FROM THE DEPARTMENT OF MOLECULAR MEDICINE AND SURGERY
SECTION OF INTEGRATIVE PHYSIOLOGY
KAROLINSKA INSTITUTET
STOCKHOLM, SWEDEN

SIGNALING TO AND FROM THE SODIUM PUMP: EFFECTS OF INSULIN AND CARDIOTONIC STEROIDS

OLGA KOTOVA

Karolinska Institutet

To my family

ABSTRACT

Insulin and proinsulin-connecting peptide (C-peptide) stimulate Na^+,K^+-ATPase activity. The molecular mechanism by which insulin and C-peptide regulate the Na-pump in differentiated primary human skeletal muscle cells (HSMCs) and primary human renal tubular cells (HRTCs) was determined. Insulin- and C-peptide stimulated Na^+,K^+-ATPase activation, as assessed by ouabain-sensitive $^{86}Rb^+$ uptake and ^3H-ouabain binding, phosphorylation of α- and translocation of α- and β-subunits to plasma membrane from intracellular stores. These effects were abolished by the MEK1/2 inhibitor (PD98059). Furthermore, inhibitors of PI3 kinase (wortmannin) and PKC (GF109203X) had similar effects. Notably, insulin-stimulated ERK1/2 phosphorylation was abolished by wortmannin and GF109203X. Insulin led to an increase in phosphorylation of the $α_1$- and $α_2$-subunits. C-peptide caused phosphorylation of the human $α_1$-subunit on a Thr-Pro amino acid motif that confers a specific ERK phosphorylation site. ERK1 and 2 kinases were able to phosphorylate the α-subunit of purified human Na-pump in $vitro$. Thus, insulin and C-peptide activate Na^+,K^+-ATPase via a MAP kinase signaling pathway.

Exercise and in $vitro$ skeletal muscle contraction leads to a significant increase in the plasma membrane abundance of the Na-pump $α_1$- and $α_2$-subunits via an ERK1/2 MAPK-dependent mechanism.

Cardiotonic steroids (CTS) initiate signaling cascade through the Na^+,K^+-ATPase, and increase growth and proliferation of different cell types. The effects of cardiotonic steroids, ouabain and marinobufagenin, on glucose metabolism in HSMC and rat skeletal muscle were explored in an effort to clarify the mechanisms of CTS signal transduction. Ouabain at a low dose increased glycogen synthesis additively to the effect of insulin. This effect was independent of PI3-kinase inhibitor LY294002, and abolished in the presence of either the MEK1/2 inhibitor PD98059 or the Src inhibitor PP2. Insulin-stimulated Akt phosphorylation was unaffected by ouabain. Ouabain increased Src-dependent tyrosine phosphorylation of the $α_1$- and $α_2$-subunits and promoted a direct interaction of α-subunits with Src and formation of signaling complex. Phosphorylation of ERK1/2, GSK3α/β and p90rsk activity, were increased in response to ouabain independently of insulin action. These effects were prevented in the presence of PD98059 and PP2. Incubation of cells with ouabain increased phosphorylation of the α-subunits at a Thr-Pro motif. Ouabain treatment decreased cell surface abundance of the $α_2$-subunit, whereas the abundance of the $α_1$-subunit was unchanged. Marinobufagenin (10 nM), an endogenous vertebrate bufadienolide cardiotonic steroid, increased glycogen synthesis in HSMC and this effect was similarly to that observed in cells exposed to 100 nM ouabain. Incubation of skeletal muscle with ouabain did not induce metabolic stress.

In conclusion, activation of a MAPK signaling cascade stimulates Na^+,K^+-ATPase activity via phosphorylation of the α-subunits and translocation to plasma membrane. Cardiotonic steroids ouabain and marinobufagenin stimulate glycogen synthesis additively to insulin in skeletal muscle. The effect of cardiotonic steroids is mediated by activation of Src-, ERK1/2-, p90rsk-, and GSK3-dependent signaling pathway.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals I-IV.

I.   Lubna Al-Khalili*, **Olga Kotova***, Hiroki Tsuchida, Ingrid Ehrén, Eric Féraille, Anna Krook, and Alexander V. Chibalin. ERK 1/2 mediates insulin stimulation of Na,K-ATPase by phosphorylation of the α-subunit in human skeletal muscle cells. *J Biol Chem* 279: 25211-25218, 2004.
     * These authors contributed equally to this study.

II. Zhihui Zhong, **Olga Kotova**, Alexandra Davidescu, Ingrid Ehrén, Karin Ekberg, John Wahren, and Alexander V. Chibalin. C-peptide stimulates Na^+,K^+-ATPase via activation of ERK1/2 MAP kinases in human renal tubular cells. *Cell Mol Life Sci* 61: 2782-2790, 2004.

III. **Olga Kotova**, Dana Galuska, Birgitta Essen-Gustavsson, and Alexander V. Chibalin. Metabolic and signaling events mediated by cardiotonic steroid ouabain in rat skeletal muscle. *Cell Mol Biol* In press.

IV. **Olga Kotova**, Lubna Al-Khalili, Sara Talia, Catherine Hooke, Olga V. Fedorova, Alexey Y. Bagrov, and Alexander V. Chibalin. Cardiotonic steroids stimulate glycogen synthesis in human skeletal muscle cells via a Src- and ERK1/2-dependent mechanism. *J Biol Chem* 281: 20085-20094, 2006.

CONTENTS

LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AMPK	AMP-activated protein kinase
BLM	Basolateral membrane
C-peptide	Proinsulin Connecting peptide
CAMK	Ca^{2+}/calmodulin-dependent protein kinase
CS	Chow-sedentary
CT	Chow-trained
CTS	Cardiotonic steroids
ET	Exercise training
ERK	Extracellular signal-regulated protein kinase
FS	Fat-sedentary
FT	Fat-trained
GLUT	Glucose transporter
GSK	Glycogen synthase kinase
HSMC	Human skeletal muscle cells
HRTC	Human renal tubular cells
IgM	Immunoglobulin M
IM	Intracellular membrane
IMP	Inosine monophosphate
i.p.	Intraperitoneal
JNK	c-Jun NH_2-terminal kinase
K^+	Potassium
KHB	Krebs-Henseleit buffer
MAPK	Mitogen-activated protein kinase
MBG	Marinobufagenin
MEK	MAPK/ERK kinase
Na^+	Sodium
Na^+,K^+-ATPase	Na^+,K^+-adenosinetriphoshatase
OK	Opossum kidney cells
p90 rsk	p90 ribosomal S6 kinase
PI3-kinase	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PMA	Phorbol meristate acetate
PTC	Proximal tubular cells
PTH	Parathyroid hormone
SGK	Serum and glucocorticoid- dependent protein kinase
SH	Src-homology domain

1 INTRODUCTION

1.1 Structure and function of Na$^+$,K$^+$-ATPase

Na$^+$,K$^+$-ATPase is an integral membrane protein, ubiquitously expressed in all cells. The Na-pump is critically involved in the maintenance of intracellular and extracellular sodium and potassium ions concentrations, cell volume and electrochemical gradients. It catalyzes the transfer of three intracellular Na$^+$ ions out of the cell, in exchange for two extracellular K$^+$ ions inside the cell per molecule of ATP hydrolyzed. The Na$^+$,K$^+$-ATPase is the only mechanism for Na$^+$ extrusion in mammalian cells, which emphasizes the importance of Na$^+$,K$^+$-ATPase as a ion transporter. The activity of the pump also provides the driving force for secondary active transport of solutes such as amino acids, phosphate, vitamins and, in epithelial cells, glucose. Na$^+$,K$^+$-ATPase promotes membrane repolarization and reuptake of extracellular potassium in excitable cells, including skeletal muscle cells (Clausen, 2003; Lingrel et al., 1994; Skou, 1998).

Na$^+$,K$^+$-ATPase belongs to P-type ATPases, a family of enzymes that become phosphorylated during transport by γ-phosphate group of ATP at an aspartic acid localized within the highly conserved sequence DKTGS/T (Lutsenko et al., 1995). This family, which contains more than 50 members, includes membrane-bound enzymes responsible for transport of heavy metal ions (P$_1$-type ATPases), other metal ions (P$_2$-type ATPases), and the K$^+$-selective Kdp-ATPase of *Escherichia coli* (P$_3$-type ATPase). Within the group of P$_2$-type ATPases, Na$^+$,K$^+$-ATPase, together with colonic or gastric H$^+$,K$^+$-ATPases, constitute a subgroup of oligomeric enzymes consisting of α and β subunits. Function of Na$^+$,K$^+$-ATPase requires the presence of both subunits. The third peptide referred to as γ-subunit appears to be involved in regulating activity of Na-pump and its interactions with Na$^+$ or K$^+$ ions (Scheiner-Bobis, 2002).
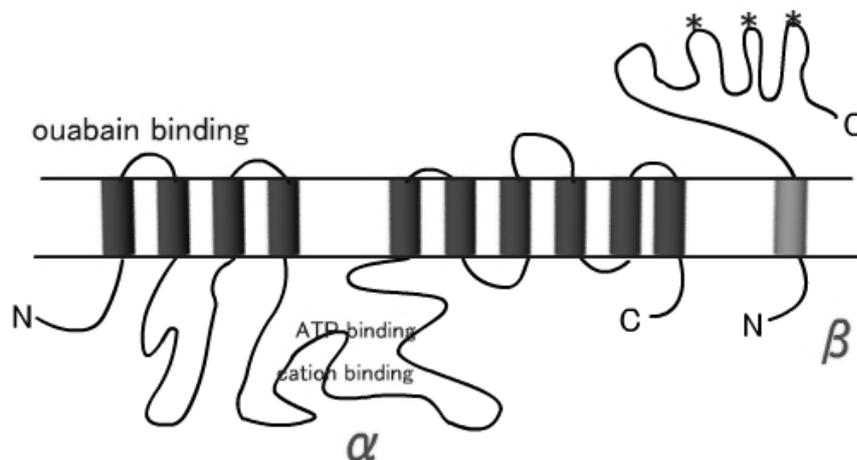
Figure 1. Na$^+$,K$^+$-ATPase α- and β-subunits structure

The catalytic α-subunit is a 112 kDa protein. There are 4 known isoforms of the α-subunit. These multiple isoforms are developmentally regulated in a tissue-specific manner. The α_1-subunit is ubiquitously expressed in mammalian tissues, and it is considered to mainly play a house-keeping role; whereas the α_2-, α_3-, α_4- isoforms are expressed in specific tissues and various membrane subdomains (Hundal et al., 1994). The α-subunit contains binding sites for Na$^+$ and K$^+$ ions, Mg^{2+}- ATP, cardiac

glycosides and vanadate and is responsible for catalytic activity of the enzyme. It contains 10 transmembrane domains, and has a NH_2- and C- terminus on intracellular side (Fig. 1). Various studies have provided evidence that ATP binding and ion occlusion occurs in the α-subunit (Scheiner-Bobis, 2002).

