific and developmentally regulated manner. The α_1 -isoform is expressed ubiquitously. The α_2 -isoform is present largely in skeletal muscle, heart, brain, adipocytes, vascular smooth muscle, and eye, as well as a number of other tissues. The α_3 -isoform is found almost exclusively in neurons and ovaries, but also occurs in white blood cells and heart of some species, such as humans. The α_4 -isoform is expressed in sperm and is specifically synthesized at the spermatogonia stage, where it is required for sperm motility. The α_2 isoform appears around birth in heart and skeletal muscle, but both the α_2 and α_3 isoforms are expressed earlier in brain development (Shamraj, Melvin et al. 1991; Lingrel, Moseley et al. 2003).

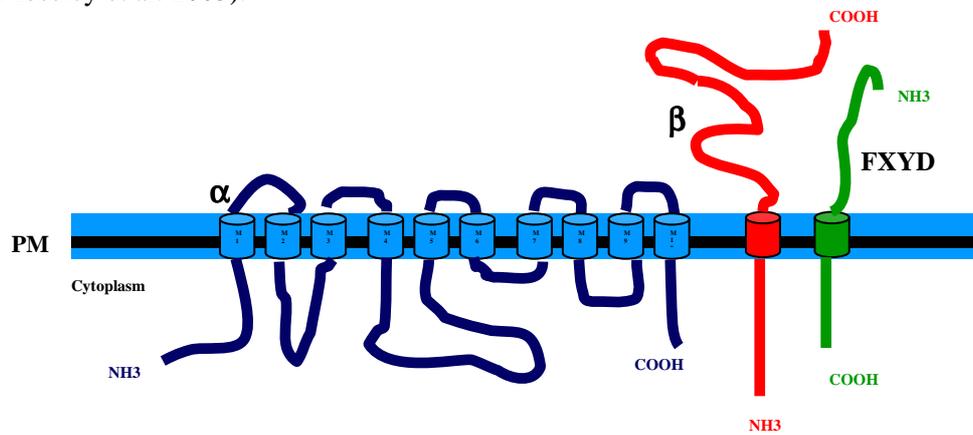


Fig. 1 Structure of Na,K-ATPase

Ouabain- a specific ligand of Na,K-ATPase

Historical notes

Ouabain is a digitalis steroid (cardiac glycosides). Digitalis steroids are prepared from the seeds and dried leaves of the genus *Digitalis* and have been used for more than 200 years as a cardiac stimulant medicine. The English physician

William Withering is credited with discovering in 1775 that the foxglove plant could help those patients suffering from abnormal fluid build-up. In 1930, Sydney Smith of Burroughs Wellcome isolated the steroid glycoside digoxin from *Digitalis lanata* and this compound and other similar derivatives have been developed into drugs still used today to treat heart failure and atrial fibrillation. Structurally related steroids, the bufadienolides, were identified in toad venom and have similar effects on the heart and respiration. In China and Japan, the dried venomous secretion of the Chinese toad, formed into round, smooth, dark brown discs and known as Cha'an Su or Senso, is still used today to treat conditions such as tonsillitis, sore throat, and palpitations. However, the medical use of digitalis steroids has mainly stemmed from their use as an herbal remedy rather than from laboratory chemistry. But upon the discovery of Na,K-ATPase, it was found that the beneficial effects of digitalis (or ouabain) on patients with congestive heart failure, was based on its ability to bind specifically to the α subunit of Na,K-ATPase and inhibit its activity. Before the discovery of Na,K-ATPase, Ringer in 1885 suggested the possibility of an endogenous compound that stimulated cardiac contraction in a manner similar to the digitalis glycosides (Ringer 1885). The modern development of the concept of endogenous digitalis-like factors began in the late 1970s with the convergence of two lines of investigation: the regulation of renal sodium excretion by extracellular fluid (ECF) volume and the pathophysiology of volume expanded models of hypertension (De Wardener 1973). It was later shown that this hormone, digitalis like compound (DLC), may act as an endogenous inhibitor of Na,K-ATPase (Overbeck, Pamnani et al. 1976). Direct cellular and molecular evidence for the presence of endogenous DLC in mammalian tissues was initially obtained in studies demonstrating that extracts from whole brain (Fishman 1979; Lichtstein and Samuelov 1980) and hypothalamus (Hauptert and Sancho 1979) inhibit Na,K-ATPase activity and ^3H -ouabain binding. Since then, much progress has been made on the origin and synthesis of endogenous ouabain, but perhaps the most significant finding is that nanomolar concentrations of ouabain can induce numerous signal transduction events in both primary and immortalized cultures of cells via the

Na,K-ATPase, without necessarily affecting Na^+ and K^+ gradients (Liu, Li et al. 2006; Nguyen, Wallace et al. 2007). However, there are still a number of aspects of endogenous DLC function that have to be more completely understood.

Ouabain chemistry and structure

According to its chemical structure, ouabain consists of two parts: steroid and sugar (rhamnose) (Fig. 2). Its steroid structure is very similar to other classical steroid hormones and is mainly responsible for the binding to Na,K-ATPase and the effect of ouabain (Robinson, Kawamura et al. 1990). The hydrophilic “tail” (rhamnose) prevents

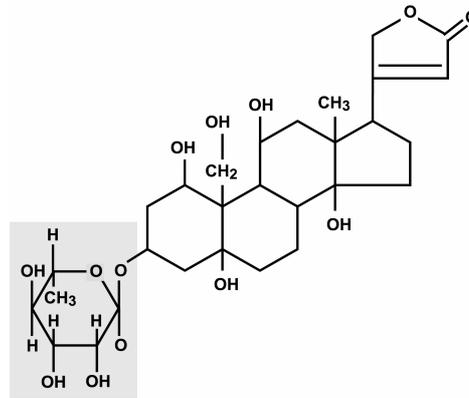


Fig. 2 Chemical structure of ouabain.

ouabain from penetrating the cell membrane. The α subunit of the Na,K-ATPase is the receptor for ouabain. Ouabain has very high affinity and specificity for the Na,K-ATPase. It is well established that the digitalis-binding site on Na,K-ATPase is composed of amino acids located between the first and second transmembrane helices facing the extracellular milieu (Fig. 3). Mutations produced in other sites (appearing as yellow rectangles in Fig. 3) also affect digitalis binding, indicating their involvement in the binding site (Mobasher, Avila et al. 2000). Recent evidence from knock-in mice with modified digitalis-binding affinity of the $\alpha 1$ - and $\alpha 2$ -subunit isoforms of Na,K-ATPase indicates that this binding site, which mediates the pharmacological effects of digitalis, is also the receptor for endogenous DLC (Dostanic-Larson, Van Huysse et al. 2005; Dostanic-Larson, Lorenz et al. 2006). It was demonstrated that the β and FXYD subunits also affect DLC binding (Blanco and Mercer 1998; Geering, Delprat et al. 2006). Thus, the particular isoform complex (out of 84 possibilities) determines the nature of the interaction between DLC and Na,K-ATPase. In agreement with this notion,

Nguyen et al., recently suggested that changes in FXYD proteins are responsible for differences in ouabain binding affinity to Na^+ , K^+ -ATPase in human kidney cells (Nguyen, Wallace et al. 2007).

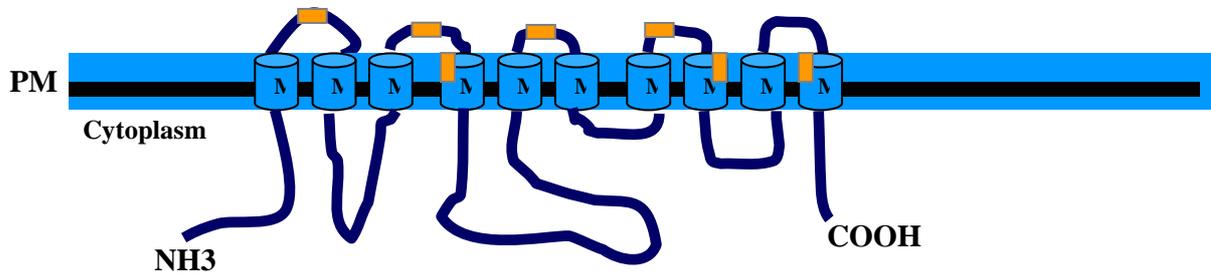


Fig. 3 Ouabain binding sites on α subunit of Na,K-ATPase.

Endogenous ouabain

Endogenous cardenolides include: ouabain found in human plasma, bovine adrenals and hypothalamus; a ouabain isomer identified in bovine hypothalamus and digoxin found in human urine. Reported endogenous bufadienolides include marinobufagenin in human plasma and in urine of patients with myocardial infarction, and 19-norbufalin and its peptide derivative in cataractous human lenses. Strong evidence points to the adrenal cortex as the site of synthesis of endogenous ouabain. (Hamlyn, Blaustein et al. 1991). Endogenous ouabain is also locally produced in other tissues such as the hypothalamus (Kawamura, Guo et al. 1999; Schoner 2000). DLC levels in mammalian plasma, as reported in the literature, are extremely variable. DLC concentrations in healthy human subjects, determined using antibodies against ouabain, were found to be 300–1000 pM (Masugi, Ogihara et al. 1986; Hamlyn and Manunta 1992; Sophocleous, Elmatzoglou et al. 2003) and 40–80 pM (Doris, Jenkins et al. 1994; Naruse, Ishida et al. 1994). The differences may be partially attributed to assay and laboratory-dependent differences in calibration, extraction recovery, and antibody. DLC

concentrations in human plasma, determined using antibodies against marinobufagenin, were estimated as 400 pM (Bagrov, Manusova et al. 2005) and using anti-digoxin antibodies, 70–1000 pM (Ijiri, Hayashi et al. 2003; Sophocleous, Elmatzoglou et al. 2003). Since anti-ouabain and anti-marinobufagenin antibodies are highly specific and do not cross react with marinobufagenin and ouabain, respectively, total DLC (free and bound) seem to be present in the human circulation at concentrations ranging from 0.5 to 2 nM. Based on studies using oocytes, all human Na,K-ATPase α subunits have a similar, high affinity (10–40 nM, in the presence of K^+) binding site for ouabain (Crambert, Hasler et al. 2000; Muller-Ehmsen, Juvvadi et al. 2001). In other species, such as rat, O'Brien et al. reported that three α -isoforms in the rat have dissociation constants of 5 μ M, 115 nM, and 1.6 nM, respectively. As a result, in these species, physiological DLC concentrations, upon a brief exposure of minutes, have a minimal effect on Na,K-ATPase activity (Muller-Ehmsen, Juvvadi et al. 2001). This fact has led to the hypothesis that the DLC-induced inhibition of ion pumping by Na,K-ATPase at the plasma membrane is not the physiological role of these steroids. However, the physiological role of endogenous ouabain has not been clearly defined. DLC parameters including water and salt homeostasis, cardiac contractility and rhythm, systemic blood pressure, cell growth and differentiation and behavior need to be studied (Fig. 4). In many cases, perturbation of the DLC system has been implicated in pathological conditions including cardiac arrhythmias (Lichtstein 1995), hypertension (Blaustein, Zhang et al. 2006), cancer (Weidemann 2005) and depressive disorders (Goldstein, Levy et al. 2006). Interestingly, a number of studies have shown that exogenous cardiac glycosides, specifically ouabain, at low concentrations can initiate signaling cascades, as well as increase Na,K-ATPase activity *in vitro*. It has been suggested that DLC affects cell growth and proliferation. Physiological concentrations of DLC have a proliferative effect on smooth muscle and endothelial cells (Aydemir-Koksoy, Abramowitz et al. 2001; Chueh, Guh et al. 2001; Abramowitz, Dai et al. 2003). In addition, ouabain has been shown to exert an anti-apoptotic effect on endothelial cells (Orlov, Thorin-

Trescases et al. 2004). To the contrary, bufadienolides were found to induce apoptosis in human leukemia cells (Watabe, Nakajo et al. 1997) and have anti-proliferative and immunosuppressive activity on T-cells (Terness, Navolan et al. 2001). Furthermore, several studies have demonstrated that digoxin and ouabain at high (μM) concentrations also induce apoptosis via activation of caspase-3, early cytochrome C release from mitochondria and generation of reactive oxygen species (ROS) in prostate cell lines PC-3, LNCaP and DU145 (Akimova, Lopina et al. 2005). These findings illustrate that ouabain indeed regulates cells growth and apoptosis and raises the question if it is a general mechanism that may take place in other tissues and if the effect of ouabain on cell apoptosis is related to cell types or cell status. The level of endogenous ouabain is known to be significantly increased in some clinical conditions where extensive cell growth and differentiation are required. Consistent with these findings, high circulating levels of ouabain are found in pregnancy (Vakkuri, Arnason et al. 2001) and postnatally (Di Bartolo, Balzan et al. 1995). Interestingly, it has been reported that endogenous ouabain levels are increased following nephrectomy (Yamada, Goto et al. 1994), a condition that is associated with compensatory growth of the remaining kidney.

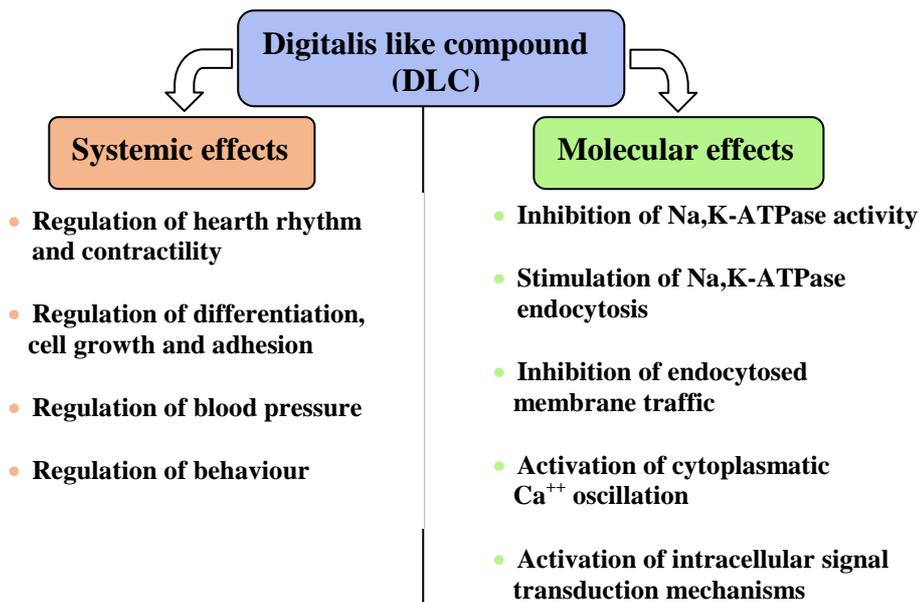


Fig. 4. Molecular and systemic effects of digitalis-like compounds.

Na,K-ATPase- a signaling transducer

It was recently demonstrated that in addition to functioning as an ion pump, Na,K-ATPase is also a signal transducer. Na,K-ATPase can serve as a scaffold and bring different proteins into a signaling complex.

There are many proteins that have been shown to associate with Na,K-ATPase. These include PKC, PKA and PTK (Therien and Blostein 2000), PI3K (Yudowski, Efendiev et al. 2000), 14-3-3 (Efendiev, Chen et al. 2005), Ankyrin (Nelson and Veshnock 1987; Devarajan, Scaramuzzino et al. 1994), AP2 (Done, Leibiger et al. 2002), PKG (Fotis, Tatjanenko et al. 1999), actin (Cantiello 1995), adducin (Ferrandi, Salardi et al. 1999), pasin (Kraemer, Koob et al. 1990), cofilin (Kim, Jung et al. 2002) and Adaptor protein 1 (Yudowski, Efendiev et al. 2000). Early studies focused on how these interactions regulate the ion pumping function of the Na,K-ATPase. Recent studies have begun to address the scaffolding function of the Na,K-ATPase.

Xie's group has demonstrated that Na,K-ATPase and Src receptor form a signaling microdomain that resides in and signals from caveolae. Caveolae are plasma membrane microdomains that look like flask-shaped vesicular invaginations of different sizes. Ouabain can regulate the interaction between Na,K-ATPase and caveolins (Wang, Haas et al. 2004) and stimulate Src kinase and tyrosine phosphorylation of EGFR, followed by activation of Ras, the Ras/Raf/Erk1/2 cascade (Akimova, Bagrov et al. 2005). Binding of Src to Na,K-ATPase inhibited Src activation whereas addition of ouabain, released the kinase domain and restored Src activity (Liang, Cai et al. 2006; Tian, Cai et al. 2006). The effects of ouabain on signal transduction are mediated by non-pumping pool of Na,K-ATPase present in the plasma membrane (Liang, Tian et al. 2007).

Ouabain activates Na,K-ATPase /IP₃R complex, triggers Ca²⁺ oscillation and activates NF-κB

Anita Aperia's group has recently demonstrated that ouabain, at nM concentrations which elicit only partial Na,K-ATPase inhibition, induces slow, regular Ca²⁺ oscillations in primary renal proximal tubule cells (Aizman, Uhlen et al. 2001). Since calcium release from the intracellular stores via inositol 1,4,5-triphosphate receptor (IP₃R) is generally required for an oscillatory calcium response, this led us to examine the interaction between Na,K-ATPase and the IP₃R. It was found that the Na,K-ATPase α subunit interacts with IP₃R to form a cell signaling microdomain. In the presence of ouabain, this generates slow Ca²⁺ oscillations. Because FRET studies suggested the close proximity between the N-terminus of Na,K-ATPase and IP₃R, it was suggested that the N-terminal tail of Na,K-ATPase plays a critical role for their interaction. Ouabain failed to induce Ca²⁺ oscillations in cells transfected with Na,K-ATPase α_1 -subunit where 38 amino acids were deleted from the N-terminus (α_1 M38). This suggests that the deleted portion of the N-terminus is important for Na,K-ATPase/IP₃R interaction. The downstream effect of this signaling pathway is activating nuclear transcription factor NF-κB (Miyakawa-Naito, Uhlen et al. 2003) (Fig. 5).

It has shown that both Na,K-ATPase and IP₃R can interact with ankyrin (Lencesova, O'Neill et al. 2004; Mohler, Davis et al. 2005). It is therefore possible that ankyrin may play a role as a scaffolding protein in such a complex. In pilot studies, we found that both Na,K-ATPase and IP₃R-immunoprecipitates contained ankyrin (Miyakawa-Naito et al., unpublished results). Ankyrins belong to a ubiquitously expressed intracellular scaffolding protein family that includes Ank-B, Ank-G and Ank-R (Mohler, Gramolini et al. 2002). Ankyrins associate with a diverse set of membrane, cytoskeletal, and cytoplasmic proteins and tether them into specialized membrane signaling domains. Both AnkB and Ank G have been

reported to interact with Na,K-ATPase, but AnkB is the only ankyrin isoform that has been reported to interact with IP₃R (Mohler, Gramolini et al. 2002).

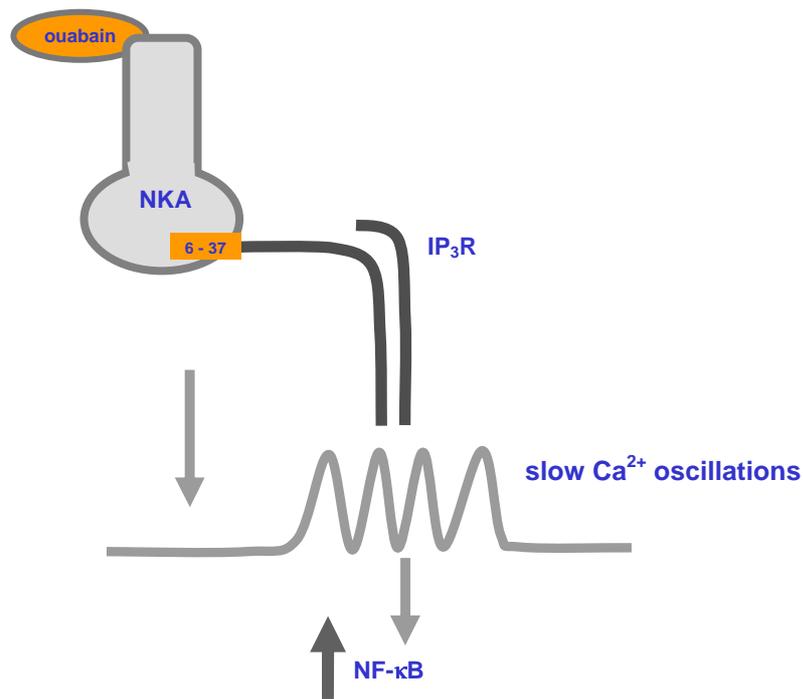


Fig.5 Ouabain signaling through Na,K-ATPase/IP₃R complex

Open questions

Is Na,K-ATPase and IP₃R interaction due to Na,K-ATPase directly binding to IP₃R or does it occur via some intermediate proteins? If Na,K-ATPase directly binds to IP₃R, what is the binding motif? If ankyrin is a member of the Na,K-ATPase/IP₃R microdomain, what is the functional role of ankyrin for ouabain/Na,K-ATPase/IP₃R signaling?

Calcium – a second messenger

Calcium is one of the major intracellular second messengers. Cytoplasmic Ca²⁺ varies in a dynamic manner as a result of Ca²⁺ release from intracellular stores (endoplasmic/sarcoplasmic reticulum, mitochondria) or regulated Ca²⁺ influx from

the extracellular space (Fig.6). To fit cell needs, the Ca^{2+} signals are shaped in space, time and amplitude. There are at least three major types of Ca^{2+} signal: sustained increase, Ca^{2+} transients and oscillations. An uncontrolled increase in Ca^{2+} is highly toxic for the cell and leads to cell death through both necrosis and apoptosis (Berridge, Bootman et al. 1998). To prevent the toxic effect of a sustained increase in Ca^{2+} cells use single Ca^{2+} spikes and Ca^{2+} oscillations as a signaling mechanism. Variations in cytoplasmic Ca^{2+} represent potent regulatory signals, activating enzymes and altering myriad protein interactions (Berridge, Bootman et al. 1998; Freedman 2006). Ca^{2+} is involved in the regulation of gene transcription, cell adhesion, cell growth, proliferation and apoptosis (Berridge and Robinson 1998).

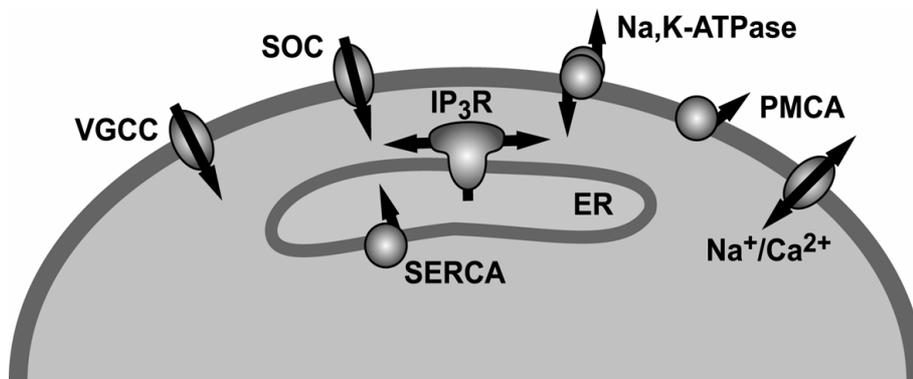


Fig.6 Calcium transport proteins

NF- κ B

NF- κ B is a family of transcription factors. Normally NF- κ B binds to I κ B in the cytoplasm which mask its nuclear localization sequence (NLS). Upon stimulation, I κ B is rapidly degraded. The free NF- κ B can then translocate into the nucleus, bind to specific DNA sequences and regulate gene transcription (Fig.7). Although NF- κ B target genes have been most intensely studied for their involvement in immunity and inflammation, this transcription factor also regulates

cell proliferation, differentiation and apoptosis. It has been suggested that NF- κ B activity is involved in the regulation of human skin epithelial and mesenchymal cells, and breast cancer cell growth (Yu, Geng et al. 2001; Hinata, Gervin et al. 2003). It is also suggested that NF- κ B activation is responsible for apoptosis resistance in HT1080I cells, NIH 3T3 cells, RelA 3T3 cells and HT-29 colon cancer cells (Chen, Wang et al. 2003; Vasudevan, Gurumurthy et al. 2004). Some evidence suggests that low frequency Ca^{2+} oscillations activate NF- κ B (Delfino and Walker, 1999). NF- κ B is also involved in kidney development by activating expression of Wt1 and Pax2 genes (Dehbi, Hiscott et al. 1998; Chen, Liu et al. 2006). Both of them play a key role during kidney development.

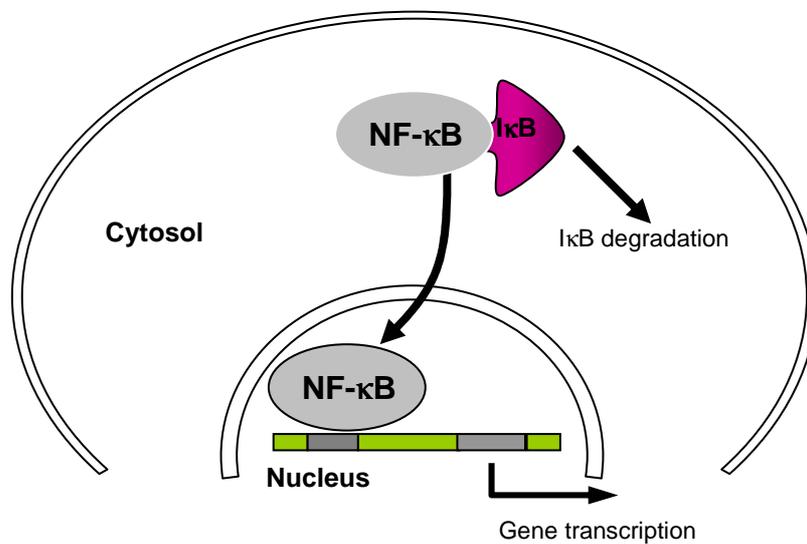


Fig. 7 NF- κ B activation

Open questions

Since the down stream effects of the signaling cascade induced by ouabain/Na,K-ATPase are not clearly understood, it is important to examine whether ouabain/Na,K-ATPase complex activation Ca^{2+} oscillations and NF- κ B can influence cell proliferation and apoptosis.

Role of ouabain/Na,K-ATPase/IP₃R for kidney development

During the kidney development, cell proliferation, differentiation and apoptosis play critical roles. During the early embryonic period, the developing kidney grows exponentially, doubling in size every 9-10 hours. This high rate of cell proliferation is accompanied by a high rate of apoptosis. Apoptosis serves to eliminate unwanted cells during organogenesis. It has been estimated that in the developing kidney, 1 cell dies for every 3-4 cells produced by division (Coles, Burne et al. 1993). This pattern requires a well controlled balance between cell proliferation and apoptosis. If this balance is broken, it causes diseases such as polycystic kidney disease, multicystic dysplasia, congenital nephrosis, renal hypoplasia, and Wilms' tumor (Sariola and Philipson 1999).

Fetal malnutrition and other factors contributing to renal growth retardation result in a reduction in nephron endowment which is correlated with a high rate to have hypertension and chronic kidney disease (Alexander 2007). The precise mechanism for the reduction in nephron number has not been established, but increased apoptosis, low cell proliferation in the developing kidney are quite related (Zandi-Nejad, Luyckx et al. 2006).