The catalytic α-subunit undergoes cyclic conformational changes, while transporting sodium and potassium ions, using the energy of ATP hydrolysis (Skou, 1988) (Fig. 2).
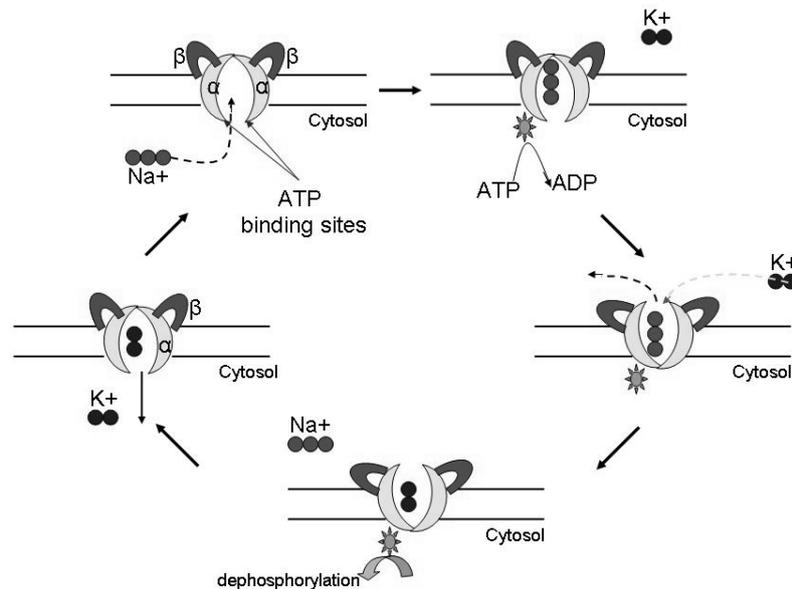
Figure 2. Scheme of Na^+-K^+ exchange by Na^+,K^+-ATPase (Albers-Post model)

The β-subunit is a highly glycosylated protein with a relative molecular mass of approximately 60 kDa. The mass of the protein moiety of the β-subunit is 36-38 kDa, depending on the isoforms: β1, β2, or β3. The β-subunit spans the membrane only once, and its N-terminus is localized on the intracellular side of the membrane. The β-subunit is essential for the ion pump properties of the Na^+,K^+-ATPase. The association between the β-subunit and the α-subunit is required for efficient assembly of functional pumps and delivery of α-subunits to plasma membrane (Fambrough et al., 1994; Sweadner, 1989). A 26-amino acid sequence in the extracellular loop of Na-pump α-subunit, located between the membrane-spanning segments 7 and 8 has been shown to bind the β-subunit and promote α-β- assembly (Lemas et al., 1994). Site-directed mutagenesis of the α-subunits revealed Val^{904}, Tyr^{898} and Cys^{908} in Na^+,K^+-ATPase α-subunit are key residues in α-β-subunit interactions (Wang S-G et al., 1998).

Na^+,K^+-ATPase has a third, non-obligatory γ-subunit (Wetzel et al., 2001). The γ-subunit alters kinetic properties of the enzyme. Two variants of the γ- subunit of the rat renal Na-pump, γ (a) and γ (b), have similar effects on Na^+,K^+-ATPase. Both increase the affinity for ATP due to a shift in the conformational equilibrium of the enzyme E(1) ↔ E(2) toward E(1).

The γ-subunit of Na-pump is a member of the family of FXYD proteins. FXYD proteins are small-membrane proteins. Recent experimental evidence suggests that at least five of the seven members of this family, FXYD1 (phospholemman), FXYD2 (γ-subunit of Na^+,K^+-ATPase), FXYD3 (Mat-8), FXYD4 (CHIF, corticosteroid hormone-induced factor), and FXYD7, are auxiliary subunits of Na^+,K^+-ATPase. They regulate Na-pump activity in tissue- and isoform-specific manner (Geering, 2006). Moreover, a mutation in

FXYD2 has been linked to cases of human hypomagnesemia, indicating that perturbations in regulation of Na-pump by FXYD proteins may be critically involved in pathophysiological states.

FXYD1 or phospholemman is mainly expressed in heart and skeletal muscle. It increases the apparent affinity of Na^+,K^+-ATPase for intracellular Na^+ and may be important for muscle contractility. FXYD2, the gamma subunit and FXYD4, or CHIF modulate the apparent affinity for Na^+ in an opposite manner and adapt to the physiological necessity of Na^+ reabsorption in different segments of the renal tubule. FXYD3 is expressed in the stomach, colon, and numerous tumors. It modulates transport properties of Na^+,K^+-ATPase, but has lower specificity of association than other FXYD proteins and an unusual membrane topology. Finally, FXYD7 is exclusively expressed in the brain and decreases affinity for extracellular K^+, which may be essential for proper neuronal excitability (Geering, 2005).

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

Since Na^+,K^+-ATPase plays a key role in maintenance of Na^+ and K^+ homeostasis, enzymatic activity and protein expression are tightly regulated in different tissues The Na-pump activity can be regulated by short-term and long-term mechanisms. Various factors can affect Na^+,K^+-ATPase activity including hormones (for example, insulin, thyroid hormone, aldosterone, catecholamines (dopamine), angiotenzine II) (Ewart et al., 1995; Feraille et al., 2001; Pedemonte et al., 2005), endogenous cardiotonic steroids (Schoner, 2002a), exercise (Clausen, 1986), skeletal muscle *in vitro* contraction (Clausen, 1996; Clausen, 1998; Nielsen et al., 1997), starvation (Matsumura et al., 1992; Schmidt et al., 1994), or high-fat diet. Alterations in Na^+,K^+-ATPase activity accompany various chronic diseases including diabetes mellitus and its complications (Hundal et al., 1993; Kjeldsen et al., 1987; Mimura et al., 1994; Wahren, 2004), kidney dysfunction (Wilson et al., 1991), cardiovascular (Schwinger et al., 2003), neurological disorders (Stevens, 1995; Wessman et al., 2004) and hypertension (Iwamoto et al., 2006).

Short-term regulation includes a) changes in affinity of the Na-pump to substrates, b) covalent modification of the catalytic α-subunit, mainly phosphorylation, and c) changes in the number of Na-pump molecules on the cell surface due to the processes of endo- and exocytosis. Long-term regulation includes changes in Na^+,K^+-ATPase gene expression and protein synthesis in response to thyroid (Azuma et al., 1993) and steroid hormones (Therien et al., 2000), exercise, starvation and hypokalemia (Schmidt et al., 1994; Thompson et al., 1996).

1.2.1. Short-term regulation

Changes in the intrinsic activity of Na^+,K^+-ATPase can be caused by changes in the pump affinity to Na^+ and K^+ ions, as well as Mg^{2+}- ATP, which can be due to Na-pump phosphorylation (Feraille et al., 2000) or changes in pH (Salonikidis et al., 2000), as well as changes in intracellular or extracellular ion concentrations, for example, during the development of membrane potential in neurons and skeletal muscle cells or during reabsorption of Na^+ into plasma in kidney cells.

Phosphorylation of Na-pump, occurs on various serine, threonine and tyrosine residues in the catalytic α-subunit. Hormonal regulation of the Na^+,K^+-ATPase has been shown to be mediated by phosphorylation of the Na-pump α-subunits by a number of protein kinases including PKC, PKA, PKG, ERK1/2, Src, CaMK, SGK.

1.2.2. Phosphorylation of Na$^+$,K$^+$-ATPase by various protein kinases

Phosphorylation of the α-subunit due to activation of PKC isoforms can lead to a decrease or increase in Na-pump activity (Fisone et al., 1995; Pedemonte et al., 1997; Pedemonte et al., 2001). In rat skeletal muscle, high-glucose induced phosphorylation of the α-subunit occurs via a PKC-dependent mechanism (Chibalin et al., 2001). In pancreatic islets and β-cells glucose inhibits Na$^+$,K$^+$-ATPase activity via Ca^{2+}-independent phospholipase A2 and PKC-dependent phosphorylation of the α-subunit (Owada et al., 1999). C-peptide stimulates Na$^+$,K$^+$-ATPase activity via PKCα -dependent phosphorylation of the Na-pump α-subunit in rat medullary thick ascending limb (Tsimaratos et al., 2003). In type 1 diabetic animal models, PKC activity is increased in glomeruli, aorta, heart, skeletal muscle and retina (Kowluru et al., 1998). Inhibition of PKCβ prevents the hyperglycemia-induced decrease in Na$^+$,K$^+$-ATPase activity in retina (Kowluru et al., 1998).

Attempts to identify PKC phosphorylation sites on the α-subunits has revealed several important amino acid residues. Due to the fact that the α-subunit has a signaling pentapeptide at N-terminal, which can be truncated during maturation of the isoform, different laboratories have used two different nomenclatures for the identification of the phosphorylation sites. In the present thesis both nomenclatures have been used.

The main phosphorylation sites of the PKC isoforms include Ser^{18} (Ser^{23}) and Ser^{11} (Ser^{16}). Simultaneous phosphorylation of Ser^{11} and Ser^{18} has been shown to promote recruitment of overexpressed rat kidney Na-pump to plasma membrane in cultured opossum proximal tubule cells (Efendiev et al., 2000). Phosphorylation of Ser^{16} on the α-subunit can contribute to an increase in Na-pump affinity to Na$^+$ ions (Feraille et al., 2000). In *Bufo marinus*, Thr^{15} and Ser^{16} of the α$_1$-isoform are phosphorylated by PKC (Beguin et al., 1994).

cAMP-dependent protein kinase (PKA), phosphorylates the α-subunit on Ser^{938} (Ser^{943}), a residue at the carboxy terminus of the enzyme that is highly conserved between different isoforms and species (Feschenko et al., 1994; Fisone et al., 1994; Mardh, 1979; Mardh, 1983). Phosphorylation at this site was confirmed by sequence analysis of tryptic peptides and site-directed mutagenesis of the wild-type enzyme with Ser^{938} mutated to Ala with subsequent expression in COS cells (Fisone et al., 1994). In the living cells, the site may have limited accessibility to PKA (Sweadner et al., 2001). In vitro phosphorylation of the Na-pump α-subunit by PKA leads to the stimulation of Na$^+$-K$^+$-ATPase activity, however, there is evidence suggesting that phosphorylation of Ser^{943} by PKA modulates the inhibitory phosphorylation of Na$^+$-K$^+$-ATPase by PKC (Cheng et al., 1997). The γ-subunit of Na$^+$-K$^+$-ATPase can be phosphorylated by protein kinase A (Cortes et al., 2006). PKA phosphorylation of the γ-subunit increases the capacity of the Na$^+$-K$^+$-ATPase to hydrolyse ATP. These results suggest that the γ-subunit can act as an intrinsic Na$^+$-K$^+$-ATPase regulator in the kidney. cAMP has also been shown to stimulate the transport of Na$^+$ and Na$^+$,K$^+$-ATPase activity in renal cortical collecting duct cells through increase in translocation of active Na-pump units from intracellular storage site to the plasma membrane (Gonin et al., 2001).

The phosphorylation of Na$^+$-K$^+$-ATPase by a cGMP-dependent protein kinase (PKG) was determined on purified enzymes from dog, sheep, pig, rat kidney and Xenopus oocytes (Fotis et al., 1999). Using specific antibodies raised against the N-terminus and C-terminus of the pump, it was shown that phosphorylation sites are located in the α-subunit intracellular loop between the 35 kDa N-terminal and the 27 kDa C-terminal fragments.

These phosphorylation sites do not appear to be easily accessible to PKG. Phosphorylation of the Na-pump by PKG leads to the stimulation of its activity.