During kidney development, a large number of genes are required. Among them, the Wilms' tumour 1 (Wt1) gene plays a critical role. It plays an important role at three different stages of kidney development: the onset of kidney formation, the progression of kidney formation and the maintenance of normal kidney function (Rivera and Haber 2005). The Wt1 proteins have been implicated in various cellular processes like proliferation, differentiation and apoptosis (Mrowka and Schedl 2000). It has been suggested that NF- κ B can activate Wt1 expression (Dehbi, Hiscott et al. 1998). Pax2, a paired-domain protein expressed in the ureteric bud, metanephric mesenchyme, and in epithelial derivatives of the metanephric mesenchyme, is a key player in kidney morphogenesis (Narlis, Grote

et al. 2007). In Pax2-deficient embryos, the kidney and genital tract never develop. It has been reported in 1995 that the renal-coloboma syndrome is caused by heterozygous mutations of Pax2 gene. In this syndrome, nephron number is strikingly reduced. The nephron deficit is caused by a loss of anti-apoptotic effect of Pax2 during kidney development (Fletcher, Hu et al. 2005). NF- κ B can mediate Pax2 gene expression (Chen, Liu et al. 2006).

Na,K-ATPase is expressed in early stage of embryogenesis,. Even at the late tail-bud stage, Na,K-ATPase is expressed in the ear vesicla and in the pronephric rudiment. Expression in the pronephric rudiment was maintained throughout embryogenesis thereafter (Eid and Brandli 2001). Most research into the role of Na,K-ATPase in embryogenesis has focused on the pump's function relation to fluid transport and ion transport (Kidder and Watson 2005; Nebel, Romestand et al. 2005). The role of Na,K-ATPase as a signaling transducer during development is not quite clear. Some research suggests that Na,K-ATPase is required for septate junction function and epithelial tube-size control which is crucial for the function of organs such as the lung, kidney and vascular system during embryogenesis (Caspers, Schwartz et al. 1987). This suggests that Na,K-ATPase as a signal transducer, may play specific roles during kidney development.

A variety of developmental processes have been reported to be regulated by release of calcium from the intracellular stores *via* IP₃R (Berridge, Lipp et al. 2000). It has been suggested that IP₃R is involved in fertilization and early cleavage divisions and is essential for determination of dorso-ventral axis formation (Saneyoshi, Kume et al. 2002).

Ouabain, as an endogenous hormone, is significantly increased during pregnancy (Vakkuri, Arnason et al. 2001), postnatally (Di Bartolo, Balzan et al. 1995) and following nephrectomy (Yamada et al., 1994).

Open questions

Since ouabain/Na,K-ATPase/IP₃R complex can trigger calcium oscillations and active NF-κB and NF-κB can activate WT1 and Pax2 expression which are important for kidney development, we hypothesize that ouabain/Na,K-ATPase/IP₃R may regulate cells proliferation and apoptosis during kidney development.

Aims of the study

The overall aim of this thesis was to explore the molecular mechanisms involved in ouabain/Na,K-ATPase/IP₃R signaling and to elucidate the importance of this signaling pathway for the regulation of cell proliferation, apoptosis and kidney development.

The main goals were:

- To explore the mechanisms by which Na,K-ATPase activates the IP₃R

Specifically I wanted:

- 1) To identify a binding motif between Na,K-ATPase and IP₃R.
 - 2) To evaluate the role of cytoskeleton protein, ankyrin, for the ouabain/Na,K-ATPase/ IP₃R complex.
- To investigate the downstream effect of ouabain/ Na,K-ATPase/ IP₃R signaling.

Specifically I wanted:

- 1) To elucidate the signaling pathway involved in NF- κ B activation.
 - 2) To study the effect of ouabain on cell proliferation and apoptosis.
 - 3) To identify the signaling pathway involved in regulating cell proliferation and apoptosis.
- To examine the role of ouabain/ Na,K-ATPase/ IP₃R mediated intracellular signaling for the regulation of embryonic kidney development.

Specifically I wanted:

- 1) To study the effect of ouabain on nephrogenesis in growth factor deprived embryonic rat kidney.
- 2) To elucidate the signaling pathway involved in ouabain's effect on nephrogenesis.
- 3) To identify specific genes involved in ouabain's effect on nephrogenesis.

Materials and Methods

Materials

Cells

This study was primarily performed using two different cell types:

- Rat proximal tubule (RPT) cells were prepared from kidney cortex of 20 day-old male Sprague-Dawley rats. The rats were anesthetized, the kidneys removed and kidney cortex was dissected. The kidney cortex was dissociated in buffered solution supplemented with collagenase and triturated with a Pasteur pipette. Cells were cultured in DMEM containing 20 mM Hepes, 24 mM NaHCO₃, 10µg/ml - penicillin, 10 µg/ml - streptomycin and 10% FBS for 24 h. For serum deprivation experiments, cells were preincubated with 0.2% FBS for 24 h. RPT cells grow as clusters and have morphology typical of epithelial cells from proximal tubule. They have a high level of Na,K-ATPase expression. They maintain a physiological apoptotic rate (0.5-1.5%) and they are sensitive to serum deprivation induced apoptosis.
- COS-7 cells are an established fibroblast-like epithelial cell line derived from African green monkey embryonic kidney (Gerard and Gluzman, 1985). COS-7 cells were purchased from ECACC (European Collection of Cell Cultures) and were maintained in Dulbecco's Modified Eagle's Medium (Sigma), supplemented with 10 % FBS and 2 mM L-glutamine. These cells are easy to handle and are easily transfected with high efficacy using different transfection methods.

Organ culture

Whole kidney rudiments were isolated from Sprague–Dawley rat embryos at day 14 of gestation (day 0 of gestation coincided with the appearance of a vaginal plug in timed pregnancies). Kidney explants were cultured intact on the top of a

Transwell filter (0.4-mm poresize, CoStar, Cambridge, MA) within individual wells of a 12-well tissue culture dish containing 800 μ l of DME/F12 media (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum. The isolated kidneys were cultured at 37°C in an atmosphere containing 5% CO₂ and 100% humidity. 1 or 10nM ouabain was added to the medium from the second day of culture. For serum deprivation-induced apoptosis, embryonic kidneys were incubated with 0.2% FBS for 24 h before the experiment.

The embryonic kidney serves as a model organ for developmental studies because of the ease with which it develops and can be studied in culture (Saxen, L 1987). It is a useful organ system to follow epithelial-branching morphogenesis, inductive-tissue interactions, differentiation, cell polarization, mesenchymal -to-epithelial transformation and pattern formation. Kidney development is regulated by sequential and reciprocal inductive-tissue interactions. How the signals that determine these interactions function and coordinate the expression of other regulatory molecules, such as transcription factors, are not fully understood.

All animal studies were approved by the Swedish Animal Ethical Committee, Karolinska Institutet.

Methods

Detection and quantification of apoptotic cells (TUNNEL staining)

For determination of apoptotic index, we used ApopTag Red In Situ Apoptosis Detection kit (Chemicon Int., USA). In this assay, DNA strand breaks are used as a biochemical marker of early apoptotic events. The assay was carried out according to the manufacturer's instructions. Briefly, cells or embryonic kidney sections (5 μ M) cultured in 10% FBS or 0.2% FBS for 24 hours and treated with different ouabain concentrations, were fixed in 0.5% paraformaldehyde for 10 min, washed twice in PBS, post-fixed in a 2:1 mixture of ethanol:acetic acid at -20°C for 5 min, and then washed again. Cells were incubated first with an equilibration buffer and then with the TdT solution at 37°C for 1 hr. The reaction was stopped

with a Stop/Wash buffer. Cells were washed twice in PBS and then incubated in the dark with the anti-digoxigenin conjugate for 30 min at RT. After incubation, cells were washed four times with PBS. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI 1.5 µg/ml) added to PBS during the last wash. Cells were mounted in Immu-Mount (Thermo Shandon, USA). Cell preparations were observed with a Leica TCS SP inverted confocal scanning laser microscope using a 40 X/1.4 N.A. oil-immersion objective. ApopTag Red fluorescence was excited at 543 nm and detected with a 560-620 nm band pass filter. Images were recorded using Leica software. DAPI stained cells were viewed using a UV light source. Cells were considered apoptotic if they exhibited DAPI and ApopTag Red staining and presented characteristic apoptotic morphology (cell shrinkage, pyknotic nuclei and apoptotic bodies).

Apoptotic index (AI) (number of apoptotic cells/total number of cells counted×100%), was determined by counting the number of ApopTag positive cells and total number of DAPI stained cells. In each preparation, 5-7 randomly selected areas were examined, and in each area, between 200-300 DAPI stained cells were counted.

TUNNEL staining is a very sensitive and specific method for quantifying apoptosis and is based on the detection of DNA strand breaks. There are however rare situations when apoptosis is induced without DNA degradation. Thus, another independent assay should be used along with the TUNNEL method to confirm apoptosis.

Measurements of Cell Proliferation

RPT cell proliferation Cells were cultured under standard conditions on 60 mm culture dishes for 24h. [³H]thymidine labeled nucleotide which can incorporate into DNA during S-phase of cell cycle, was added to cells at 1 µCi/well and for 24 h in DMEM 5% FBS in the absence or presence of different ouabain concentrations. Cells were subsequently washed twice with PBS and then lysed in 1M NaOH. The cell lysate was used to measure radioactivity by

scintillation counting (LKB, Wallac, Turku, Finland). Protein content was determined using a kit from Bio-Rad (Bio-Rad Laboratories CA) following the manufacturer's instructions. All experiments were performed in triplicate.

[³H]-thymidine incorporation method is a direct and sensitive method to measure cell proliferation. It is a radiolabeled nucleotide and ³H has a long half-life and the radioactive waste must be properly managed.

COS-7 cell proliferation was determined as described above with some modifications. COS-7 cells (10000 cells/well) were cultured in 96-well flat bottom tissue culture plates in DMEM medium supplemented with 10% FBS for 24h. The medium was changed to DMEM with 1% FBS for an additional 24h with or without different ouabain concentrations and cells were incubated with 2.5 μCi/well of [³H]-thymidine during the final 5 h of culture. Cells were harvested by using an automatic harvester and [³H]-thymidine incorporation was measured by liquid scintillation counting.

WST-1 assay (Chemicon Int., USA) measures the increase in metabolic activity and is an index of expansion in the number of viable cells. Cells (10000 cells/well) were cultured in a 96-well plate (100μl of culture medium/well) for 24 h and exposed for 24 h to different ouabain concentrations or to medium alone (control). WST-1 reagent (20 μl) was added directly to culture wells and incubated for 1 hour. The absorbance was measured at 450 nm with a 96-well plate reader. The optical density values were normalized to baseline values and presented as percentage of control.

WST-1 assay is a very simple, easy, and fast method. But it is not a direct method and WST-1 is not metabolized by all cell types. It should be accompanied with other methods.

Trypan blue dye exclusion test was used to evaluate cell viability. Trypan blue dye can enter into cells with a damaged plasma membrane. After treatment with indicated ouabain concentrations for 24 hours, cells were harvested and the

relative number of viable cells was determined by microscopic examination. Trypan blue dye is a classical standard method to distinguish viable from dead cells. It's a quick and cheap and fast method. But it only stains necrotic cells or very late apoptotic cells (secondary necrosis). It is not suitable for detection of apoptosis.

BrdU incorporation was used to determine embryonic kidney cells proliferation. BrdU, an analogue of thymidine that is incorporated into DNA during the S-phase of the cell cycle (Dolbeare, 1995), served as an indicator of cell cycle entry into mitosis. The cell proliferation kit (RPN20, Amersham Biosciences, UK) was used as suggested by the manufacturer with minor modifications. Briefly, cultured kidneys were incubated for 3 h in the presence of 10 μ M BrdU and washed with PBS to remove unincorporated BrdU, fixed with 3% paraformaldehyde at RT for 3h, cryopreserved and cryosectioned. Sections (5 μ M) were detected with the mouse anti-BrdU antibody (1:1000) provided in the kit. Secondary antibody was anti-mouse Alexa 488 (1:1000). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI 1.5 μ g/ml). Images were recorded using an inverted Zeiss LSM 510 confocal microscope equipped with a C-Apochromat 10x/NA 0.45 objective. Alexa 488 (green) was imaged using a 488nm argon laser with primary dichroic mirror HFT 405/488/561, secondary beam splitter NFT 565 and a LP 505 long pass filter. DAPI stained cells were viewed using a UV light source. Cells were considered proliferative if they exhibited DAPI and BrdU green staining.

BrdU index (BI) (number of proliferative cells/total number of cells counted \times 100%), was determined by counting the number of BrdU positive cells and DAPI stained cells. In each preparation, 5-7 randomly selected areas were examined, and in each area around 1000 DAPI stained cells were counted. All sections were blinded before viewing by the investigator.

BrdU incorporation is a specific and direct method to detect cells proliferation. It is radioactive free. This BrdU incorporation kit is designed for

immuno-histochemical staining of BrdU and is useful to measure cell proliferation in organs or tissues.

NF- κ B activity

NF- κ B translocation to nucleus was used as an index of NF- κ B activation and was studied using immunocytochemistry and subcellular fractionation.

Immunocytochemistry Cells were fixed with 2% paraformaldehyde and blocked with 5% normal goat serum and 0.1% triton. NF- κ B was probed with rabbit polyclonal anti-NF- κ B p65 antibody (1:200) (Santa Cruz Biotechnology, Inc.) and secondary antibody anti-rabbit Alexa 546 (1:3000). Green fluorescent protein was probed with mouse polyclonal anti-GFP antibody (1:200) (IBD) and goat anti-mouse Alexa 546 (1:6000) (Molecular Probes) as a secondary antibody. The immunolabelled cells were observed with a Leica TCS SP inverted confocal scanning laser microscope using 40 X/1.4 N.A. objectives. Preparations, where the primary antibody was omitted from the staining protocol, were used as negative controls. NF- κ B translocation to nucleus was semi-quantitatively calculated as the ratio between the mean fluorescent signal intensity in a given area in the nucleus and cytosol. In each preparation, 6-7 images containing around 100 cells in each field of view were randomly recorded by confocal microscopy and all cells in these fields were analyzed. Calculations were performed using a software package from Scion Image (Scion Corporation, USA) by a person that was blind to the protocol performed.

Immunocytochemistry is an effective technique to visualize the distribution of specific proteins in the cell. The specificity of the antibody is very important for this technique. The sample preparation and fixation protocol are also very important. The calculation of the ratio between nuclear and cytosolic staining is time consuming.

Subcellular Fractionation RPT cells or embryonic kidneys were washed with ice-cold PBS, then 200 μ l of Buffer A (10 mM HEPES [pH 7.9], 10 mM KCl,

2 mM MgCl₂, 0.1 mM EDTA, cocktail of protease inhibitors and 2% NP 40) was added. The mixture was vortexed and incubated on ice for 10 min and centrifuged at 500 x g for 5 min; the supernatant was collected and represents cytosolic protein. The pellet was resuspended in 65 µl of buffer B (50 mM HEPES, 10% glycerol, 300 mM NaCl, 50 mM KCl, and a cocktail of protease inhibitors). The mixture was vortexed and incubated on ice for 30 min and centrifuged at 13,000 x g for 10 min; the supernatant was nuclear protein. The protein content was measured using BioRad protein assay reagent. When the extracts were not used immediately, they were stored at -80°C.

Western blot Cell fractions were probed with rabbit polyclonal anti-NF-κB p65 (1:2000) (Santa Cruz Biotechnology, Inc.). IκB was probed with a rabbit antibody (1:2000) (Santa Cruz Biotechnology, Inc.).

Western blot is a very simple and fast way to measure NF-κB protein expression. It allows measurement of total nuclear NF-κB content and as such includes NF-κB that is not necessarily activating gene expression.

TransAM™ NFκB p65 Kit (Active motif, USA) was performed following the manual. 0.4 ng nuclear extracts were processed in each well of a 96-well dish. The activated NF-κB present in nuclei binds to an oligonucleotide containing an NF-κB consensus binding site which was immobilized in the well. By using an antibody directed against the NF-κB p65 subunit, the NF-κB complex which bound to the oligonucleotide was detected. Secondary antibody which was conjugated to horseradish peroxidase (HRP) was added and chemiluminescence was determined by luminometry. Nuclear extracts from TPA-stimulated Jurkat cells were used as a positive control. To demonstrate binding specificity, an NF-κB wild-type consensus oligonucleotide and a mutated consensus NF-κB oligonucleotide were used.

TransAM™ NF-κB p65 Kit measures NF-κB which enter into the nuclear and binds to its target consensus sequence necessary for gene activation. Compared to western blot, it is more specific and easier.

Ratiometric imaging

Cells or whole kidney explant cultures were incubated with 3-10 uM Fura-2 acetylmethyl ester (Fura-2/AM) (Molecular Probes) for 40-120 min before the $[Ca^{2+}]_i$ measurements. Ratiometric imaging was performed using a heated chamber (FCS2, Bioptechs) mounted on a Zeiss Axiovert 135 microscope using a 40 X/1.4 epifluorescence oil-immersion objective. Fura-2/AM loaded cells were excited at wavelength 340/10-nm and emission fluorescence was collected via a GenIISys image intensifier system connected to a CCD-camera (MTI CCD72; Dage-MTI) and acquisition software from Inovision Corporation. Cells were excited every 30 s. All experiments were performed using PBS medium (100 mM NaCl/4 mM KCl/20 mM Hepes/25 mM NaHCO₃/1 mM CaCl₂/1.2 mM MgCl₂/1 mM NaH₂PO₄·H₂O/10 mM D-glucose).

Whole-mount immunostaining

Kidney explants after 2 or 3 days of culture were fixed on filters with 100% methanol for 10 minutes and rinsed with a solution of 0.1% Tween-20 in PBS (PBST) for 10 minutes. Anti-E-cadherin (Sigma, 1:500), and anti-Wt1 (Santa Cruz, 1:200) primary antibodies were diluted in PBST with 2% serum and incubated overnight at 4 °C. The explants were washed with PBST three times for 10 minutes each with another wash with PBST overnight at 4 °C. Explants were then incubated with the secondary antibodies Alexa 488 anti-rat (Molecular Probes, 1:200), and Alexa 546 anti-mouse (Molecular Probes, 1:400) diluted in PBST with 2% fetal calf serum for 3 hours at room temperature. The explants were then washed three times for 10 minutes in PBST and mounted with mounting medium.

Confocal microscopy and glomerular and ureter tip counts

Following whole-mount immunostaining, explants were imaged with an inverted Zeiss LSM 510 confocal microscope equipped with a C-Apochromat

10x/NA 0.45 objective. Alexa 488 (green) was imaged using a 488nm argon laser with primary dichroic mirror HFT 405/488/561, secondary beam splitter NFT 565 and a LP 505 long pass filter. Alexa 546 (red) was imaged using a 561 nm diode laser, HFT 405/488/561 dichroic mirror, NFT 565 beam splitter, and a LP 575 long pass filter. Glomeruli were detected by Wt1 staining (red) and ureteric bud was detected by E-cadherin staining (green). By regulating the focal plane, we imaged with main ureteric branching at the largest cross-section for each kidney. Glomeruli and ureter terminal tips were counted in the largest sagittal cross-section from each kidney. The number of glomeruli and ureter terminal tips per maximal cross-section was taken as a reflection of total kidney development. For each group, around 8-10 explants were counted and the number in the control kidney was adjusted to 100%. All sections were blinded and counted by investigator.

RNA extraction

Total RNA was extracted from kidneys cultured for 2 days according to the manufacturer's recommendations for Aurum Total RNA Mini Kit (Bio-Rad). In each experimental group, 6 kidneys were used. RNA quality was evaluated using real time PCR quantification of 18S mRNA with or without iScript Reverse Transcriptase for each RNA sample. Average Δ Ct value for this control was found around 27.2 ± 4 . Average Δ Ct value for no-template controls and minus RT controls was found around 27.2 ± 4 .

Real time RT PCR

Reverse transcriptase PCR and PCR was performed using iScript One-Step RT-PCR Kit with SYBR Green (Biorad) following the instruction from the manufacturer with a few modifications. A *iCycler iQ* PCR system (Bio-Rad) was used to amplify primed message and detect fluorescence incorporation of the SYBR Green reagent. The volume of samples was 20 μ l: PCR primers corresponding gene of interest are shown in Table 1. PCR quantification was performed in quadruplet for each sample.

Concentration of RNA samples was estimated and normalized using commercial 18S Classic II primers (100nM) and 18S Classic II competitors (300nM) (Quantum RNA 18S Internal Standards kit, Ambion). 18S stands for a ribosomal 18S RNA. Quantum RNA 18S Internal 5 Standards (Ambion) were used as the “housekeeping” gene to normalize for variations in RNA quality and starting quantity, and random tube-to-tube variation in RT and PCR reactions. Real time RT PCR gene of interest values were normalized to 18S Classical II values.

Rat Wt1 mRNA structure (accession number X69716) was used to select Wt1 specific primers (13). The rat Wt1 primers should recognize all known isoforms of Wt1.

rWT1.0616U 5'-TGCCACACCCCTACCGACAGTT-3' 150 nM, Ta 65°C

rWT1.0756L 5'-CTTCAAGGTAGCTCCGAGGTTTCATC-3'

Rat Pax2 specific primers were selected using the predicted rat Pax2 mRNA structure (accession number XM_239083) (14). The rat Pax2 PCR fragment should correspond to the human Pax2 mRNA splicing variants *A* and *E* (accession number NM_003987 & NM_003990 respectively).

rPAX2.0654U 5'-TACTGATCCTGCCACATTAGA-3' 150 nM, Ta 55°C

rPAX2.0850L 5'-GGATAGGAAGGACGCTCAAAGACT-3'

Primers for real time RT PCR experiments were designed using PrimerSelect software (DNASTAR Inc, Madison, USA). Primer concentration and annealing temperature were optimized prior real time RT PCR quantification. Agarose gel analysis revealed single PCR product at the end of quantification in each assay. Structure of all PCR fragments was confirmed by sequence analysis.

Results and Comments

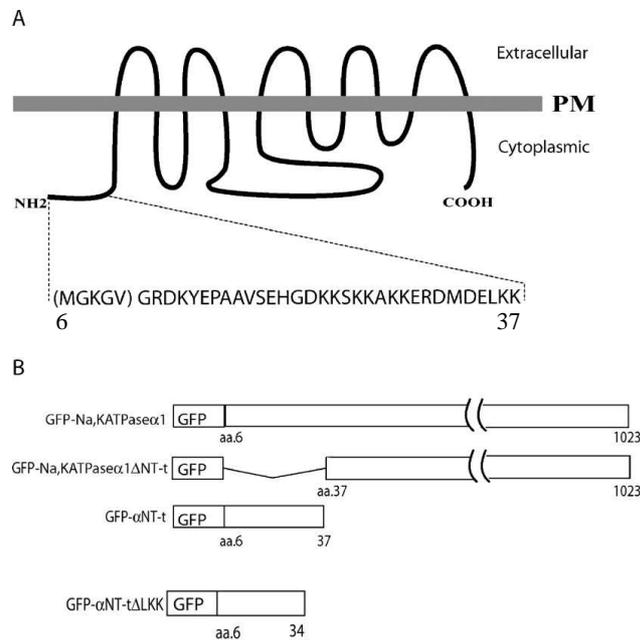
Na,K-ATPase directly binds to IP₃R to form a signaling micro-domain and ankyrin B tethers to the Na,K-ATPase /IP₃R complex (Papers I and II)

We have previously shown that Na,K-ATPase α subunit interacts with inositol 1,4,5,-triphosphate receptor (IP₃R) to form a signaling micro-domain. The N-terminal tail of Na, K-ATPase plays a crucial role for this interaction (Miyakawa-Naito et al., 2003). The exact mechanisms by which Na,K-ATPase activates the IP₃R remain to be elucidated. Additional studies were required to further investigate the exactly binding motif between Na,K-ATPase α subunit and IP₃R and how Na,K-ATPase/IP₃R interaction occurs, directly or via some intermediate proteins. The role of the scaffolding protein, ankyrin B (Ank-B), for this interaction was also studied.

The N-terminal tail of Na,K-ATPase α -subunit binds IP₃R. An LKK motif present in the N-terminal tail of Na,K-ATPase is essential for this interaction (Paper I)

First, we determined the interaction domain between IP₃R and Na,K-ATPase. By using a GST-pull down assay, we found that GST-IP₃R (encompassing amino acids 1-604) pulled down Na,K-ATPase α_1 , α_2 , and α_3 isoforms. It has been suggested that the N-terminal tail of the Na,K-ATPase α -subunit plays an important role for Na,K-ATPase signaling (Miyakawa-Naito et al., 2003). Next we examined whether N-terminal tail of the Na,K-ATPase α -subunit could assemble with GST-IP₃R (1-604). To test this, we generated a truncated form of the Na,K-ATPase α -subunit, where the first 32 residues of the N-terminal tail had been deleted (GFP-Na,K-ATPase $\alpha_1\Delta$ NT-t) as well as a small peptide fragment comprised solely of the N-terminal tail of Na,K-ATPase α_1 fused to GFP (GFP- α NT-t) and expressed them in COS-7 cells. We found that GST-IP₃R (1-604) assembled with GFP- α NT-

t but that GFP-Na,K-ATPase $\alpha 1\Delta NT-t$ did not. This demonstrated that the N-terminal tail of Na,K-ATPase $\alpha 1$ -subunit played an important role for the interaction. The N-terminal tail of different isoforms and species display little homology, except for 3 amino acid residues, LKK, which are conserved in all of the Na,K-ATPase α -subunit isoforms. To study the role of the LKK residues for Na,K-ATPase-IP₃R interaction, a peptide fragment corresponding to the N-terminal tail of the rat Na,K-ATPase $\alpha 1$ -subunit in which the LKK residues were deleted was generated (GFP- $\alpha NT-t\Delta LKK$). This fragment did not assemble with GST-IP₃R (1-604). To determine whether the Na,K-ATPase α -subunit can bind directly to the IP₃R, IP₃R (1-604) was tagged with His (InsP₃R (1-604)-His). Purified GST- $\alpha NT-t$ did bind to purified IP₃R (1-604)-His.



A, schematic structure of the rat Na,K-ATPase $\alpha 1$ and the amino acids sequence of the N-terminal cytoplasmic tail. B, GFP-Na,K-ATPase $\alpha 1$ or its truncations: GFP-Na,K-ATPase $\alpha 1\Delta NT-t$, GFP- $\alpha NT-t$ and GFP- $\alpha NT-t\Delta LKK$.

Ankyrin B tethers to the Na,K-ATPase /IP₃R complex by directly binding to the N-terminal domains of Na,K-ATPase and IP₃R (Paper II)

To test whether Ank-B may associate with Na,K-ATPase/IP₃R complex, immuno-precipitation assays were performed. Ank-B co-precipitated with $\alpha 1$ subunit of Na,K-ATPase and with IP₃R. Since the N-terminal tail of Na,K-ATPase $\alpha 1$ subunit interacts with IP₃R(1-604), we tested if Ank-B may bind directly to Na,K-ATPase α subunit N-terminus and IP₃R (1-604). Purified Ank-B was incubated with purified GST-tagged N-terminal tail of Na,K-ATPase α subunit or with purified GST-tagged IP₃R (1-604). We found that Ank-B bound directly to both these peptides. To study whether Ank-B may modulate the function of the ouabain/ Na,K-ATPase /IP₃R signalosome, we used siRNA technology. Since this study was carried out on monkey-derived COS-7 cells, the siRNA duplexes were based on the monkey Ank-B sequence. Forty-eight hours after siRNA transfection, Western blot detected that the Ank-B expression was consistently reduced to 10-20% of the value in non-transfected cells. We found that Na,K-ATPase-IP₃R interaction was dramatically diminished in Ank-B silenced cells compared to rat siRNA control cells.