Hormonal action on Na^+-K^+-ATPase have been shown to be modulated in kidney proximal tubule cells, fibroblasts, vascular smooth muscle cells via ERK1/2 MAP-kinase (Isenovic et al., 2004; Khundmiri et al., 2004; Kitamura et al., 2001). In alveolar epithelial cells fibroblast growth factor 10 stimulates Na^+,K^+-ATPase activity due to increased cell surface abundance and protein synthesis of Na-pump subunits via the Grb 2-SOS/Ras/MAPK pathway (Upadhyay et al., 2003). In vascular smooth cells angiotensine II up-regulates Na^+,K^+-ATPase activity and increases the α_1-subunit gene transcription via a PI3-kinase/MAPK signaling pathway (Isenovic et al., 2004). In contrast, parathyroid hormone inhibits the Na-pump activity in opossum kidney cells transfected with rat α_1-subunit, through phosphorylation of Ser^{11} via a PKC- and ERK 1/2-dependent mechanism (Khundmiri et al., 2004).

In *in vitro* phosphorylation experiments in skeletal muscle, the α-subunit of Na^+,K^+-ATPase has been reported to act as a substrate for the tyrosine-specific protein kinase c-Src (Al-Khalili et al., 2003a). Moreover, ouabain infusion in rats induces cardiac and renal hypertrophy and hypertension (Ferrandi et al., 2004). These *in vivo* effects of ouabain were associated with an enriched presence of the α_1- β_1- and γ-subunits of Na-pump together with c-Src and EGF receptor molecules in renal caveolae membranes and activation of ERK1/2 MAP-kinase. Co-immunoprecipitation experiments demonstrated a direct interaction of the Na^+,K^+-ATPase with c-Src and c-Src-dependent Tyr phosphorylation of the α_1- subunit (Ferrandi et al., 2004). In central neurons Na^+,K^+-ATPase activity has been shown to be regulated by specific Src tyrosine kinases via mechanism involving protein-protein interactions. Such modulation of the Na-pump activity may play a role in apoptosis in neurons (Wang et al., 2005). Ca^{2+}/calmodulin-dependent protein kinase (CaMK) has been shown to phosphorylate Na^+,K^+-ATPase in cardiac sarcolemma. Such phosphorylation leads to a significant reduction in Na-pump activity (Netticadan et al., 1997).

In *Xenopus* oocytes, expressing active serum and glucocorticoid-dependent kinase SGK1, Na^+,K^+-ATPase activity was found to be increased (Setiawan et al., 2002). This observation indicates SGK1 may participate in the regulation of renal tubular Na^+ transport. Co-expression of Na^+,K^+-ATPase (rat α_1-subunit and *Xenopus laevis* β_1-subunit) and *Xenopus* SGK1 in *Xenopus* oocytes increases the Na-pump current, which appears to be secondary to the increase in Na^+,K^+-ATPase cell-surface expression as visualized by Western blot analysis of cell surface-biotinylated proteins (Zecevic et al., 2004).

1.2.3. Insulin action on Na^+,K^+-ATPase

Skeletal muscle contains one of the largest pools of Na^+,K^+-ATPase in the body, and therefore plays a central role in the clearance of $[K^+]$ from the blood during ingestion or infusion of K^+ (McDonough et al., 2002). Pathological hyperkalemia and impaired K^+-tolerance frequently occur in people with metabolic disorders.

Skeletal muscle is one of the most important target tissues for insulin, the hormone which plays a major role in the control of glucose transport and metabolism, as well as K^+ uptake and maintenance of the plasma K^+ concentration (Clausen, 1986). In particular, an increase in the uptake of K^+ by various tissues is a well-known effect of insulin and it has been mainly ascribed to the stimulation of the Na^+-K^+-ATPase in skeletal muscle (Ewart et al., 1995; Sweeney et al., 1998a), and not secondary to an increase in $[Na^+]_i$ via the Na^+-H^+ anti-porter stimulation (Weil et al., 1991). The

molecular signaling mechanism by which insulin modulates Na^+,K^+-ATPase activity is incompletely known. The Na^+,K^+-ATPase α-subunits are phosphorylated in response to insulin in skeletal muscle in isoform-specific manner (Chibalin et al., 2001). Insulin-stimulated phosphorylation of the α-subunits of Na^+,K^+-ATPase may constitute an important regulatory mechanism of the Na^+,K^+-ATPase activity and the Na^+- and K^+-gradient in skeletal muscle. Insulin stimulation of the Na-pump is PKC-dependent (Sampson et al., 1994; Sweeney et al., 1998a) and is partly mediated by an increase in the cell surface appearance of Na^+,K^+-ATPase in rat skeletal muscle (Al-Khalili et al., 2003b; Hundal et al., 1992). However, the human α-subunit of Na^+,K^+-ATPase lacks Ser^{18} (Ser^{23}), a specific PKC phosphorylation site, highlighting important species differences in the regulation of the Na-pump (Ewart et al., 1995; Lingrel et al., 1994). Only rodent Na^+,K^+-ATPase has been shown to be regulated by PKC (Beguin et al., 1994; Beguin et al., 1996). *In vitro* phosphorylation of GST fusion proteins containing the N-terminal of the Na^+,K^+-ATPase α-subunit indicates that the rat $α_2$ and the human $α_1$ subunit are poor substrates for PKC (Beguin et al., 1996). Evidence for PKC-mediated regulation of the human isoforms of the Na-pump remains a matter of debate (Beguin et al., 1996).

Computer-based screening of possible phosphorylation sequence motifs in human Na^+,K^+-ATPase, reveals that in human the $α_1$-subunit Thr^{81} is a possible site for ERK 1/2, Ser^{943} is a possible site for cAMP-dependent protein kinase (PKA), and Ser^{491} is a possible site for calmodulin-dependent kinase II. In the human $α_2$ –subunit, Thr^{414} is a possible site for Akt/PKB kinase, Ser^{936} (a homologue for Ser^{943} in $α_1$- subunit) is a possible site for PKA, and Thr^{79} is a possible site for ERK 1/2. Importantly, the predicted ERK phosphorylation sites are conserved between human, rat, mouse and chicken (Obenauer et al., 2003; Yaffe et al., 2001).

Inhibition of phosphatidylinositol (PI) 3-kinase and atypical PKC isoform (PKCζ), abrogates the effect of insulin on Na^+-K^+-ATPase activity in 3T3-L1 adipocytes (Sweeney et al., 1998a; Sweeney et al., 1998b). Signaling cascades affected by these kinases converge on the phospholipase A2 (PLA_2) pathway, indicating that the regulation of Na^+-K^+-ATPase by insulin may involve arachidonic acid. Insulin stimulates Na^+,K^+-ATPase activity and mediates phosphorylation of the α-subunit on serine, threonine, and tyrosine residues (Tyr^{10}) (Chibalin et al., 2001; Feraille et al., 1999). Tyrosine phosphorylation of the α-subunits on Tyr^{10} in response to insulin is likely to participate in physiological control of sodium reabsorption in PCT. In rat *soleus* muscle insulin stimulates α-subunit phosphorylation on tyrosine residues (Chibalin et al., 2001). Tyrosine phosphorylation of the α-subunit may be an important mechanism for facilitating insulin-mediated translocation of the Na^+,K^+-ATPase to the plasma membrane and the effect to increase Na-pump activity.

An important mechanism accounting for the insulin-induced short-term regulation of the Na^+,K^+-ATPase is the insulin-mediated translocation of the sodium pumps from intracellular storage sites to the cell surface. The first evidence was obtained in experiments with frog skeletal muscle (Grinstein et al., 1974; Omatsu-Kanbe et al., 1990). Such rapid translocation is considered to be the main mechanism for the pump stimulation in skeletal muscle. Utilising subcellular fractionation, insulin-induced translocation of $α_2$- $β_1$-dimers to plasma membrane from intracellular compartments in rat skeletal muscle has been reported (Hundal et al., 1992; Marette et al., 1993). Insulin-induced membrane translocation of $α_1$- and $α_2$-subunits, as assessed by cell surface biotinylation, in rat epitrochlearis muscle and cultured human skeletal muscle cells has also been reported (Al-Khalili et al., 2003b). Na^+,K^+-ATPase α-subunit translocation

was abolished by the PI3-kinase inhibitor wortmannin, as well as by the PKC inhibitor GF109203X. Thus, insulin mediates Na^+,K^+-ATPase α_1- and α_2-subunit translocation to the skeletal muscle plasma membrane via PI 3-kinase- and PKC-dependent mechanism. Experiments on rat skeletal muscle have shown that the effect of insulin on cell-surface expression of the pumps is specific to oxidative slow-twitch skeletal muscle, rather than glycolytic fast-twitch muscle, such that insulin-induced redistribution of the α_2- and β_1-isoforms of Na^+,K^+-ATPase from an intracellular pool to the plasma membrane was observed (Lavoie et al., 1996).

In addition to skeletal muscle, a short-term insulin-mediated up-regulation of Na^+-K^+-ATPase activity has been observed in the kidney. In studies addressing the regulation of Na^+-K^+-ATPase in kidney cortical tubules, insulin increased the apparent affinity of the enzyme for Na^+ (Feraille et al., 1994; Feraille et al., 1995). Much earlier, similar effects of insulin were observed in rat *soleus* muscle (Clausen et al., 1977), where insulin stimulated Na^+-K^+-ATPase activity possibly through an increase in the apparent affinity of the enzyme for Na^+.

Insulin has also long-term effects on Na^+-K^+-ATPase expression. These effects are complex and there has been the evidence for insulin-mediated increases and decreases in the Na-pump activity, with the latter being particularly relevant for diabetes (Sweeney et al., 1998a).

1.2.4. Changes in cell surface abundance of Na-pump subunits due to other stimuli

Activation of various membrane receptors facilitates the trafficking of Na^+-K^+-ATPase molecules between plasma membrane and intracellular compartments. These effects are accompanied by a time-dependent interaction of the Na^+-K^+-ATPase α-subunit with specific intracellular signaling molecules, either at the plasma membrane (endocytosis) or at the endosome's membranes (recruitment). Most of these studies have been performed in rat renal epithelial cells in which the α_1- isoenzyme is present. Studies in neurons from the neostriatum in which all three α-subunit isoforms are present indicate that neurotransmitter-dependent regulation of Na^+-K^+-ATPase activity displays isoform specificity and suggests a complex organization of intracellular signaling networks controlling Na^+-K^+-ATPase traffic in mammalian cells (Teixeira et al., 2003).

1.2.5. Endocytosis of the Na-pump

1.2.5.1. Dopamine

Locally synthesized dopamine is a natriuretic factor in the kidney proximal tubule and acts in a paracrine and autocrine fashion (Aperia, 1994). Dopamine was shown to be the inhibitor of Na^+-K^+-ATPase activity in the kidney proximal convoluted tubule (PCT) (Aperia et al., 1987), but similar effects have also been observed in other regions of kidney, namely, the medullary thick ascending limb (mTAL) and cortical collecting duct (CCD), as well as in cultured Madin-Darby canine kidney (MDCK) cells, neurons, arteries, retinal cells, aortic smooth muscle, small intestine, and lung (Therien et al., 2000). The overall consensus is that dopamine inhibits Na^+-K^+-ATPase, and this represents a physiologically important mechanism for regulating salt reabsorption in the kidney during high salt intake. Illustrating this point is the observation that mechanisms of dopamine-dependent sodium pump modulation are often compromised in aged and hypertensive rats.