Summary

The present results demonstrated that the N-terminal tail of Na,K-ATPase directly binds to IP₃R to form a signaling microdomain. The well conserved amino acid residues LKK in the N-terminal domain of Na,K-ATPase play a crucial role for this binding. Ank-B supports Na,K-ATPase and IP₃R binding.

Ouabain activates NF- κ B through Na,K-ATPase and IP₃R binding (Papers I, II, III)

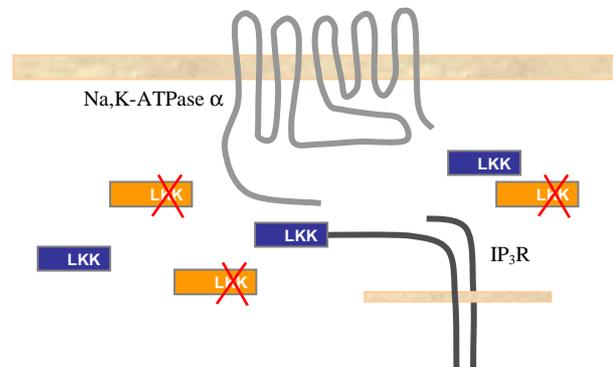
We have shown previously that 250 μ M ouabain triggers a translocation of NF- κ B to the nucleus within 30 min and that this effect depends on the interaction between Na,K-ATPase and IP₃R (Miyakawa-Naito et al., 2003). Here we studied

the effect of low, physiological concentrations of ouabain on NF- κ B activity. The involvement of the well conserved amino acid residues LKK in the N-terminal domain of Na,K-ATPase α subunit and Ank-B were also examined for their role in ouabain mediated activation of NF- κ B.

Ouabain activates NF- κ B (Paper III)

Under nonstimulated conditions, NF- κ B is located predominantly in the cytoplasm in association with the inhibitory protein I κ B. Upon activation, I κ B dissociates from this complex and NF- κ B is translocated to the nucleus (Senftleben, Cao et al. 2001). By using immunocytochemistry, we found that NF- κ B immunosignal was preferentially detected in the cytoplasm of control cells. After 24 h exposure to low (1 or 10 nM) ouabain, the nuclear/cytosolic ratio of the immunosignal was significantly increased indicating NF- κ B activation. In another protocol, subcellular fractionation was performed on cells that were exposed to 10 nM of ouabain. Immunoblotting of nuclear and cytosolic fractions showed a significant increase in nuclear NF- κ B and a decrease in cytosolic I κ B. Short-term exposure to a low ouabain concentration (10 nM) failed to activate NF- κ B. The effect was detected initially after 2 h and the maximum was achieved after 12 h.

Mechanism of ouabain-induced NF- κ B activation (Papers I, II)



Schematic model to explain a peptide corresponding to N-terminal tail of Na,K-ATPase α 1 with the well conserved amino acid residues LKK (GFP-Na,K-ATPase α 1NT-t) (blue box)

can bind to IP₃R and block Na,K-ATPase/IP₃R binding but N-terminal tail of Na,K-ATPase α 1 without LKK (GFP-Na,K-ATPase α 1NT-t Δ LKK) (yellow box) doesn't block.

First, we tested the impact of the well conserved amino acid residues LKK in the N-terminal domain of Na,K-ATPase α subunit on ouabain mediated activation of NF- κ B. Renal proximal tubule (RPT) cells expressing GFP- α NT-t, GFP- α NT-t Δ LKK, or GFP only were incubated with 10 nM ouabain for 24 h. NF- κ B activation was estimated by measuring the ratio of NF- κ B nuclear to cytosolic signal. In cells expressing GFP only or GFP- α NT-t Δ LKK, exposure to ouabain caused a significant increase in NF- κ B nuclear to cytosolic ratio. In contrast, the ratio was not affected in cells expressing GFP- α NT-t exposed to ouabain. This peptide can compete with Na,K-ATPase for binding to IP₃R and block Na,K-ATPase/IP₃R binding. To test whether Ank-B can modulate the NF- κ B response to ouabain, control and Ank-B silenced cells were exposed to a low dose of ouabain for 24 h. The translocation of NF- κ B from the cytoplasm to the nucleus was studied either with immunofluorescent labeling of NF- κ B in fixed cells or by subcellular fractionation and detection of activated NF- κ B. In both non-transfected cells and rat siRNA transfected cells as control groups, we observed a significant translocation of NF- κ B to nucleus after ouabain treatment. In contrast, we did not observe any changes in the subcellular distribution of NF- κ B in Ank-B silenced cells.

Summary

The present results demonstrated that physiological concentration of ouabain can activate NF- κ B. The well conserved amino acid residues LKK in the N-terminal domain of Na,K-ATPase α subunit and Ank-B play a crucial role for ouabain-mediated activation of NF- κ B.

Anti-apoptotic effect of ouabain is the down stream effect of ouabain/ Na,K-ATPase /IP₃R signaling pathway (Papers I, II, III)

Our previous studies revealed that low dose ouabain can activate Na,K-ATPase/IP₃R binding and give rise to slow, regular Ca²⁺ oscillations that activates NF-κB. NF-κB is involved in the regulation of cell growth, differentiation and apoptosis (Delfino and Walker 1999). Therefore we proposed that ouabain *via* Na,K-ATPase /IP₃R signaling pathway may protect cells from apoptosis.

Low doses of ouabain protect from serum deprivation–triggered apoptosis (Paper III)

The effect of ouabain on serum deprivation–triggered apoptosis was studied in RPT cells that were grown in medium supplemented with 10 or 0.2% FBS for 24h. Cells that were grown in 10% FBS had a low incidence of apoptosis. Serum deprivation (0.2% FBS for 24 h) caused a dramatic increase in the number of apoptotic cells. Ouabain in nanomolar concentrations completely abolished the apoptotic effect of serum deprivation.

Anti-apoptotic effect of ouabain is the downstream result of ouabain/ Na,K-ATPase/IP₃R signaling pathway (Papers I, II, III)

First we examined if deletion of the N-terminal of Na,K-ATPase α subunit could prevent the anti-apoptotic effect of ouabain. RPT cells were transfected with GFP-Na,K-ATPaseα₁ΔNT-t or GFP-Na,K-ATPaseα₁. In both groups, cells had similarly low apoptotic levels when grown in full serum condition. After 24 h of serum deprivation, apoptotic cells were increased to the same extent. In cells that expressed GFP-Na,K-ATPaseα₁, ouabain had significant protective effects on serum deprivation–triggered apoptosis. In cells that expressed GFP-Na,K-ATPaseα₁ΔNT-t, the anti-apoptotic effect of ouabain was abolished. Next, we examined the involvement of the amino acid residues LKK in the N-terminal of Na,K-ATPase α subunit for the anti-apoptotic effect of ouabain. Ouabain failed to

protect from serum deprivation-induced apoptosis in RPT cells expressing GFP- α NT-t which binds to IP₃R and blocks Na,K-ATPase α subunit binding to IP₃R.

We have shown that depletion of intracellular Ca²⁺ stores in the endoplasmic reticulum (ER) abolishes ouabain-induced Ca²⁺ signaling (Aizman, Uhlen et al. 2001). In the studies shown in paper III, RPT cells were pretreated for 24 h with a sarco-ER Ca²⁺-ATPase inhibitor, cyclopiazonic acid (CPA; 0.5 μ M), to deplete the intracellular stores of calcium. This treatment completely abolished the anti-apoptotic effect of ouabain. Regulated Ca²⁺ release from intracellular ER Ca²⁺ stores occurs via IP₃ receptors (IP₃R) or via ryanodine receptors. IP₃R are expressed abundantly in RPT cells, whereas ryanodine receptors do not seem to be of any functional importance in these cells (Aizman, Uhlen et al. 2001). The membrane-permeable substance 2-APB is an inhibitor of IP₃R-evoked Ca²⁺ release as well as a blocker of store-operated calcium-channels. IP₃R has been reported to be blocked completely by 1 to 20 μ M 2-APB, whereas store-operated calcium-channel activity is inhibited by 50 to 100 μ M 2-APB (Bootman, Collins et al. 2002). Exposure of RPT cells to 5 μ M 2-APB completely prevented the anti-apoptotic effect of ouabain in serum-deprived cells. Taken together, these results strongly indicate that ouabain-mediated protection from serum deprivation-triggered apoptosis depends on calcium release from the intracellular stores.

To examine the role of NF- κ B activation for ouabain-mediated protection from serum deprivation-triggered apoptosis, RPT cells were exposed to helenalin, an NF- κ B inhibitor. Helenalin (1 μ M) abolished the antiapoptotic effect of ouabain

Ouabain Stimulates Cell Proliferation (Paper III)

To study the rate of DNA synthesis, we determined the rate of [³H] thymidine incorporation. Ouabain 0.1 to 10 nM significantly stimulated RPT cell proliferation. Ouabain-mediated stimulation of cell proliferation was calcium dependent. Pretreatment of RPT cells with 5 μ M 2-APB or 0.5 μ M CPA completely abolished the stimulatory effect of ouabain on cell proliferation. These

effects of low-dose ouabain were not unique for rat kidney cells because they were also observed in COS-7 monkey kidney cells

Summary

Physiological concentrations of ouabain can rescue cells from serum-deprivation induced apoptosis and stimulate cell proliferation. These effects of ouabain require direct binding of Na,K-ATPase α subunit and IP₃R which triggers calcium oscillations and activation of NF- κ B. Thus, we present a down stream effect of ouabain/ Na,K-ATPase /IP₃R signaling.

Ouabain rescues nephrogenesis in growth- factor deprived embryonic rat kidney (Paper IV)

Normal kidney development is critically dependent on a well-controlled balance between cell proliferation and apoptosis. Aberrations in these processes lead to a variety of kidney malformations (Sariola and Philipson 1999). The inductive factors Wt1 and Pax2, which are of critical importance for kidney development, are known to be activated by NF- κ B (Dehbi, Hiscott et al. 1998; Chen, Liu et al. 2006). Circulating levels of ouabain are reported to be high during pregnancy and in the fetus (Vakkuri, Arnason et al. 2001). The ouabain level is increased following nephrectomy (Yamada et al., 1994). Taken together, we suggested that ouabain might have a positive effect on kidney development. To address this question, we established a model of cultured rat embryonic kidney.

Ouabain protects from apoptosis and rescues nephrogenesis in growth factor deprived kidneys

Kidneys dissected from rat 14 days post conception (dpc) embryos were studied after 72 h in culture. Under normal conditions (SS), the apoptotic cells were low. Serum deprivation (SD) significantly increased the apoptotic cells. Low ouabain concentrations completely abolished serum-deprivation triggered apoptosis. Next we examined the role of ouabain for the formation of new

nephrons. Embryonic 14 dpc kidneys cultured for 3 days were fixed and immunostained for Wt1 to identify newly formed glomeruli and for E-cadherin to identify the branching ureter. In each group, nephrons normally contained a single glomerular structure connected to the ureter bud via the distal tubule. In SD kidneys, the number of newly formed nephrons was significantly reduced as compared to SS kidneys. Exposure to ouabain significantly attenuated the response to SD.

The rescue effects of ouabain depend on intracellular calcium stores and NF- κ B activity

We have previously shown in RPT cells that ouabain in doses that do not fully inhibit Na,K-ATPase activity, triggers an interaction with the IP₃R, that results in slow calcium oscillation (Aizman, Uhlen et al. 2001; Miyakawa-Naito, Uhlen et al. 2003). An oscillatory response to ouabain was also observed in embryonic kidney cells exposed to ouabain. To test the role of IP₃R triggered calcium oscillations for the effect of ouabain, embryonic kidneys were exposed to CPA. CPA exposure completely abolished the anti-apoptotic effect of ouabain. CPA also abolished the rescuing effect of ouabain on nephrogenesis. We have found that ouabain activated NF- κ B is required for the anti-apoptotic effect of ouabain on RPT cells. Here we also found ouabain activated NF- κ B in embryonic kidney cells. Inhibition of NF- κ B activation by helenalin also abolished the rescuing effect of ouabain on apoptosis and nephrogenesis in embryonic kidney.

Ouabain restores Wt1 and Pax2 genes expression

A number of well-characterized genes are essential for normal kidney morphogenesis. The Wilms' tumor suppressor gene, Wt1, is necessary for ureteric bud outgrowth and survival of the metanephric blastema (Rivera and Haber 2005). Pax2 is another key player in kidney morphogenesis. In Pax2-deficient embryos, the kidney and genital tract never develop (Narlis, Grote et al. 2007). It has been reported by several groups that NF- κ B can activate Wt1 and Pax2 expression (Dehbi, Hiscott et al. 1998; Chen, Liu et al. 2006). Thus it is

interesting to study whether ouabain exposure may have an effect on Wt1 and Pax2 mRNA expression. To do so, we used real-time RT-PCR (qRT-PCR). In SD kidneys exposed to ouabain, the expression of Wt1 mRNA and Pax2 mRNA was significantly higher than in kidneys exposed to SD alone.

Ouabain stimulates cell proliferation during embryonic rat kidney development

To study cell proliferation, we determined DNA synthesis with BrdU incorporation. The majority of proliferative cells were observed in the periphery of the kidney and most of them appeared to be blast cells both in the control and in the ouabain treatment group. Ouabain 1nM significantly stimulated kidney cell proliferation.