In the kidney, inhibition of Na-pump in the proximal segments of the nephron is mediated through the dopamine receptors DA_1 and DA_2, and involves G protein-coupled, PKC-dependent pathways (Aperia et al., 1987; Bertorello et al., 1989). In distal

segments (mTAL and CCD), mainly DA_1 receptors and PKA-associated pathways are involved (Satoh et al., 1992).

In rat proximal renal tubular cells dopamine inhibits Na^+-K^+-ATPase activity (Pedemonte et al., 2005) via activation of PKC signaling pathway, which leads to phosphorylation of Na^+-K^+-ATPase α-subunits on specific serine residues (Ser^{18} and Ser^{11} of the rat enzyme) (Chibalin et al., 1998a; Chibalin et al., 1999), stimulation of PI3-kinase activity (Chibalin et al., 1998b), recruitment of PI3-kinase to specific proline-rich motifs of the α-subunit (Yudowski et al., 2000), which involves the interaction with 14-3-3 protein (Efendiev et al., 2005), recruitment of the adaptor protein 2 (AP2) to its binding site on the Na-pump α-subunit at Tyr^{537} (Done et al., 2002), phosphorylation of the AP2-μ2-subunit (Chen et al., 2006) and subsequent clathrin-dependent endocytosis of Na-pump molecules (Chibalin et al., 1997).

1.2.5.2. Parathyroid hormone

Parathyroid hormone has been shown to inhibit Na-pump activity via PKC- and ERK-dependent pathways, phosphorylation of α-subunits on Ser^{11} and subsequent clathrin-dependent endocytosis of Na-pump molecules (Khundmiri et al., 2004).

1.2.5.3. Hypoxia

In alveolar epithelial cells, hypoxia inhibits Na^+-K^+-ATPase activity due to clathrin-dependent endocytosis of Na-pump. The mechanism of endocytosis includes generation of reactive oxygen species, activation of PKC ζ and phosphorylation of the Na-pump α-subunit on Ser^{18} (Dada et al., 2003).

1.2.6. Recruitment of Na^+-K^+-ATPase molecules to plasma membrane

In addition to insulin, several other stimuli cause translocation of the Na-pump molecules to the plasma membrane from intracellular storage sites. In alveolar type 2 epithelial (AT2) cells, contrary to the kidney, dopamine and its dopaminergic D1 agonist, fenoldopam, increases Na^+-K^+-ATPase activity (Ridge et al., 2002). This effect was parallel to an increase in Na^+-K^+-ATPase α_1 and β_1 protein abundance in the basolateral membrane (BLM) of AT2 cells. Increased cell surface content of Na-pump subunits was mediated by exocytosis of the Na^+-K^+-pump from endosomal compartments into the BLM. The molecular mechanism of Na-pump exocytosis includes activation of novel diacylglycerol-sensitive protein kinase C isoforms, namely PKC δ and ϵ (Bertorello et al., 2003; Ridge et al., 2002).

In alveolar type 2 epithelial cells, stimulation of β-adrenergic receptors by isoproterenol increases Na^+-K^+-ATPase activity through a PKA-dependent mechanism. This stimulation, is independent of phosphorylation of the Na-pump α -subunit by PKA, and is the result of an increased number of α-subunits recruited from late endosomes into the plasma membrane (Bertorello et al., 1999).

In cultured PTC (OK) cells, phorbol ester (PMA) stimulation activates the PKC β isoform and leads to a simultaneous phoshorylation of the Na-pump on Ser^{11} and Ser^{18}, which promotes the recruitment of Na-pump molecules to the plasma membrane (Efendiev et al., 2000).

In rat proximal convoluted tubules, forskolin increases Na^+-K^+-ATPase activity by approximately 40% via a protein kinase A (PKA) mediated mechanism by increasing the number of Na^+-K^+-pumps in the plasma membrane (Carranza et al., 1998). In mouse renal cortical collecting duct cells cAMP stimulates Na^+-K^+-ATPase activity through

stimulation of translocation of Na-pump units from intracellular storage sites to the plasma membrane (Gonin et al., 2001).

1.2.6.1. Corticosteroids: long- and short-term regulation of Na-pump

Steroid hormones, in particular, corticosteroids, have specific long- and short-term regulatory effects on the Na^+-K^+-ATPase. Long-term effects are generally mediated by changes in mRNA/protein synthesis induced by direct interactions of receptor/corticosteroid complexes with nuclear DNA. Though many types of corticosteroids have been shown to mediate the regulation of the Na^+-K^+-ATPase, the mineralocorticoid aldosterone and glucocorticoid dexamethasone are most widely studied (Therien et al., 2000).

Corticosteroids are synthesized and released by adrenal cortex. Aldosterone, in particular, has an important role in regulation of Na^+ and K^+ transport in the kidney, and its physiological role is considered to be in the long-term adaptation to decreases in Na^+ or increases in K^+ intake (Bonvalet, 1998). The main effect of aldosterone and dexamethasone on Na^+-K^+-ATPase is to sustain a long-term increase in expression of Na- pumps. This effect has been widely observed in different mammalian tissues. Steroid hormones can increase mRNA expression of α- and β- subunit genes (Bhutada et al., 1991; Dorup et al., 1997; Welling et al., 1993).

Aldosterone has specific short-term effects on Na^+-K^+-ATPase activity. The first type of stimulatory effect of aldosterone is dependent on increases in intracellular Na^+ concentration, and it is inhibited by amiloride (Rayson et al., 1985). The second type of aldosterone-mediated up-regulation of Na^+-K^+-ATPase has been observed in cortical collecting tubules, where aldosterone administration to adrenalectomized rats or stimulation of cultured mouse collecting duct principal cells mpkCCD increased basolateral cell surface abundance of Na-pump molecules (Summa et al., 2001).

1.2.6.2. Exercise

Exercise stimulates Na^+,K^+-ATPase activity. There has been a number of reports, elucidating the mechanisms of such stimulation. Acute exercise has been shown to cause translocation of the Na-pump to plasma membrane as assessed in human muscle biopsies (McKenna et al., 1993). Exercise-training induces an increase in vesicular membrane content of α_2-, total α- and β_1- subunits in giant sarcolemmal vesicles prepared from human *vastus lateralis* muscle (Juel et al., 2000). Treadmill running increases the plasma membrane content of α_1- and α_2- subunits of Na^+,K^+-ATPase, as well as α_1- and β_2- mRNAs in rat skeletal muscle (Tsakiridis et al., 1996). These results suggest that translocation of pump subunits to plasma membrane is an important mechanism involved in short-term up-regulation of Na^+,K^+-ATPase, associated with skeletal muscle activity.

1.3. Na^+,K^+-ATPase as a receptor for cardiotonic steroids and signal transducer

Na^+,K^+-ATPase as an ion pump has been studied extensively since its discovery in 1957 (Skou, 1957; Skou, 1998). Although early findings suggested a role for Na^+,K^+-ATPase in regulation of cell growth and expression of various genes, in recent years there has been a growing interest in molecular mechanisms of Na^+,K^+-ATPase-mediated signal transduction (Xie et al., 2002). What can be an endogenous ligand for Na^+,K^+-ATPase as a receptor molecule?

Digitalis-like steroids and related agents have been used to treat congestive heart failure (CHF) ever since the publication in 1785 of Withering's monograph on the foxglove (Blaustein et al., 2003). All digitalis-like cardiotonic steroids (CTS) or cardiac glycosides enhance heart contraction through a mechanism involving inhibition of Na^+,K^+-ATPase, an increase in the activity of the Na^+-Ca^{2+}-exchanger, and finally, an increase in $[Ca^{2+}]_i$. Ouabain (g-strophanthin), one of the cardiotonic steroids, is known to be the specific inhibitor of the Na-pump (Kawamura et al., 1999). Ouabain as, well as other CTS, binds to one of the extracellular loops of Na-pump and competes with K^+ for binding. Upon binding, CTS stabilize E2P confirmation of the pump, thus making Na-pump inactive.

Cardiac glycosides differ in their binding affinity to the Na-pump α-subunit. With a given cardiac glycoside and given combination of ligands, the inhibitory effects vary for different tissues. For enzymes from most tissues, the $K_{0.5}$ for ouabain for inhibition of Na^+,K^+-ATPase activity is in the range of 10^{-7}-10^{-5} M. Na^+,K^+-ATPase from rat heart or crab nerves is less sensitive (Skou, 1988). The α-subunits differ in their sensitivity to ouabain. The $α_2$ subunit is an ouabain-sensitive subunit, while the $α_1$-subunit is more ouabain-resistant.

Attempts to find endogenous digitalis-like factors in mammals led to isolation of endogenous ouabain, as well as several additional cardiotonic steroids of cardenolide and bufadienolide type from blood, adrenal glands, and hypothalamus (Fig. 3) (Hamlyn et al., 1991; Schneider et al., 1998; Schoner, 2000; Schoner, 2001; Schoner, 2002a).

OUABAIN
R = Rhamnose
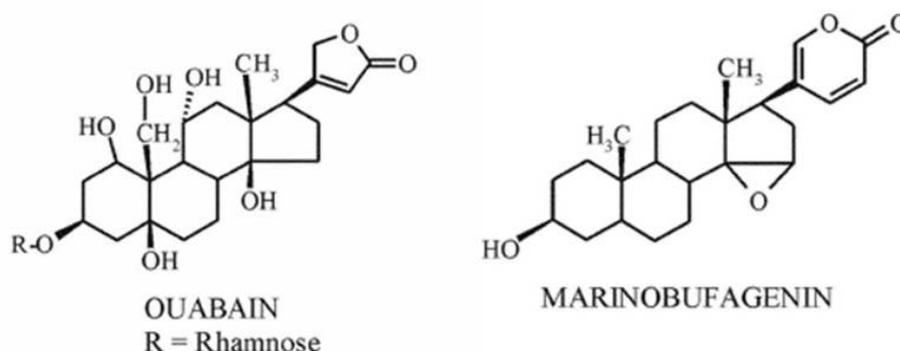
MARINOBUFAGENIN

Figure 3. Structure of cardiotonic steroids ouabain and marinobufagenin

The concentration of endogenous ouabain is elevated in circulation in response to an in increase in Na^+ intake, hypoxia, and physical exercise. Endogenous ouabain rapidly changes in humans and dogs upon physical exercise and is under control of epinephrine and angiotensin II (Bauer et al., 2005). Adrenal cortical cells in tissue culture release ouabain in response to angiotensin II and epinephrine. Ouabain levels in blood are elevated in 50% of Caucasians with low-renin hypertension (Hamlyn et al., 1982). Infusion over several weeks of low concentrations of ouabain induces hypertension in rats (Manunta et al., 1994). The search for a specific binding globulin for cardiac glycosides in bovine plasma resulted in identification of d allotype of μ-chain of IgM, which hydrophobic surfaces interact with cardiotonic steroids and cholesterol. Such IgM complexes might be involved in hepatic elimination of cardiotonic steroids (Schoner et al., 2003).

The discovery of ouabain as a new adrenal hormone affecting Na^+ metabolism and the development of the new ouabain antagonist PST 2238 allows for new

possibilities for the therapy of hypertension and congestive heart failure, as well as better understanding of the disease on the physiological and endocrinological level.

Bagrov and co-workers purified bufodienolide, from urine of patients with myocardial infarction and observed that it is indistinguishable from amphibian marinobufagenin (Bagrov et al., 1998). Marinobufagenin (MBG) may show natriuretic properties, because it inhibits the α_1- isoform of Na$^+$,K$^+$-ATPase, the main Na-pump isoform in the kidney, much better than any other pump isoform (Fridman et al., 2002; Schoner, 2002a). Earlier, the same authors demonstrated that the amphibian Bufo marinus toad venom contained bufalin and marinobufagenin, which exhibited significant vasoconstrictor properties and directly inhibited the Na-pump in vascular smooth muscle membranes (Bagrov et al., 1993; Bagrov et al., 1995). Recently, marinobufagenin has been shown to be involved in the pathogenesis of diabetes mellitus. Renal excretion and plasma levels of MBG were increased and erythrocyte Na$^+$,K$^+$-ATPase activity inhibited in rats with type 1 and type 2 diabetes (Bagrov et al., 2005). In rat aorta, marinobufagenin induced vasoconstriction via inhibition of vascular smooth muscle Na$^+$,K$^+$-ATPase, which mainly contains the α_1- isoform, and ouabain had its predominant effect on pumps localized in nerve endings, enriched in α_3- subunit (Fedorova et al., 1997). Thus, α-isoforms of Na-pump exhibit differential responsiveness to endogenous digitalis-like factors.

A biosynthetic pathway for steroid derivatives closely related to plant cardiac glycosides in mammalian tissues has been suggested. A gene array analysis has revealed a unique steroid biosynthetic circuit, existing in Milan hypertensive rat brain, which could account for overproduction of hypothalamic ouabain-like compound in this species (Murrell et al., 2005).

The above mentioned effects of endogenous cardiac glycosides are observed at concentrations that do not inhibit the Na- pump. Na$^+$,K$^+$-ATPase functions as the receptor of cardiotonic steroids and signal transducer (Xie et al., 2002), which plays a role in tissue proliferation, heart contractility, arterial hypertension, and natriuresis via various intracellular signaling pathways (Schoner, 2002b; Schoner et al., 2005).

Research, carried out mostly on cardiac myocytes, shows that Na$^+$,K$^+$-ATPase interacts with neighboring membrane proteins and participates in organized cytosolic cascades of signaling proteins (Liu et al., 2003; Liu et al., 2005; Wang et al., 2004; Xie et al., 2003b). The signaling pathways that are rapidly elicited by the interaction between ouabain with Na$^+$,K$^+$-ATPase, and are independent of changes in intracellular Na$^+$ and K$^+$ concentrations, include activation of Src kinase (Tian et al., 2006), transactivation of the epidermal growth factor receptor by Src (Haas et al., 2000), activation of Ras and p42/44 mitogen-activated protein kinases (Haas et al., 2002; Mohammadi et al., 2001; Mohammadi et al., 2003; Plourde et al., 2006), and increased generation of reactive oxygen species by mitochondria. In cardiac myocytes, the resulting downstream events include the induction of some early response proto-oncogenes, activation of transcription factors, activator protein-1 and nuclear factor kappa-B, regulation of a number of cardiac growth-related genes, and stimulation of protein synthesis and myocyte hypertrophy. For these downstream events, the induced reactive oxygen species and the rise in intracellular Ca^{2+} are essential second messengers (Liu et al., 2000; Xie et al., 1990; Xie et al., 1999). Digitalis drugs promote cardiac hypertrophy (Cutilletta et al., 1977; Pierdomenico et al., 2001). Similarly to other hypertrophic stimuli, ouabain regulates the transcription of several hypertrophic marker genes in cardiac myocytes (Huang et al., 1997a; Huang et al., 1997b; Peng et al., 1996). In cells other than cardiac myocytes, the proximal pathways linked to Na$^+$,K$^+$-ATPase through protein-protein interactions are similar to those reported in myocytes,

but downstream events and physiological consequences may be significantly different (Dmitrieva et al., 2003; Rajasekaran et al., 2001; Xie et al., 2002; Xie, 2003a).

In renal cells exposed to ouabain, Na^+,K^+-ATPase forms a cell signaling microdomain with the inositol 1,4,5-trisphosphate receptor, which generates slow Ca^{2+} oscillations (Miyakawa-Naito et al., 2003). In opossum proximal tubule cells ouabain induces cell proliferation via the Ca^{2+}-dependent phosphorylation of Akt/PKB kinase (Khundmiri et al., 2006).

Metabolic events modulated by cardiotonic steroids are poorly studied. Ouabain, in a wide concentration range (0.1- 4 mM), has been shown to stimulate renal gluconeogenesis from pyruvate, lactate, propionate and fructose by 10-40%, which is similar to epinephrine stimulation (Friedrichs et al., 1973). In rat diaphragm (Clausen, 1965; Clausen, 1966), ouabain increases glycogen content and glucose incorporation into glycogen. The mechanisms of cardiotonic steroids action in skeletal muscle, the most abundant tissue in the mammalian body, still require further clarification.

2   AIMS

The main aims of this thesis are:

1. To study the involvement of the MAP-kinase signaling pathway in the regulation of Na^+,K^+-ATPase activity.

2. To study the function of Na^+,K^+-ATPase as a cardiotonic steroid receptor and signal transducer in skeletal muscle.

3   MATERIALS AND METHODS

3.1. Antibodies and reagents

Mouse monoclonal and rabbit polyclonal antibodies to α_1-subunit of Na^+,K^+-ATPase were a kind gift from Dr. M. Caplan (Yale University, New Haven, CT, USA). Mouse monoclonal antibody to α_2-subunit of Na^+,K^+-ATPase was a kind gift from Dr. K. Sweadner (Massachusetts Central Hospital, MA, USA), rabbit polyclonal antibody to α_2-subunit of Na^+,K^+-ATPase was the kind gift from Dr. T. Pressley (Texas Tech University Health Sciences Center, Lubbock, USA). Rabbit polyclonal anti-NK1 antibodies were kind gifts of Dr. E. Feraille (University of Geneve, Switzerland). Rabbit polyclonal antibodies to β1 and β2 subunits were from Dr. P. Martin-Vasallo (University La Laguna, Tenerife, Spain). Rabbit polyclonal antibodies to phospho-GSK3α/β (Ser^{21} of GSK3α and Ser^9 of GSK3β), phospho-c-Src (Tyr^{416}), phospho-p90rsk (Thr^{573}), phospho-PKC α/β_{II} ($Thr^{638/641}$), δ (Thr^{505}) and ζ/λ ($Thr^{410/403}$), phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}), phospho-AMPKα (Thr^{172}), phospho-CaMKII (Thr^{286}) and monoclonal antibody to phospho-Thr-Pro motif were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit polyclonal antibodies to phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and phospho-Akt/PKB (Ser^{473}) were from New England Biolabs Inc. (Beverly, MA). Rabbit polyclonal antibodies to phospho-ACC (Ser^{79}) were from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal antibody to c-Src was from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibody to phospho-Tyr was from BD Transduction Laboratories. Rabbit polyclonal antibody to p90rsk was a kind gift from Dr. D. Alessi (University of Dundee, Scotland, UK). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G was obtained from Bio-Rad Laboratories (Hercules, CA). Reagents for enhanced chemiluminescence were obtained from Amersham (Arlington Heights, IL). Protein A sepharose CL-4B was from Amersham Biosciences AB (Uppsala, Sweden) and protein A, horseradish peroxidase linked was from Amersham Biosciences UK Ltd (Buckinghamshire, England). Protein L-agarose and protein G-sepharose were from Sigma (St. Louis, MO, USA). Human insulin (Actrapid) was from Novo Nordisk AS (Copenhagen, Denmark). Human recombinant C-peptide was obtained from Schwarz Pharma (Monheim, Germany). Scrambled C-peptide (the same amino acid residues as in C-peptide, but in random order) was from Sigma Genosys (Cambridge, UK). Ouabain was obtained from Sigma (St Louis, MO, USA). Marinobufagenin was purified from *Bufo marinus* toad venom as described previously (Bagrov et al., 1993). Recombinant ERK1 and ERK2 kinases, recombinant PKCζ and MEK1/2 inhibitor U0126 were from Upstate Cell Signaling Solutions (Charlottesville, VA). MEK1/2 inhibitor PD98059 (2'-amino-3'-methoxyflavone), c-Src inhibitor PP2 (4-amino-5-[4-chlorophenyl]-7-[t-butyl] pyrazolo[3,4-d]-pyrimidine), PI3-kinase inhibitors LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) and wortmannin, PKC inhibitor GF109203X and purified rat brain PKC were from Calbiochem (San Diego, CA). Streptavidin-agarose beads and EZ-link Sulfo-NHS-SS-biotin were from Pierce, (Rockford, IL, USA). Crosstide (Gly-Arg-Pro-Arg-Thr-Ser-Ser-Phe-Ala-Glu-Gly) was from Sigma-Aldrich. Solvent NEF-910 G was from Du Pont de Memours GmbH (Hamburg, Germany). Cell culture media and reagents were from Invitrogen (Sweden).

Dimethylsulfoxide (Calbiochem) was used as a solvent for protein kinases inhibitors. All other reagents were of analytical grade (Sigma, St.Louis, MO).

3.2. In silico screen of possible phosphorylation sites of Na$^+$,K$^+$-ATPase α-subunits

Protein sequences of α$_1$ and α$_2$-subunits of Na$^+$,K$^+$-ATPase of human, rat, mouse and chicken origin were analyzed by motif-based profile scanning programs Scansite 2.0 (http://scansite.mit.edu) and PhosphoBase2.0 (http://www.cbs.dtu.dk/databases/ PhosphoBase). The statistical stringency criteria for predicted phosphorylation sites were set at high, medium, and low levels according to the program's user recommendations.

3.3. Subject characteristics

Skeletal muscle biopsies (rectus abdominus) were obtained with the informed consent of the donors during scheduled abdominal surgery. Subjects (3 male and 3 female) had no known metabolic disorders. Mean age was 54.5 ± 6.5 years (BMI 26 ± 1.5 and fasting blood glucose 5.2 ± 0.3). The Ethical Committee at the Karolinska Institutet approved the study protocols.

3.4. Animals

Male Wistar rats (110 – 120 g) were purchased from B & K Universal (Sollentuna, Sweden) and housed at the animal facility at the Karolinska Institutet. Rats were maintained on a 12-h light-dark cycle and given free access to standard rodent chow and water. Rats were studied after a 4-5 hour fast. The regional animal ethical committee approved all experimental procedures.

3.5. Cell culture

Human skeletal muscle satellite cells were isolated from muscle biopsies and cultured, as previously described (Al-Khalili et al., 2003a). Cells from passages 3–4 were used for the experiments. Glucose uptake in HSMC was measured, as previously described (Al-Khalili et al., 2003a).

Human renal tubular cells were cultured from the unaffected outer cortex of renal tissue obtained from non-diabetic patients undergoing elective nephrectomy for renal cell carcinoma. Tissue collection was undertaken with the informed consent of the subject and approval by the Karolinska Institutet ethics committee. Cells from passages 2-3 were used for the experiments.