Summary (Paper IV)

The present results demonstrate that ouabain can rescue nephrogenesis and protect from apoptosis in growth factor deprived embryonic kidney. These rescuing effects of ouabain are exerted by calcium release from intracellular stores, activation of NF- κ B and increase of Wt1 and Pax2 expression. Our findings underline the importance of the Na,K-ATPase -IP₃R signaling pathway for kidney development. Ouabain also stimulates embryonic kidney cell proliferation during kidney development.

Discussion and Future Perspectives

In the present thesis we focused on novel aspects of Na,K-ATPase function as a signal transducer. The present study provides documentation that Na,K-ATPase can regulate cellular functions via direct interaction with an intracellular signaling molecule. Here we demonstrate that Na,K-ATPase directly binds to IP₃R to form a signaling microdomain and that Ankyrin B (AnkB) tethers to this signaling microdomain. Physiological concentrations of ouabain activate this signaling pathway. The downstream effects of this signaling pathway are stimulation of cell proliferation, protection from apoptosis and rescue of growth factor deprivation induced inhibition of embryonic kidney nephrogenesis.

Na,K-ATPase directly binds to IP₃R to form a signaling microdomain and ankyrin B tethers to this complex

Na,K-ATPase is a main determinant of the sodium/potassium gradient across the plasma membrane in most eukaryotic cells (Skou 1998; Kaplan 2002). The minimal functional unit is a heterodimer composed of a catalytic α -subunit and a β -subunit (Beguín, Hasler et al. 1998). Recent work from many laboratories has suggested that Na,K-ATPase is an important signaling receptor. More and more work has begun to define the Na,K-ATPase signalosome and to map the functional domains that are involved in the organization of the individual signaling molecules.

In our lab, we have demonstrated that Na,K-ATPase α subunit can interact with IP₃R to form a signaling microdomain and that the N-terminal tail of Na,K-ATPase α subunit plays a crucial role for this interaction. The α subunit of Na,K-ATPase exists in at least four isoforms (Shamraj, Melvin et al. 1991; Lingrel, Moseley et al. 2003). All isoforms of rat Na,K-ATPase α -subunit interact with the IP₃R. We further demonstrate that the interaction domain of Na,K-ATPase, that facilitates IP₃R association, is located in the N-terminal tail of the α subunit. Although all Na,K-ATPase α -isoforms share more than 80% homology, the N-

terminal tail shows little homology between different isoforms (Ohta, Noguchi et al. 1991). One exception is the lysine-rich motif (LKK) in the N-terminal tail that is highly conserved in almost all species. This well conserved motif is found to be essential for the binding between the Na,K-ATPase α -subunit and the IP₃R, indicating the universal importance of this protein-protein interaction.

It is known that Na,K-ATPase and IP₃R are components of a complex made up of several proteins, possessing both scaffolding and signaling functions. It has been demonstrated that both Na,K-ATPase and IP₃R interact with ankyrin B (Lencesova, O'Neill et al. 2004; Mohler, Davis et al. 2005). Ank-B belongs to a scaffolding protein family that associates with a diverse set of membrane, cytoskeletal and cytoplasmic proteins and that tethers them into specialized membrane signaling domains (Mohler, Gramolini et al. 2002). It is therefore possible that Ank-B may play a role as a scaffolding protein in Na,K-ATPase and IP₃R binding. Here we show that Ank-B is an active partner of this signaling microdomain. We demonstrate that Ank-B directly binds to N-terminal tail of Na,K-ATPase α subunit and N-terminus of IP₃R. These two N-termini, which are the exact domains involved in binding between Na,K-ATPase and IP₃R, represent novel binding sites for Ank-B. The binding of Ank-B to the N-termini of Na,K-ATPase α_1 and IP₃R plays an important role in bringing Na,K-ATPase α_1 and IP₃R together. In Ank-B silenced cells, the interaction between Na,K-ATPase α_1 and IP₃R, is reduced as indicated by the co-IP studies. These data indicate that Ank-B serves to tether Na,K-ATPase and IP₃R.

In a series of studies, Xie ZJ and colleagues demonstrated that Na,K-ATPase associates with Src (Haas, Askari et al. 2000). Our findings indicate that Na,K-ATPase binds to IP₃R. These data suggest that Na,K-ATPase can associate with different proteins to form a signaling microdomain. The remaining question is the role of Ank-B for the association between Na,K-ATPase and Src. Recently Xie's group presented evidence suggesting the existence of two pools of Na,K-ATPase in the plasma membrane, a pumping and a nonpumping pool (Liang, Tian et al.

2007). One could therefore speculate that it is mainly the non-pumping pool of Na,K-ATPase that associates with Ank-B and that it is the caveolae structures, rather than Ank-B, that stabilize and anchor non-pumping Na,K-ATPase in the plasma membrane.

Physiological concentrations of ouabain activate Na,K-ATPase-mediated signaling

Ouabain is a highly specific ligand of the Na,K-ATPase α -subunit. Several lines of evidence suggest that ouabain-like compounds (ELOC) are mammalian steroid hormones. ELOC are synthesized in the adrenals and hypothalamus (Kawamura, Guo et al. 1999; Schoner 2000). Circulating levels of ELOC in human have been reported to range between 40 pM and 1nM (Ijiri, Hayashi et al. 2003; Sophocleous, Elmatzoglou et al. 2003). These concentrations of ELOC should have a minimal effect on Na,K-ATPase activity and ion transport (Muller-Ehmsen, Juvvadi et al. 2001). In contrast, a number of studies have shown that ouabain can, at such low concentrations, initiate signaling cascades (6, 24) and modulate cell growth (Aydemir-Koksoy, Abramowitz et al. 2001; Chueh, Guh et al. 2001; Abramowitz, Dai et al. 2003) and apoptosis (Orlov, Thorin-Trescases et al. 2004). The circulating levels of ELOC have been reported to be increased in conditions where extensive cell growth and differentiation are required. High circulating levels of ELOC are found in pregnancy and postnatally (Vakkuri, Arnason et al. 2001). High levels have also been reported following nephrectomy, a condition that is associated with compensatory growth of the remaining kidney (Yamada et al., 1994).

In our previous work, we have demonstrated that ouabain, in doses resulting in only partial inhibition of Na,K-ATPase activity, increases the interaction between Na,K-ATPase and IP₃R, induces slow calcium oscillations and activates transcription factor NF- κ B. Inhibition of intracellular calcium stores abolish the effect of ouabain on NF- κ B activation (Aizman, Uhlen et al. 2001; Miyakawa-

Naito, Uhlen et al. 2003). Here we demonstrate that long time exposure to a low dose of ouabain (10 nM for 24 h) activates NF- κ B. We also demonstrate that the activation of NF- κ B by ouabain depends on the direct interaction of Na,K-ATPase and IP₃R. We have shown that the well conserved motif (LKK) is essential for the binding between the Na,K-ATPase α -subunit and the IP₃R. Cells overexpressing a peptide corresponding to α NT-t, which binds to IP₃R and blocks Na,K-ATPase /IP₃R binding, suppress ouabain's effect. Ank-B is also involved in the regulation of ouabain's effect on NF- κ B activity. Ouabain can not activate NF- κ B in Ank-B silenced cells. This suggests that Ank-B is required for the stabilization of the signaling function of the Na,K-ATPase /IP₃R complex.

Xie ZJ and colleagues have demonstrated that binding of ouabain to Na,K-ATPase leads to Src release from a Na,K-ATPase /Src complex (Haas, Wang et al. 2002; Tian, Cai et al. 2006). Most of their work was performed on LLC-PK1 (the pig renal proximal tubule cell line) and MDCK (canine renal distal tubule cell line, Madin-Darby canine kidney) cells. The question remains as to whether ouabain triggers different signaling microdomains in a cell specific manner or whether ouabain can trigger different signaling pathways at the same time with different downstream effects. Notably, a recent study from Xie's group demonstrated a cross-talk between the Ras/MAPK-ERK kinase/mitogen-activated protein kinase cascade and an IP₃R–calcium signaling pathway in ouabain-stimulated cells (Yuan, Cai et al. 2005).

The downstream effect of ouabain/Na,K-ATPase/IP₃R signaling is protection from apoptosis

We found that ouabain can activate the interaction between Na,K-ATPase and IP₃R, and induce regular, low-frequency intracellular calcium oscillations, that elicit activation of the transcription factor NF- κ B (Aizman, Uhlen et al. 2001). Calcium is one of the major intracellular second messengers. Calcium is involved in the regulation of gene transcription, cell adhesion, cell growth, proliferation and

apoptosis (Berridge et al., 1998). NF- κ B is a pleiotropic regulator of many genes that are involved in regulation of cell growth, differentiation, and apoptosis. NF- κ B is known to inhibit apoptosis through induction of antiapoptotic proteins and/or suppression of proapoptotic genes (Yu, Geng et al. 2001; Hinata, Gervin et al. 2003). We demonstrate that exposure to low doses of ouabain can protect renal epithelial cells from serum-starvation triggered apoptosis. To get serum-starvation triggered apoptosis, renal epithelial cells were cultured in low (0.2%) FBS for 24h. This results in a 6-fold increase in apoptosis. Exposure to physiologically nanomolar ouabain concentrations completely prevents this apoptosis.

The anti-apoptotic effect of ouabain depends on ouabain/ Na,K-ATPase/IP₃R signaling. First, we demonstrate that the interaction between ouabain-bound Na,K-ATPase and IP₃R is essential for the antiapoptotic effect of ouabain. The NH₂ terminus tail of the Na,K-ATPase plays a key role for the interaction with IP₃R (Miyakawa-Naito, Uhlen et al. 2003). In cells that express Na,K-ATPase α_1 -subunit with a truncated NH₂ terminus tail, ouabain fails to protect from serum deprivation-triggered apoptosis. Furthermore, ouabain-dependent protection from apoptosis is completely abolished in cells overexpressing α NT-t corresponding to the N-terminal tail of Na,K-ATPase α -subunit.

Our results also indicate that the antiapoptotic effect of ouabain depends on IP₃R-mediated calcium release from intracellular stores and subsequently activation of NF- κ B. Depletion of intracellular ER calcium stores and inhibition of the IP₃R abolish the antiapoptotic effect of ouabain. NF- κ B activation is also required for ouabain protection against serum deprivation-triggered apoptosis. The selective NF- κ B inhibitor helenalin prevents ouabain-mediated NF- κ B activation and completely abolishes the antiapoptotic effect of ouabain.

These data indicate that the anti-apoptotic effect of ouabain is a downstream effect of ouabain/ Na,K-ATPase/IP₃R signaling.

The calcium-dependent antiapoptotic effect of ouabain may seem paradoxical in view of the fact that a number of recent studies have demonstrated that calcium release from the ER *via* the IP₃R results in a mitochondrial overload of calcium and cell death (Patterson, Boehning et al. 2004). This proapoptotic effect is triggered by the binding of cytochrome C to IP₃R, which leads to an uncontrolled release of calcium (Boehning, Patterson et al. 2003). In contrast, ouabain-triggered calcium release occurs as highly constant and repetitive calcium transients, with a periodicity in the minute range. Therefore, the pre- or proapoptotic effect of IP₃R activation depends on the pattern of calcium release. The question of whether ouabain may exert a feedback control on the proapoptotic effects of cytochrome C remains to be determined. It should be noted, though, that ouabain failed to protect from apoptosis induced by 0.5 μM staurosporine, a broad-spectrum inhibitor of protein kinases and potent apoptosis inducer. Staurosporine mediated apoptosis is associated with a massive release of cytochrome C.

Ouabain stimulates cell proliferation

Low physiological concentrations of ouabain are found to stimulate the proliferation of kidney cells from rat and monkey. Our observation that ouabain stimulates cell growth is in line with findings from several other laboratories, that showed that ouabain stimulates growth of smooth muscle cells and renal epithelial cells from both rodent and human species (Aydemir-Koksoy, Abramowitz et al. 2001; Chueh, Guh et al. 2001; Abramowitz, Dai et al. 2003). These effects have been attributed to the activation of several intracellular signaling pathways, among them activation of Ras/MAPK-ERK kinase/mitogen-activated protein kinase cascade (Gong, He et al. 2006). Here we provide evidence that the proliferation effect of ouabain can also be transduced *via* a signaling pathway, which involves calcium release from intracellular stores *via* the IP₃R.

Ouabain rescues embryonic kidney nephrogenesis

Here we report that activation of ouabain/ Na,K-ATPase/IP₃R signaling rescues nephrogenesis in the growth factor deprived embryonic rat kidney. Nephrogenesis is normalized, the apoptotic rate is decreased and the expression of Wt1 and Pax2, factors that are required for the growth and continued patterning of the kidney, are enhanced.

The organotypic culture of rat embryonic kidney rudiments used in this study is a classical model for studies of kidney development. To perturb kidney development, the explant kidneys are deprived of growth factors for 1 day. This results in a 4-fold increase of apoptotic cells. Exposure to physiological doses of ouabain completely protects from the apoptosis triggered by growth factor deprivation. It has been suggested that large-scale apoptosis is a normal feature of kidney development to eliminate unwanted cells during organogenesis (Coles, Burne et al. 1993). Thus it is notable that ouabain does not decrease the apoptotic rate below that seen in control kidneys. Apoptosis is a double-edged sword during kidney development. If the apoptotic rate is increased above normal, it will cause tubular atrophy and hypoplastic kidneys (Hughes and Savill 2005). Fetal malnourishment, which is a common cause of retarded kidney development and which predisposes to hypertension and chronic renal disease, has been reported to be associated with a high rate of apoptosis (Zandi-Nejad, Luyckx et al. 2006).

The protective effects of ouabain can be attributed to activation of a ouabain/Na,K-ATPase/IP₃R signaling pathway. Here we find that ouabain triggers slow calcium oscillations and activates NF-κB in embryonic kidney cells. The effects of ouabain on apoptosis and nephrogenesis are both dependent on the release of calcium from the intracellular stores, underlining the importance of IP₃R activation for these effects. We provide evidence that the effect of ouabain on the growth factor deprived apoptosis in kidney cells is NF-κB dependent. Both the anti-apoptotic effect and the normalization of nephron development are abolished when activation of NF-κB is prevented.

NF- κ B has been reported to modulate the expression of several inductive factors for kidney development, including *Wt1* and *Pax2* (Dehbi, Hiscott et al. 1998; Chen, Liu et al. 2006). Here we find that ouabain increased mRNA expression of both *Wt1* and *Pax2* in the growth factor deprived kidney. *Wt1*, the Wilms' tumor suppressor gene, plays an important role at three different stages of kidney development, including the onset of kidney formation, the progression of kidney formation and the maintenance of normal kidney function (Mrowka and Schedl 2000; Rivera and Haber 2005). The *Wt1* proteins can influence cell proliferation, differentiation and apoptosis (Mrowka and Schedl 2000). *Pax2* is a key player in kidney morphogenesis. In *Pax2*-deficient embryos, the kidney and genital tract never develop (Narlis, Grote et al. 2007). The renal-coloboma syndrome is caused by mutations of *Pax2* gene. In this syndrome, the nephron number is strikingly reduced. It has been reported that the nephron deficit is caused by a loss of the anti-apoptotic effects of *Pax2* (Fletcher, Hu et al. 2005). Thus it is likely that the rescuing effect of ouabain on nephron formation can, at least partially, be attributed to increased expression of *Wt1* and *Pax2*.

The embryonic kidney grows exponentially during the early period of nephrogenesis (Bard 2002). The proliferation rate is particularly high in the blast cells at the rudiment periphery (Coles, Burne et al. 1993). By using BrdU incorporation assays, we show that ouabain stimulates cell proliferation during kidney development and that this stimulatory effect is most pronounced in the blast cells at the rudiment periphery. Our results indicate that ouabain plays a role as a kidney growth factor.

Several lines of evidence suggest that suboptimal intrauterine conditions leading to growth retardation will predispose to hypertension and chronic kidney disease in adult life (Zandi-Nejad, Luyckx et al. 2006; Alexander 2007). Embryonic kidney growth retardation will result in low nephron number, which is considered as a major risk factor for hypertension (Zandi-Nejad, Luyckx et al. 2006). In our model, growth factor deprivation results in enhanced apoptosis and

fewer nephrons. Exposure to nM concentrations of ouabain completely abolishes these effects. It should be of utmost importance to find therapeutic tools that protect the embryonic kidney from growth retardation in conditions such as fetal malnutrition. Future animal studies of the effect of maternal ouabain administration on kidney development, performed on experimentally growth retarded fetuses, should provide proof of this concept, and open up a new avenue for prevention of hypertension and chronic renal disease.

Acknowledgements

The work presented here has been performed at the Pediatric Unit, Department of Woman and Child Health, Arstrid Lindgren Children's Hospital, Karolinska Institutet during the years 2002-2007. This work could not have been accomplished without help, encouragement and support of my colleagues, friends and family. I would like to take this opportunity to express my sincere gratitude to all those people who have directly or indirectly contributed to my study.

My supervisors: Oleg Aizman for leaving me work independently and giving me more chances to challenge myself. Professor Anita Aperia for accepting me as a post graduate student in her lab, for believing in my scientific capability, for sharing her great knowledge in science with me and for always encouraging me and supporting me.

My co-authors and co-workers: Sergey Zelenin for always being so positive and creative. Ilaria Perini for weeks counting cells in darkness with patience and for reading my thesis very carefully and pointing out my small problems. Markus Kruusmägi for helping me to get beautiful images with the confocal microscope and teaching me how to use the confocal microscope and helping me to manage my computer. Eivor Zettergren Markus for sharing your excellent cell culture experience with me. Ann-Christine Eklöf for helping me to get better rats or extra rats and helping me to solve small or big problems like tax, visa, apartment. Raija Wallenborg for expert secretarial help especially during my dissertation stage. Mona Agrén for being the first lady to teach me experimental technique-western blot in this lab and making me feel convenience with myself. Louise Gustafsson for doing good transfection for my cells. Lill-Britt Svensson for teaching me to do dissection with very small kidneys. Josephin Sundborger for helping with animal experiments.

Extra thanks go to Mark Lal, for proof-reading of my thesis and his valuable comments.

I also would like to thank all my colleagues in the lab: Eli Gunnarson, Xiang Hua, Maria Sol Kruse, Lena Scott, Nermin sourial Bassillious, Nina Illarionova, Farah Khan, Marina Zelenin, Zuzana Spicarova, Sandeep Kumar, Ulla Holtbäck, Seth Malmersjö, Per Uhlen, Songbai Zhang, Xiaoli Liu, Yanhong Li, Yutong Song and Susanna Rydholm, Susanne Crambert, Evgenija Burlaka, Pernilla Grillner, Thomas Liebmann, Andreas Ringman, Linda Westin. Thanks for all your help and support during my PhD study.

I want to acknowledge Andrei Chagin at the 8th floor for giving me useful suggestions and help.

I also would like to thank Astrid Häggblad for your kindness help and support during my PhD study.

I want to thank my Chinese colleagues at KI: Mi Hou, Lin Ma, Hong Qi, Anquan Li, Yue Min, Liang Wu, Shaolian Yang and Harvest Gu. It's a great pleasure to meet you and know you in Sweden. Thanks for your help and frendship.

I wish to thank my parents, my father, Ziyuan Li for teaching me all valuable things in the life and giving me support whenever I need. My mother, Guiyu Zhao for your love, support and teaching me as an independent female. My sister Jieping Li and her husband Yan Shi for their help and support. I had a very pleasure time in Guang Zhou during I went back to China for vocation and you took care me as a baby, Thanks a lot. My brother Dan Li for your encoungment.

Above all, I would like to express my special thanks to my son Ruimin Fang for being with me for 5 years and making my life full of interesting and surprising

without lonely. For working together with me at lab during the day or at night and making me working efficiency and creative. My husband Huisheng Fang for encouraging me to do research work but not only just clinical work and for supporting me whenever I need your help and encouraging me to finish my PhD study in Sweden.

This work was supported by grants from the Swedish Research Council, Karolinska Institutet research foundation, Sällskapet Barnavård, the Persson Family Foundation, the Märta and Gunnar V. Philipson Foundation, the Foundation for Strategic Research, the Japan Science and Technology Agency, and the Ministry of Education, Science, Sports, Culture, and Technology of Japan.

References

- Abramowitz, J., C. Dai, et al. (2003). "Ouabain- and marinobufagenin-induced proliferation of human umbilical vein smooth muscle cells and a rat vascular smooth muscle cell line, A7r5." *Circulation* **108**(24): 3048-53.
- Aizman, O., P. Uhlen, et al. (2001). "Ouabain, a steroid hormone that signals with slow calcium oscillations." *Proc Natl Acad Sci U S A* **98**(23): 13420-4.
- Akimova, O. A., A. Y. Bagrov, et al. (2005). "Cardiotonic steroids differentially affect intracellular Na⁺ and [Na⁺]_i/[K⁺]_i-independent signaling in C7-MDCK cells." *J Biol Chem* **280**(1): 832-9.
- Akimova, O. A., O. D. Lopina, et al. (2005). "Search for intermediates of Na⁺,K⁺-ATPase-mediated [Na⁺]_i/[K⁺]_i-independent death signaling triggered by cardiotonic steroids." *Pathophysiology* **12**(2): 125-35.
- Alexander, B. T. (2007). "Divergent origins of slow fetal growth: relevance to adult cardiovascular disease." *Hypertension* **50**(3): 465-6.
- Aydemir-Koksoy, A., J. Abramowitz, et al. (2001). "Ouabain-induced signaling and vascular smooth muscle cell proliferation." *J Biol Chem* **276**(49): 46605-11.
- Bagrov, Y. Y., N. B. Manusova, et al. (2005). "Endogenous digitalis-like ligands and Na/K-ATPase inhibition in experimental diabetes mellitus." *Front Biosci* **10**: 2257-62.
- Bard, J. B. (2002). "Growth and death in the developing mammalian kidney: signals, receptors and conversations." *Bioessays* **24**(1): 72-82.
- Beguin, P., U. Hasler, et al. (1998). "Membrane integration of Na,K-ATPase alpha-subunits and beta-subunit assembly." *J Biol Chem* **273**(38): 24921-31.
- Berridge, K. C. and T. E. Robinson (1998). "What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience?" *Brain Res Brain Res Rev* **28**(3): 309-69.
- Berridge, M. J., M. D. Bootman, et al. (1998). "Calcium--a life and death signal." *Nature* **395**(6703): 645-8.
- Berridge, M. J., P. Lipp, et al. (2000). "The versatility and universality of calcium signalling." *Nat Rev Mol Cell Biol* **1**(1): 11-21.
- Blanco, G. and R. W. Mercer (1998). "Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function." *Am J Physiol* **275**(5 Pt 2): F633-50.
- Blaustein, M. P., J. Zhang, et al. (2006). "How does salt retention raise blood pressure?" *Am J Physiol Regul Integr Comp Physiol* **290**(3): R514-23.
- Boehning, D., R. L. Patterson, et al. (2003). "Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis." *Nat Cell Biol* **5**(12): 1051-61.
- Bootman, M. D., T. J. Collins, et al. (2002). "2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca²⁺ entry but an inconsistent inhibitor of InsP₃-induced Ca²⁺ release." *Faseb J* **16**(10): 1145-50.

- Cantiello, H. F. (1995). "Actin filaments stimulate the Na(+)-K(+)-ATPase." Am J Physiol **269**(5 Pt 2): F637-43.
- Caspers, M. L., R. D. Schwartz, et al. (1987). "Autoradiographic visualization and characterization of [3H]ouabain binding to the Na⁺,K⁺-ATPase of rat brain and pineal." Brain Res **409**(2): 335-42.
- Chen, F., M. Wang, et al. (2003). "Phosphorylation of PPARgamma via active ERK1/2 leads to its physical association with p65 and inhibition of NF-kappabeta." J Cell Biochem **90**(4): 732-44.
- Chen, Y. W., F. Liu, et al. (2006). "Reactive oxygen species and nuclear factor-kappa B pathway mediate high glucose-induced Pax-2 gene expression in mouse embryonic mesenchymal epithelial cells and kidney explants." Kidney Int **70**(9): 1607-15.
- Chueh, S. C., J. H. Guh, et al. (2001). "Dual effects of ouabain on the regulation of proliferation and apoptosis in human prostatic smooth muscle cells." J Urol **166**(1): 347-53.
- Clausen, T., C. Van Hardeveld, et al. (1991). "Significance of cation transport in control of energy metabolism and thermogenesis." Physiol Rev **71**(3): 733-74.
- Coles, H. S., J. F. Burne, et al. (1993). "Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor." Development **118**(3): 777-84.
- Crambert, G., U. Hasler, et al. (2000). "Transport and pharmacological properties of nine different human Na, K-ATPase isozymes." J Biol Chem **275**(3): 1976-86.
- De Wardener, H. E. (1973). "The natriuretic hormone." Proc Eur Dial Transplant Assoc **10**(0): 3-19.
- Dehbi, M., J. Hiscott, et al. (1998). "Activation of the wt1 Wilms' tumor suppressor gene by NF-kappaB." Oncogene **16**(16): 2033-9.
- Delfino, F. and W. H. Walker (1999). "Hormonal regulation of the NF-kappaB signaling pathway." Mol Cell Endocrinol **157**(1-2): 1-9.
- Devarajan, P., D. A. Scaramuzzino, et al. (1994). "Ankyrin binds to two distinct cytoplasmic domains of Na,K-ATPase alpha subunit." Proc Natl Acad Sci U S A **91**(8): 2965-9.
- Di Bartolo, V., S. Balzan, et al. (1995). "Evidence for an endogenous ouabain-like immunoreactive factor in human newborn plasma coeluted with ouabain on HPLC." Life Sci **57**(15): 1417-25.
- Done, S. C., I. B. Leibiger, et al. (2002). "Tyrosine 537 within the Na⁺,K⁺-ATPase alpha-subunit is essential for AP-2 binding and clathrin-dependent endocytosis." J Biol Chem **277**(19): 17108-11.
- Doris, P. A., L. A. Jenkins, et al. (1994). "Is ouabain an authentic endogenous mammalian substance derived from the adrenal?" Hypertension **23**(5): 632-8.
- Dostanic-Larson, I., J. N. Lorenz, et al. (2006). "Physiological role of the alpha1- and alpha2-isoforms of the Na⁺-K⁺-ATPase and biological significance of

- their cardiac glycoside binding site." Am J Physiol Regul Integr Comp Physiol **290**(3): R524-8.
- Dostanic-Larson, I., J. W. Van Huysse, et al. (2005). "The highly conserved cardiac glycoside binding site of Na,K-ATPase plays a role in blood pressure regulation." Proc Natl Acad Sci U S A **102**(44): 15845-50.
- Efendiev, R., Z. Chen, et al. (2005). "The 14-3-3 protein translates the Na⁺,K⁺-ATPase {alpha}1-subunit phosphorylation signal into binding and activation of phosphoinositide 3-kinase during endocytosis." J Biol Chem **280**(16): 16272-7.
- Eid, S. R. and A. W. Brandli (2001). "Xenopus Na,K-ATPase: primary sequence of the beta2 subunit and in situ localization of alpha1, beta1, and gamma expression during pronephric kidney development." Differentiation **68**(2-3): 115-25.
- Ferrandi, M., S. Salardi, et al. (1999). "Evidence for an interaction between adducin and Na(+)-K(+)-ATPase: relation to genetic hypertension." Am J Physiol **277**(4 Pt 2): H1338-49.
- Fishman, M. C. (1979). "Endogenous digitalis-like activity in mammalian brain." Proc Natl Acad Sci U S A **76**(9): 4661-3.
- Fletcher, J., M. Hu, et al. (2005). "Multicystic dysplastic kidney and variable phenotype in a family with a novel deletion mutation of PAX2." J Am Soc Nephrol **16**(9): 2754-61.
- Fotis, H., L. V. Tatjanenko, et al. (1999). "Phosphorylation of the alpha-subunits of the Na⁺/K⁺-ATPase from mammalian kidneys and Xenopus oocytes by cGMP-dependent protein kinase results in stimulation of ATPase activity." Eur J Biochem **260**(3): 904-10.
- Freedman, B. D. (2006). "Mechanisms of calcium signaling and function in lymphocytes." Crit Rev Immunol **26**(2): 97-111.
- Geering, K., B. Delprat, et al. (2006). "FXYP proteins: new regulators of Na-K-ATPase [FXYP proteins: novel regulators of Na,K-ATPase]." Am J Physiol Renal Physiol **290**(2): F241-50.
- Goldstein, I., T. Levy, et al. (2006). "Involvement of Na(+), K(+)-ATPase and endogenous digitalis-like compounds in depressive disorders." Biol Psychiatry **60**(5): 491-9.
- Gong, X., X. He, et al. (2006). "Stromal cell derived factor-1 acutely promotes neural progenitor cell proliferation in vitro by a mechanism involving the ERK1/2 and PI-3K signal pathways." Cell Biol Int **30**(5): 466-71.
- Haas, M., A. Askari, et al. (2000). "Involvement of Src and epidermal growth factor receptor in the signal-transducing function of Na⁺/K⁺-ATPase." J Biol Chem **275**(36): 27832-7.
- Haas, M., H. Wang, et al. (2002). "Src-mediated inter-receptor cross-talk between the Na⁺/K⁺-ATPase and the epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases." J Biol Chem **277**(21): 18694-702.