3.6. Measurement of ouabain-sensitive ^{86}Rb$^+$ uptake

Na$^+$,K$^+$-ATPase transport activity was measured as ouabain-sensitive ^{86}Rb$^+$ uptake, under conditions of initial rate, as previously described (Gonin et al., 2001), (Cheval et al., 1990). For paper I, myotubes (day 6) were pre-incubated in serum-free DMEM with or without ouabain (0.2 mM) and kinase inhibitors for 30 min at 37 ^{0}C. Thereafter, myotubes were incubated in the presence or absence of insulin (20 min) and/or inhibitors.

For ouabain study, paper IV, ^{86}Rb$^+$ uptake that was inhibited by 100 μM ouabain was taken as the maximal rate of active uptake.

For paper II, human renal tubular cells (HRTCs) were pre-incubated in serum-free RPMI 1640 with or without ouabain (0.2 mM) and kinase inhibitors for 30 min at 37^{0}C, with subsequent incubation with or without C-peptide (10 min) and/or inhibitors.

For all the studies, Na^+,K^+-ATPase transport activity was determined after addition of 50 µl of medium containing tracer amounts of $^{86}RbCl$ (100 nCi/sample; Amersham Biosciences, UK) for 5 min. Incubation was stopped by cooling on ice, and dishes were washed three times with an ice-cold washing solution containing 150 mM cholinechloride, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 2 mM $BaCl_2$, and 5 mM HEPES, pH 7.4. Cells were lysed in 750 µl lysis buffer A and radioactivity was measured by liquid scintillation. Protein content was determined in parallel using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Ouabain-sensitive $^{86}Rb^+$ uptake was calculated as the difference between the mean values measured in triplicate samples incubated with or without 0.2 mM ouabain. Data were expressed as % of control. Basal ouabain-sensitive $^{86}Rb^+$ uptake was 3.1 ±0.3 pM Rb^+ per microgram of protein per min for human myotubes and 16.3±2.4 pmol Rb^+ per microgram of protein per min for HRTCs.

3.7. [^{3}H]-ouabain binding

Measurement of ouabain binding sites on cell surface of differentiated myotubules was performed with [^{3}H]-ouabain, as previously described (Aydemir-Koksoy et al., 2001a). Myotubes were pre-incubated in serum-free DMEM in the presence or absence of insulin (20 min) and/or inhibitors. Thereafter, cells were washed and incubated with [^{3}H]-ouabain-binding buffer (OBB), containing 2 mM HEPES, pH 7.4, 0.25 µM [^{3}H]-ouabain (specific activity: 16.5 Ci/mmol, Amersham Biosciences, UK), 120 mM NaCl, 0.05 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM glucose. After 15 min of incubation with [^{3}H]-ouabain, plates were washed with [^{3}H]-ouabain-free OBB, and cells were lysed in 750 µl of lysis buffer A (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 20 mM Tris, pH 8.0, 1% Triton X-100, 10 % v/v glycerol, 10 mM NaF, 0.5 mM Na_3VO_4, 0.2 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin and 1 µM microcystin) and radioactivity was measured by liquid scintillation. Protein content was determined in parallel using BCA assay. Unlabeled ouabain (1 mM) was used as the background control for non-specific binding. Specific binding was calculated by subtracting the background control from [^{3}H]-ouabain. Data were expressed as % of control.

3.8. Cell surface biotinylation of HSMC

Myotubes (6 day) were pre-incubated in PBS in the presence or absence of insulin (20 min) and/or inhibitors or 100 nM ouabain (2 hrs) and thereafter exposed to EZ-link Sulfo-NHS-SS-biotin at the final concentration of 1.5 mg/ml in PBS-CM at 4^0C for 60 min with gentle shaking. Cell surface biotinylation was performed as described (Al-Khalili et al., 2003b). After streptavidin precipitation, samples were analyzed by SDS-PAGE with subsequent Western blot with appropriate antibodies.

3.9. Metabolic labeling of myotubes or human renal tubular cells with ^{32}Pi

^{32}Pi metabolic labeling was performed (Chibalin et al., 2001) to investigate in vivo phosphorylation of α-subunits of Na^+,K^+-ATPase. Myotubes (day 6) were incubated at 37 ^{0}C for 3 h in serum free DMEM containing ^{32}Pi (1 mCi/ml); HRTCs were incubated for the same time in serum-free RPMI 1640 containing ^{32}Pi. Insulin and/or inhibitors or C-peptide and/or inhibitors were added during the last 20 or 50 min of incubation time. Incubation was terminated by cooling on ice. Myotubes or HRTCs were lysed in buffer A and α-subunits were immunoprecipitated with polyclonal anti-NK1 rabbit antibodies. The

bead pellets were mixed with Laemmli buffer (60 µl) (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, and 10 mM DTT), separated by SDS-PAGE, and transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, MA). Phosphoproteins were analyzed using Bio-Imaging Analyzer BAS-1800II (Fuji Photo Film Co., Ltd, Japan), and quantification was performed using the Image Gauge software, version 3.4 (Fuji Photo Film Co., Ltd, Japan). In each experiment, the amount of radioactivity incorporated into α-subunit was corrected for the amount of protein detected by Western blot analysis. The quantitative data is reported as percent of basal.

3.10. Immunoprecipitation

Immunoprecipitation of Na^+,K^+-ATPase α-subunit was performed as described previously (Chibalin et al., 2001). Myotubes or HRTCs were lysed in 500 µl of ice-cold lysis buffer A. Insoluble material was removed by centrifugation (12,000 x g for 10 min at 4^0C). Aliquots of supernatant (300 µg of protein) were immunoprecipitated overnight at 4^0C with 50 µl of polyclonal anti-NK1 rabbit antibodies or with 30 µl of anti-phospho-Thr-Pro mouse IgM. Immunoprecipitates were collected on protein A-sepharose or protein L-agarose beads, respectively. Beads were washed four times in lysis buffer A; twice in 0.1 M Tris (pH 8.0) and 0.5 M LiCl; once in 10 mM Tris (pH 7.6), 0.15 M NaCl, and 1 mM EDTA; and once in 20 mM HEPES, 5 mM $MgCl_2$, and 1 mM dithiothreitol. Pellets were resuspended in Laemmli sample buffer.

For ouabain study, papers IV and III, cell lysates or soleus muscle lysates (500 µl) were incubated with 40 µl of protein A-sepharose beads, rotating for 30 min at 4^0C. After brief centrifugation, supernatants were collected and immunoprecipitated with 1) antibodies to c-Src overnight at 4^0C; 2) antibodies to p-Tyr overnight at 4^0C. Immunoprecipitates were collected on protein A-sepharose beads for 2 hrs at 4^0C. 3) HSM myotubes lysates (300 µg of protein) were immunoprecipitated for 2.5 h at 4^0C with anti-phospho-Thr-Pro mouse IgM. Immunoprecipitates were collected on protein L-agarose beads. For immunoprecipitations, beads were washed 3 times in lysis buffer A and twice in ice-cold PBS. Pellets were resuspended in Laemmli sample buffer.

3.11. In vitro phosphorylation of Na^+,K^+-ATPase α-.subunits by ERK or PKC

Na^+,K^+-ATPase holoenzyme was purified from human renal tubular cells and rat kidney cortex as described (Jorgensen, 1988). Quality of purification was verified by SDS-PAGE (7.5% gel) followed by Coomassie staining. Na^+,K^+-ATPase activity has previously been determined under Vmax conditions (Chibalin et al., 1998a). For in vitro phosphorylation, 5 µg of Na^+,K^+-ATPase protein preparation were re-suspended in 40 µl of phosphorylation media (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1mM EDTA, 5mM NaF, 1 mM β-glycerophosphate, 200 µM ouabain, 0.5 mM DTT, 1.5 mM $CaCl_2$, in the presence or absence of 0.5% Triton X-100), followed by addition of 0.1 µg of PKC or 0.1 µg of ERK1 and 0.1 µg of ERK2. The phosphorylation reaction for both kinases was initiated by addition of 10 µl of ATP (final concentration 0.1 mM containing 3 µCi/pmol $[^{32}P]$-γ-ATP, Amersham Biosciences, UK) and allowed to proceed for 1 hour at 30^0C. Reactions were terminated by the addition of 20 µl 4x Laemmli sample buffer. Samples were incubated for 20 min at 56^0C, thereafter iodoacetamide was added to final concentration of 20 mM. The phosphorylated samples were resolved by SDS-PAGE, transferred to PVDF membranes and ^{32}P-labeled Na^+,K^+-ATPase α-subunits were

identified by Bio-Imaging Analyzer BAS-1800 II. PVDF membranes were subjected to Western blotting with appropriate antibodies or to phosphoamino acid analysis.

3.12. Preparation of basolateral membranes and endosomes from HRTC

Cells were pre-incubated for 30 min either with 0.2% DMSO or 20 mM PD98059. After pre-exposure to inhibitors, cells were incubated with C-peptide (5 nM) and scrambled C-peptide (5 nM) for 15 min. After treatment, cells were washed twice with ice-cold PBS, and harvested by scraping into 500 µl ice-cold buffer (12 mM HEPES, 300 mM mannitol, 1 M Tris, pH 7.6, 0.5 mM Na_3VO_4, 0.2 mM PMSF, 5 mg/ml leupeptin, 5 mg /ml aprotinin, and 1 mM microcystin). Cells were homogenized by pellet pestle twice for 1 min, and passaged through an insulin syringe with a 21G needle 10–12 times into an eppendorf tube. Homogenates were pre-cleaned by centrifugation at 2,500 g for 15 min. Supernatants were collected. Pellets were re-suspended in 500 µl lysis buffer, and spun down at 2,500 g for 15 min. Supernatants were collected and transferred to the tubes, containing the first 500 µl, and centrifuged at 20,000 g for 20 min, $+4^0C$ on a TLA 100.2 rotor. Basolateral membranes (BLMs) were further purified (Hammond et al., 1994), using Percoll gradient. The yellow layer of the pellet was re-suspended again in the supernatant (carefully removed from the brown pellet containing mitochondria and cell ghosts) and centrifuged at 48,000 g for 30 min. Supernatant was discarded, and pellet was re-suspended in 1 ml buffer (300 mM mannitol and 12 mM HEPES, pH 7.6, adjusted with Tris) by gentle pipetting. To form a percoll gradient, 0.19 g undiluted percoll (Pharmacia Biotech) was added to 1 ml suspension (0.8–1.0 mg of protein). Suspension was gently mixed and centrifuged at 48,000 g for 30 min, and the ring of BLMs, light endosomal fraction (the top one-third of the tube), and pellets were collected and frozen at -20^0C. Protein content was determined in parallel using the BCA protein assay and samples, containing BLMs and light endosomes (10 µg protein) were resolved on SDS-PAGE.