- Hamlyn, J. M., M. P. Blaustein, et al. (1991). "Identification and characterization of a ouabain-like compound from human plasma." Proc Natl Acad Sci U S A **88**(14): 6259-63.
- Hamlyn, J. M. and P. Manunta (1992). "Ouabain, digitalis-like factors and hypertension." J Hypertens Suppl **10**(7): S99-111.
- Hauptert, G. T., Jr. and J. M. Sancho (1979). "Sodium transport inhibitor from bovine hypothalamus." Proc Natl Acad Sci U S A **76**(9): 4658-60.
- Hinata, K., A. M. Gervin, et al. (2003). "Divergent gene regulation and growth effects by NF-kappa B in epithelial and mesenchymal cells of human skin." Oncogene **22**(13): 1955-64.
- Hughes, J. and J. S. Savill (2005). "Apoptosis in glomerulonephritis." Curr Opin Nephrol Hypertens **14**(4): 389-95.
- Ijiri, Y., T. Hayashi, et al. (2003). "Digitalis-like immunoreactive substances in maternal and umbilical cord plasma: a comparative sensitivity study of fluorescence polarization immunoassay and microparticle enzyme immunoassay." Ther Drug Monit **25**(2): 234-9.
- Kaplan, J. H. (2002). "Biochemistry of Na,K-ATPase." Annu Rev Biochem **71**: 511-35.
- Kawamura, A., J. Guo, et al. (1999). "On the structure of endogenous ouabain." Proc Natl Acad Sci U S A **96**(12): 6654-9.
- Kidder, G. M. and A. J. Watson (2005). "Roles of Na,K-ATPase in early development and trophectoderm differentiation." Semin Nephrol **25**(5): 352-5.
- Kim, M., J. Jung, et al. (2002). "Identification of the cofilin-binding sites in the large cytoplasmic domain of Na,K-ATPase." Biochimie **84**(10): 1021-9.
- Kraemer, D., R. Koob, et al. (1990). "Two novel peripheral membrane proteins, pasin 1 and pasin 2, associated with Na⁺,K⁺-ATPase in various cells and tissues." J Cell Biol **111**(6 Pt 1): 2375-83.
- Lencesova, L., A. O'Neill, et al. (2004). "Plasma membrane-cytoskeleton-endoplasmic reticulum complexes in neurons and astrocytes." J Biol Chem **279**(4): 2885-93.
- Liang, M., T. Cai, et al. (2006). "Functional characterization of Src-interacting Na/K-ATPase using RNA interference assay." J Biol Chem **281**(28): 19709-19.
- Liang, M., J. Tian, et al. (2007). "Identification of a pool of non-pumping Na/K-ATPase." J Biol Chem **282**(14): 10585-93.
- Lichtstein, D. (1995). "Na⁺, K⁺-ATPase and heart excitability." Adv Exp Med Biol **382**: 23-30.
- Lichtstein, D. and S. Samuelov (1980). "Endogenous 'ouabain like' activity in rat brain." Biochem Biophys Res Commun **96**(4): 1518-23.
- Lingrel, J., A. Moseley, et al. (2003). "Functional roles of the alpha isoforms of the Na,K-ATPase." Ann N Y Acad Sci **986**: 354-9.
- Lingrel, J. B. and T. Kuntzweiler (1994). "Na⁺,K⁺-ATPase." J Biol Chem **269**(31): 19659-62.

- Liu, L., J. Li, et al. (2006). "Involvement of Na⁺/K⁺-ATPase in hydrogen peroxide-induced hypertrophy in cardiac myocytes." Free Radic Biol Med **41**(10): 1548-56.
- Masugi, F., T. Ogihara, et al. (1986). "Circulating factor with ouabain-like immunoreactivity in patients with primary aldosteronism." Biochem Biophys Res Commun **135**(1): 41-5.
- Miyakawa-Naito, A., P. Uhlen, et al. (2003). "Cell signaling microdomain with Na,K-ATPase and inositol 1,4,5-trisphosphate receptor generates calcium oscillations." J Biol Chem **278**(50): 50355-61.
- Mobasher, A., J. Avila, et al. (2000). "Na⁺, K⁺-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions." Biosci Rep **20**(2): 51-91.
- Mohler, P. J., J. Q. Davis, et al. (2005). "Ankyrin-B coordinates the Na/K ATPase, Na/Ca exchanger, and InsP3 receptor in a cardiac T-tubule/SR microdomain." PLoS Biol **3**(12): e423.
- Mohler, P. J., A. O. Gramolini, et al. (2002). "Ankyrins." J Cell Sci **115**(Pt 8): 1565-6.
- Mrowka, C. and A. Schedl (2000). "Wilms' tumor suppressor gene WT1: from structure to renal pathophysiologic features." J Am Soc Nephrol **11 Suppl 16**: S106-15.
- Muller-Ehmsen, J., P. Juvvadi, et al. (2001). "Ouabain and substrate affinities of human Na⁽⁺⁾-K⁽⁺⁾-ATPase alpha(1)beta(1), alpha(2)beta(1), and alpha(3)beta(1) when expressed separately in yeast cells." Am J Physiol Cell Physiol **281**(4): C1355-64.
- Narlis, M., D. Grote, et al. (2007). "Pax2 and pax8 regulate branching morphogenesis and nephron differentiation in the developing kidney." J Am Soc Nephrol **18**(4): 1121-9.
- Naruse, T., T. Ishida, et al. (1994). "Relationship between hypotensive effects and plasma concentrations of clonidine in spontaneously hypertensive rats: continuous treatment and sudden termination of clonidine infusion." Gen Pharmacol **25**(7): 1421-5.
- Nebel, C., B. Romestand, et al. (2005). "Differential freshwater adaptation in juvenile sea-bass *Dicentrarchus labrax*: involvement of gills and urinary system." J Exp Biol **208**(Pt 20): 3859-71.
- Nelson, W. J. and P. J. Veshnock (1987). "Ankyrin binding to (Na⁺ + K⁺)ATPase and implications for the organization of membrane domains in polarized cells." Nature **328**(6130): 533-6.
- Nguyen, A. N., D. P. Wallace, et al. (2007). "Ouabain binds with high affinity to the Na,K-ATPase in human polycystic kidney cells and induces extracellular signal-regulated kinase activation and cell proliferation." J Am Soc Nephrol **18**(1): 46-57.
- Ohta, T., S. Noguchi, et al. (1991). "The 'lysine cluster' in the N-terminal region of Na⁺/K⁽⁺⁾-ATPase alpha-subunit is not involved in ATPase activity." Biochim Biophys Acta **1059**(2): 157-64.

- Orlov, S. N., N. Thorin-Trescases, et al. (2004). "Na⁺/K⁺ pump and endothelial cell survival: [Na⁺]_i/[K⁺]_i-independent necrosis triggered by ouabain, and protection against apoptosis mediated by elevation of [Na⁺]_i." Pflugers Arch **448**(3): 335-45.
- Overbeck, H. W., M. B. Pamnani, et al. (1976). "Depressed function of a ouabain-sensitive sodium-potassium pump in blood vessels from renal hypertensive dogs." Circ Res **38**(6 Suppl 2): 48-52.
- Patterson, R. L., D. Boehning, et al. (2004). "Inositol 1,4,5-trisphosphate receptors as signal integrators." Annu Rev Biochem **73**: 437-65.
- Ringer, S. (1885). "An Experimental Investigation to ascertain the Action of Veratria on a Cardiac Contraction." J Physiol **6**(4-5): 150-292 2.
- Rivera, M. N. and D. A. Haber (2005). "Wilms' tumour: connecting tumorigenesis and organ development in the kidney." Nat Rev Cancer **5**(9): 699-712.
- Robinson, W. E., Jr., T. Kawamura, et al. (1990). "Human monoclonal antibodies to the human immunodeficiency virus type 1 (HIV-1) transmembrane glycoprotein gp41 enhance HIV-1 infection in vitro." Proc Natl Acad Sci U S A **87**(8): 3185-9.
- Saneyoshi, T., S. Kume, et al. (2002). "The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in *Xenopus* embryos." Nature **417**(6886): 295-9.
- Sariola, H. and L. Philipson (1999). "Bridge over troubled waters." Nat Med **5**(1): 22-3.
- Schoner, W. (2000). "Ouabain, a new steroid hormone of adrenal gland and hypothalamus." Exp Clin Endocrinol Diabetes **108**(7): 449-54.
- Senftleben, U., Y. Cao, et al. (2001). "Activation by IKK α of a second, evolutionary conserved, NF-kappa B signaling pathway." Science **293**(5534): 1495-9.
- Shamraj, O. I., D. Melvin, et al. (1991). "Expression of Na,K-ATPase isoforms in human heart." Biochem Biophys Res Commun **179**(3): 1434-40.
- Skou, J. C. (1998). "Nobel Lecture. The identification of the sodium pump." Biosci Rep **18**(4): 155-69.
- Skou, J. C. and M. Esmann (1992). "The Na,K-ATPase." J Bioenerg Biomembr **24**(3): 249-61.
- Sophocleous, A., I. Elmatzoglou, et al. (2003). "Circulating endogenous digitalis-like factor(s) (EDLF) in man is derived from the adrenals and its secretion is ACTH-dependent." J Endocrinol Invest **26**(7): 668-74.
- Terness, P., D. Navolan, et al. (2001). "The T-cell suppressive effect of bufadienolides: structural requirements for their immunoregulatory activity." Int Immunopharmacol **1**(1): 119-34.
- Therien, A. G. and R. Blostein (2000). "Mechanisms of sodium pump regulation." Am J Physiol Cell Physiol **279**(3): C541-66.
- Tian, J., T. Cai, et al. (2006). "Binding of Src to Na⁺/K⁺-ATPase forms a functional signaling complex." Mol Biol Cell **17**(1): 317-26.

- Vakkuri, O., S. S. Arnason, et al. (2001). "Radioiodinated tyrosyl-ouabain and measurement of a circulating ouabain-like compound." *Clin Chem* **47**(1): 95-101.
- Wang, H., M. Haas, et al. (2004). "Ouabain assembles signaling cascades through the caveolar Na⁺/K⁺-ATPase." *J Biol Chem* **279**(17): 17250-9.
- Vasudevan, K. M., S. Gurumurthy, et al. (2004). "Suppression of PTEN expression by NF-kappa B prevents apoptosis." *Mol Cell Biol* **24**(3): 1007-21.
- Watabe, M., S. Nakajo, et al. (1997). "Treatment of U937 cells with bufalin induces the translocation of casein kinase 2 and modulates the activity of topoisomerase II prior to the induction of apoptosis." *Cell Growth Differ* **8**(8): 871-9.
- Weidemann, H. (2005). "Na/K-ATPase, endogenous digitalis like compounds and cancer development -- a hypothesis." *Front Biosci* **10**: 2165-76.
- Yamada, K., A. Goto, et al. (1994). "Role of ouabainlike compound in rats with reduced renal mass-saline hypertension." *Am J Physiol* **266**(4 Pt 2): H1357-62.
- Yu, Q., Y. Geng, et al. (2001). "Specific protection against breast cancers by cyclin D1 ablation." *Nature* **411**(6841): 1017-21.
- Yuan, Z., T. Cai, et al. (2005). "Na/K-ATPase tethers phospholipase C and IP3 receptor into a calcium-regulatory complex." *Mol Biol Cell* **16**(9): 4034-45.
- Yudowski, G. A., R. Efendiev, et al. (2000). "Phosphoinositide-3 kinase binds to a proline-rich motif in the Na⁺, K⁺-ATPase alpha subunit and regulates its trafficking." *Proc Natl Acad Sci U S A* **97**(12): 6556-61.
- Zandi-Nejad, K., V. A. Luyckx, et al. (2006). "Adult hypertension and kidney disease: the role of fetal programming." *Hypertension* **47**(3): 502-8.