3.13. Western blot analysis

HSMC or HRTCs or parts of frozen skeletal muscle samples after pulverization in liquid N_2 were lysed in 500 or 300 µl of lysis buffer A on the rotation wheel at 4^0C during 1 h. Then samples were centrifuged at 12 000 g for 10 min, 4^0C. Supernatants were collected and protein concentration was measured, using BCA^{TM} protein assay kit or Bradford reagent. Aliquots of cell or muscle lysates (30 µg of protein), fractions of BLMs and light endosomes (10 µg protein), or immunoprecipitates were re-suspended in Laemmli sample buffer. Proteins were separated by SDS-PAGE (7.5 or 10% resolving gel), transferred to PVDF membranes and blocked with 7.5% non-fat milk in TBS-T. Membranes were incubated with primary antibodies overnight at 4^0C on a shaking platform. Thereafter, membranes were washed with TBS-T and incubated with anti-rabbit or anti-mouse secondary antibody or protein A conjugate with HRP. Proteins were visualized by ECL and quantified by densitometry.

3.14. Phosphoamino acid and phosphopeptide analysis

Phosphorylated α-subunits were immunoprecipitated, resolved by SDS-PAGE, transferred to PVDF membranes and the ^{32}P-labeled Na^+,K^+-ATPase α-subunits were identified on the membrane by Bio-Imaging Analyzer BAS-1800 II and excised. Thereafter, phosphorylated α-subunits were trypsinized, hydrolyzed in 6 M HCl and

analyzed by two-dimensional high voltage electrophoresis on cellulose thin layer plates. Phosphoamino acid analysis was performed as described by Boyle et al. (Boyle et al., 1991). Phosphoamino acids, on thin-layer electrophoresis plates, were analyzed using the Bio-Imaging Analyzer BAS-1800 II.

3.15. Glucose incorporation into glycogen in human skeletal muscle cells

Myoblasts were seeded 1500 cells/well in 6 well plates and differentiated at 70-80 % confluence. Differentiated myotubes (5-7 days of differentiation) were serum starved (DMEM + 0% FBS) overnight prior the experiment to reduce the basal level of insulin- and cytokine-dependent kinase activity. Cells were pre-incubated with 1) 22 μM PD98059, 20 μM PP2, 10 μM LY294002, or DMSO for 20 min, then incubated with 100 nM ouabain for 10 min and finally stimulated with 120 nM insulin for 20 min at 37°C in 950 μl serum free DMEM; or 2) cells were incubated with ouabain or marinobufagenin for 30 min; or 3) cells were pre-incubated with 22 μM PD98059, 20 μM PP2, 10 μM LY294002, or DMSO for 20 min, then ouabain, marinobufagenin, insulin or DMSO were added. Thereafter, 50 μl of the isotope solution (D-[U-^{14}C]-glucose with 1 μCi/ml; final specific activity, 0.18 μCi/μmol in DMEM) was added and plates were incubated for 30 min. Reactions were terminated by placing the plates on ice. Media was aspirated and wells were washed with ice cold PBS 3-4 times. Plates were frozen directly after at -80^{0}C or myotubes were solubilized with 1 ml 0.03% SDS for 1 h at room temperature. Aliquots (0.85 ml) of the suspension was transferred to 10 ml tubes and 100 μl (2 mg) of carrier glycogen was added. The remained suspension was used for protein concentration determination. Samples were boiled on a water bath for 30 min. A 3 ml solution of 98% ethanol was added to precipitate glycogen. Samples were incubated overnight at 4^{0}C with slight agitation and centrifuged at 5000 g for 35 min at 4^{0}C. Pellet was washed once with 70% ethanol, samples were centrifuged at 5000 g for 10 min, and ethanol was aspirated off. Pellet was solubilized in 200 μl dH$_2$O and transferred to 4 ml scintillation vials. Samples and aliquots of the media were counted on the liquid scintillation counter (1214 Rackbeta, Wallac, Turku, Finland).

3.16. p90 rsk activity assay

Cultured myotubes, treated with 100 nM ouabain for 2 hours, or rat soleus muscles, incubated with 200 μM ouabain, in the presence or absence of inhibitors PD98059 and PP2, were analyzed. Myotube lysates (100 μg protein/sample) or muscle lysates (250 μg protein/ sample) were immunoprecipitated at 4^{0}C overnight with anti-p90 rsk antibody, previously equilibrated with protein G-sepharose in lysis buffer A. Immunoprecipitates were washed 3 times in lysis buffer A, containing 0.5 M NaCl and twice in buffer B (50 mM Tris-HCl, pH 7.5, 0.03% Brij-35, 0.1 mM EGTA, 0.1% β-mercaptoethanol). Samples were re-suspended in 30 μl of kinase buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 17 μM cAMP-dependent protein kinase inhibitor peptide, 16.7 mM Mg(Ac)$_2$, 50 μM crosstide, and 2 μCi of [γ-^{32}Pi]ATP) and incubated at 30^{0}C for 10 min. Reactions were terminated on ice by addition of sample buffer (125 mM Tris, 6 M urea, pH 6.8). Reaction products were resolved on 40% acrylamide gel, and ^{32}Pi incorporation into peptide substrate was analyzed by exposing gels on phosphoimager (Fuji BAS-1800II).

For ouabain signaling study, paper III, additional methods were used:

3.17. Glucose transport

All incubation media were prepared from a pre-gassed (95% O_2/5% CO_2) stock of Krebs-Henseleit bicarbonate buffer (KHB), supplemented with 5 mM HEPES and 0.1% RIA grade bovine serum albumin (fraction V, RIA grade, Sigma). Pre-incubation media contained 5 mM glucose and 15 mM mannitol. Rat soleus muscles were pre-incubated in the presence or absence of insulin (0.6 nM or 12 nM) and ouabain (200 µM) for 40 min, then rinsed in the media, containing 20 mM mannitol for 10 min and kept in the hot media with 8 mM 3-O-methylglucose with 438 µCi/mmol [^{3}H]-3-O-methylglucose for 12 min. Muscles were incubated in 2 ml of media on a shaking water bath at 30^0C. The gas phase in the incubation vial was maintained at 95% O_2/5% CO_2. After incubation in the hot media muscles were frozen in liquid N_2 and then processed as described previously (Wallberg-Henriksson et al., 1987). When present, insulin and ouabain were maintained throughout the experiment.

3.18. Photolabelling of cell-surface glucose transporters

Total cell-surface glucose transporters labeling was performed using biotinylated ATB-BMPA {4,4'-O-[2-[2-[2-[2-[2-[6-(biotinyl-amino)hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2-trifluoro-ethyl) benzoyl]amino-1,3-propanediyl bis-D-mannose}. The utility of this compound has been described previously (Koumanov et al., 1998). Following 40 min of pre-incubation as described for glucose transport, soleus muscles were transferred to a dark room and incubated for 8 min at 18^0 C in the media containing 1 mCi/ml ATB-[2-^{3}H] BMPA. Muscles were irradiated with UV light for 2 x 3 min. Thereafter, muscles were blotted, trimmed free of connective tissue and frozen in liquid N_2. Muscles were processed and samples were analyzed for the determination of cell-surface glucose transporters. Thereafter, proteins were separated by SDS-PAGE and photolabelled glucose transporters were visualized using antibodies to GLUT1 or GLUT4.

3.19. Glucose oxidation

Incubation media were prepared from a pre-gassed (95% O_2/5% CO_2) stock of Krebs-Henseleit bicarbonate buffer (KHB) (Krebs et al., 1932) supplemented with 5 mM HEPES and 0.1% RIA grade bovine serum albumin (fraction V, RIA grade, Sigma). Incubation media contained 5 mM glucose, 15 mM mannitol and 8 mM [U-^{14}C]-glucose (0.2µCi/ml). Rats were anesthetized with 60 mg/kg body weight of sodium pentobarbital. Soleus muscles were dissected for *in vitro* incubation at 30^0 C in the presence or absence of insulin (12 nM), ouabain (200 µM), MEK1/2 inhibitor PD98059 (22 µM) or c-Src inhibitor PP2 (20 µM) for 1 h in a sealed flask with a rubber stopper containing a hanging center-well. When present, insulin, ouabain and kinase inhibitors were maintained throughout the experiment. Glucose oxidation and glucose incorporation to glycogen were assesses as described (Barnes et al., 2005). After1 h muscles were frozen in liquid N_2 for further analysis of [U-^{14}C]-glucose incorporation into glycogen and vials were resealed immediately. 0.2 ml of solvent NEF-910 G was injected into the central-well and 0.5 ml of 15% PCA was injected into the media. Vials were incubated for an additional 1 hour. The central-well was removed and placed into the scintillation vial, containing 4 ml of scintillation fluid and 100 µl of 5 N HCl. Vials were covered with lids, and placed in the

cool dark room for 1 h. Samples were counted for [14C] in the β-counter. Glucose oxidation rate was expressed as nmol/g/h.

3.20. Glucose incorporation into glycogen

Muscles, frozen in glucose oxidation experiment, were weighed and dissolved in 0.5 ml of 1 N NaOH at 70^0 C for 30 min in thermomixer. 0.5 ml of 20% TCA was added and samples were vortexed and centrifuged at 3500 g at 10^0C for 15 min. Supernatant was transferred to the new tube, and 200 µl of glycogen solution (100 mg of glycogen in 5 ml of distilled water) and 2 ml of 95% ethanol were added. Each tube was vortexed and placed at -20 ^{0} C for at least 1 h or until the contents was fully dissolved, then centrifuged at 2000 g for 15 min. The supernatant was discarded and 0.7 ml of distilled water was added into the tube to dissolve the pellet. Aliquots (500 µl) were put into scintillation vials and counted in the β-counter. Glucose incorporation into glycogen was expressed as nmol/g/h.

3.21. Adenine nucleotides measurements

Freeze-dried muscle samples were dissected free from connective tissue, blood and fat and then weighed (around 1.5 mg) and extracted in 1.5 M PCA before being neutralized with $KHCO_3$. Concentrations of ATP, ADP, AMP and IMP in the samples as well as in external standards were analyzed with HPLC using a reverse phase column (Hypersil Elite, C18, 250 x 4.6mm, 5um), as described (Sellevold et al., 1986). Separation of nucleotides was achieved with a flow rate of 1.0 ml/min, UV-detection at 254 nm and an oven temperature of 40^0C.

3.22. High-fat diet and exercise training protocol (unpublished study)

Female Wistar rats were divided into 4 groups: sedentary/standard rodent chow fed group (CS), sedentary/high fat diet (55% of calories from fat) fed group (FS), trained/standard rodent chow fed group (CT), trained/ high fat diet fed group (FT). Animals were kept on different diets for 4 and 12 weeks. After 3 or 11 weeks of feeding the CT and FT groups were trained for 5 days. Rats were acclimated to swimming for 10 min per day for 2 days. The swimming protocol was a modification of a previously published procedure (Ren et al., 1994). Rats swam in groups of six, in plastic barrels 45 cm in diameter, filled to a depth of ≈50 cm. Water temperature was maintained was 34–35°C. Animals performed two 3-h exercise bouts, separated by one 45-min rest period. After the last exercise bout, animals were fed ad libitum. Approximately 16 h after the last exercise bout, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight) and epitrochlearis and hindlimb muscles were removed.

3.23. Subcellular fractionation of rat skeletal muscle membranes

Overnight-fasted male Wistar rats (120–130 g) were anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (5 mg/100 g body weight). Animals were injected with either insulin (0.75 U of insulin/100 g body weight, i.p.) or an equal volume of saline (control group) 30 min prior to being sacrificed. Hindlimb muscles were removed and frozen in liquid nitrogen. Cell surface and intracellular membrane (IM) fractions were isolated from 350 mg of gastrocnemius muscle by differential centrifugation and discontinuous sucrose gradients (Chibalin et al., 2001; Hundal et al., 1992). After

centrifugation of the discontinuous sucrose gradients three protein fractions were collected: fraction 25 (on top of the 25% layer), fraction 30 (from the interphase 25–30%), and fraction 35 (from the interphase 30–35%). Protein concentration was determined using BCA assay (Pierce, Rockford, IL, USA). Fraction 25 corresponds to a cell surface membrane fraction enriched with PM, whereas fraction 35 corresponds to IMs (Hundal et al., 1992; Klip et al., 1987; Lavoie et al., 1996). Protein recovery yield (calculated as percent of protein content in crude membranes in five membrane preparations) was 3.4±0.3% for PM and 9.8±0.8% for IM fraction, respectively. Protein recovery was not affected by insulin.

3.24. In vitro muscle contraction (unpublished study)

Following 20 min pre-incubation in the presence or absence of MEK1/2 inhibitor PD98059 (20 μM), rat epitrochlearis muscles were placed inside a temperature controlled (30^0 C) stimulation chamber, and immersed in 4 ml of KHB identical to pre-incubation condition. Each muscle was positioned between two platinum electrodes with the distal tendon mounted to the bottom of the chamber. The proximal tendon was connected to a jeweler's chain, which was fixed to an isometric force transducer (Harvard Apparatus Inc., South Natick, MA, USA). Resting tension was adjusted to 0.5 g. Isometric tension development during the contraction protocol was recorded using a compact 2-Channel Student Oscillograph (Harvard Apparatus Inc.). Isometric muscle contraction was achieved via electrical stimulation. Muscles were stimulated at a frequency of 100 Hz (0.2 ms pulse duration, 10 V amplitude), delivered at a rate of one 0.2 s contraction every 2 s (0.2 s / 2s) for 10 min. The pulses were generated by a Tektronix TM 503 Power Module (Beaverton, OR, USA) and amplified on a 4-Channel Power Amplifier (Somedic Inc., Sollentuna, Sweden). Basal muscles were treated as described above minus electrical stimulation. Thereafter, muscles were subjected to cell surface biotinylation procedure, (the protocol is described earlier), and then frozen in liquid N_2.

3.25. Statistics

Data were presented as mean ± SE. Comparisons between groups were performed using Student's t-test. Significance was established at $p < 0.05$.

4   RESULTS AND DISCUSSION

4.1. Regulation of Na$^+$, K$^+$-ATPase by ERK1/2

4.1.1. Insulin effects on Na$^+$,K$^+$-ATPase in human skeletal muscle cells (paper I)

Insulin stimulates Na$^+$,K$^+$-ATPase activity and induces the translocation of Na$^+$,K$^+$-ATPase molecules to the plasma membrane in skeletal muscle. We determined the molecular mechanism by which insulin regulates Na$^+$,K$^+$-ATPase in differentiated primary human skeletal muscle cells (HSMCs).

Insulin increased ouabain-sensitive ^{86}Rb$^+$ uptake and [^{3}H]-ouabain binding in intact cells (paper I, Fig. 1 A and B). These effects were completely abolished by 20 μM PD98059, a specific MEK1/2 inhibitor, 10μM GF109203X, a PKC inhibitor, and 100 nM wortmannin, a PI 3-kinase inhibitor. Importantly, a lower concentration of GF109203X (1 μM), in the range known to inhibit conventional and novel PKC isoforms, but not atypical isoforms (Martiny-Baron et al., 1993), was ineffective in altering insulin stimulation of ^{86}Rb$^+$ uptake and [^{3}H]-ouabain binding. These data suggest the involvement of the MAPK signaling pathway, PI3-kinase, and the classical and novel PKC isoforms as signal transducers that mediate insulin-stimulatory effects on Na$^+$,K$^+$-ATPase activity.

Insulin increased the plasma membrane content of the Na$^+$,K$^+$-ATPase α$_1$- and α$_2$-subunits (paper I, Fig. 4 A and B), as well as phosphorylation of the α-subunits, as assessed by metabolic labeling of myotubes with ^{32}P$_i$ (paper I, Fig. 5 A). These effects were abolished by exposure of muscle to 20 μM PD98059, 10μM GF109203X, and 100 nM wortmannin. Notably, insulin-stimulated ERK1/2 phosphorylation was abolished when HSMCs were incubated in the presence of wortmannin and 10 μM GF109203X (paper I, Fig. 2). The involvement of PI 3-kinase and PKC in the regulation of insulin-stimulated translocation of rat Na$^+$,K$^+$-ATPase α$_1$-subunits in α$_1$-overexpressing HEK-293 cells was shown earlier by Sweeney et al. (Sweeney et al., 2001a). PKC-dependent phosphorylation of the Na-pump in response to insulin in rat skeletal muscle was also shown earlier (Chibalin et al., 2001), but the role of different PKC isoforms was unspecified. Sweeney et al. demonstrated involvement of PI 3-kinase and PKC ζ in insulin stimulation of K$^+$ uptake in 3T3 L1 fibroblasts (Sweeney et al., 1998b). MAPK-dependent phosphorylation of the α-subunits has not been yet identified as a mechanism to mediate insulin-action on Na$^+$,K$^+$-ATPase activity in skeletal muscle.

Phosphoamino acid analysis revealed that insulin stimulated the phosphorylation of the α-subunits on serine and threonine residues, and induced phosphorylation on tyrosine residues. In the presence of the MEK1/2 inhibitor PD98059, phosphorylation of serine and threonine residues was markedly decreased (paper I, Fig. 5B) and phosphorylation of tyrosine residues was unaffected.

These data suggest that in human skeletal muscle cells, insulin-stimulated Na$^+$,K$^+$-ATPase activation, phosphorylation, and translocation of the α-subunits to the plasma membrane are ERK1/2- dependent. ERK1/2 MAP kinase is involved in Na$^+$,K$^+$-ATPase α-subunit phosphorylation primarily on threonine and serine residues. In contrast, insulin-induced tyrosine phosphorylation of the Na$^+$,K$^+$-ATPase α-subunit is MAP kinase independent.

A phosphorylation motif scan of the human α_1-subunit was performed using the Scansite 2.0 program (Yaffe et al., 2001). The sequence analysis of the Na^+,K^+-ATPase α_1-subunit, performed at a high stringency level, revealed three phosphorylation sites: Ser^{943} as possible site for cAMP-dependent protein kinase (PKA), Thr^{81} as possible site for ERK, and Ser^{491} as possible site for calmodulin-dependent kinase II (CaMKII). The Scan of the human α_2-subunit protein sequence suggested Ser^{936} (homologue for Ser^{943} in α_1-subunit) as possible site for PKA, Thr^{79} as possible site for ERK and Thr^{414} as possible site for Akt/PKB kinase. Sequence scans of the α_1- and α_2-subunits performed at medium and low stringency levels predicted Thr^{85}, Thr^{86}, Thr^{226}, Ser^{228}, Thr^{282} and Thr^{788} as possible ERK phosphorylation sites in human α_1-subunit, and homologues aminoacid residues Thr^{83}, Thr^{84}, Thr^{224}, Ser^{226} and Thr^{781} as possible ERK phosphorylation sites in α_2-subunit, respectively. Importantly, the predicted ERK phosphorylation sites are conserved between human, rat, mouse and chicken.

In our study, insulin stimulated phosphorylation of the α_1- and α_2-subunits of Na-pump on Thr-Pro amino acid motifs, which form specific ERK substrates (paper I, Fig. 6 A-C). Furthermore, recombinant ERK1 and -2 kinases were able to phosphorylate the α-subunit of purified human kidney Na^+,K^+-ATPase *in vitro*, mainly on threonine and less on serine amino acid residues (paper I, Fig. 7 A-C). Phosphorylation was enhanced in the presence of the non-ionic detergent Triton X-100. Addition of the detergent to the buffer may increase accessibility of Na^+,K^+-ATPase amino acid residues to phosphorylation by ERK1/2. Data obtained by amino acid sequence analysis of phosphopeptides indicates that Ser^{228} is one of the amino acids that are phosphorylated by ERK in the α_1-subunits *in vitro*. However, the N-terminal part of the Na^+, K^+-ATPase α-subunit was proteolized to small peptides that were unidentifiable by mass spectrometry. Thus, we were unable to confirm phosphorylation of Thr^{81} and Thr^{85}, or Thr^{86}, experimentally.

In conclusion, in skeletal muscle cells, insulin stimulates Na^+,K^+-ATPase activity via phosphorylation of the catalytic α-subunits by ERK1/2 MAPK-dependent mechanism and translocation of the α-subunits to plasma membrane.

In order to further delineate the mechanism for MAP kinase- dependent activation of the sodium pump, we investigated the further involvement of ERK signaling cascade in the regulation of Na^+,K^+-ATPase in response to different stimuli and in different tissues.

4.1.2. Connecting peptide, C-peptide, effects on Na^+,K^+-ATPase in human renal tubular cells (paper II)

Proinsulin connecting peptide (C-peptide) is known to stimulate Na^+,K^+-ATPase activity (Ohtomo et al., 1996). C-peptide is also a potent activator of ERK1/2 MAP kinase (Kitamura et al., 2003; Zhong et al., 2005). We determined the molecular mechanism by which C-peptide stimulates Na^+,K^+-ATPase in primary human renal tubular cells (HRTC). Incubation of HRTCs with 5 nM human C-peptide at 37^0C for 10 min stimulated $^{86}Rb^+$ uptake by 40 % (p<0.01). C-peptide stimulation of $^{86}Rb^+$ uptake was abolished by pre-incubation of HRTC with pertussis toxin (PTX), an inhibitor of G_i protein activation, as well as by the MEK 1/2 inhibitor PD 98059, and the PKC inhibitor GF109203X (1 μM) (paper II, Fig. 1D). These data implicate activation of G-protein coupled receptor, as

well as PKC- and MAPK-dependent pathways in the regulation of C-peptide stimulation of Na^+,K^+-ATPase.

In parallel with ouabain-sensitive $^{86}Rb^+$ uptake, C-peptide increased Na^+,K^+-ATPase α-subunit phosphorylation, as assessed by metabolic labeling of cells with $^{32}P_i$ and this effect was abolished in the presence of PD 98059 (paper II, Fig. 2A). Sequence analysis of the Na^+,K^+-ATPase α-subunits revealed several potential ERK phosphorylation sites (paper II, Fig. 2D). C-peptide stimulated phosphorylation of the human Na^+,K^+-ATPase α_1-subunit on Thr-Pro amino acid motif, which forms a specific ERK substrate (paper II, Fig. 2B). Phosphoamino acid analysis revealed that C-peptide, similarly to insulin, stimulated phosphorylation of the α-subunits mostly on threonine residues and less on serine, though tyrosine phosphorylation was unaffected (paper II, Fig. 2C). In the presence of the MEK1/2 inhibitor, PD98059 phosphorylation of threonine and serine residues was markedly decreased (paper II, Fig. 2C). Thus, we confirmed the role of ERK1/2 MAPK in Na-pump activation and phosphorylation of the α-subunit.
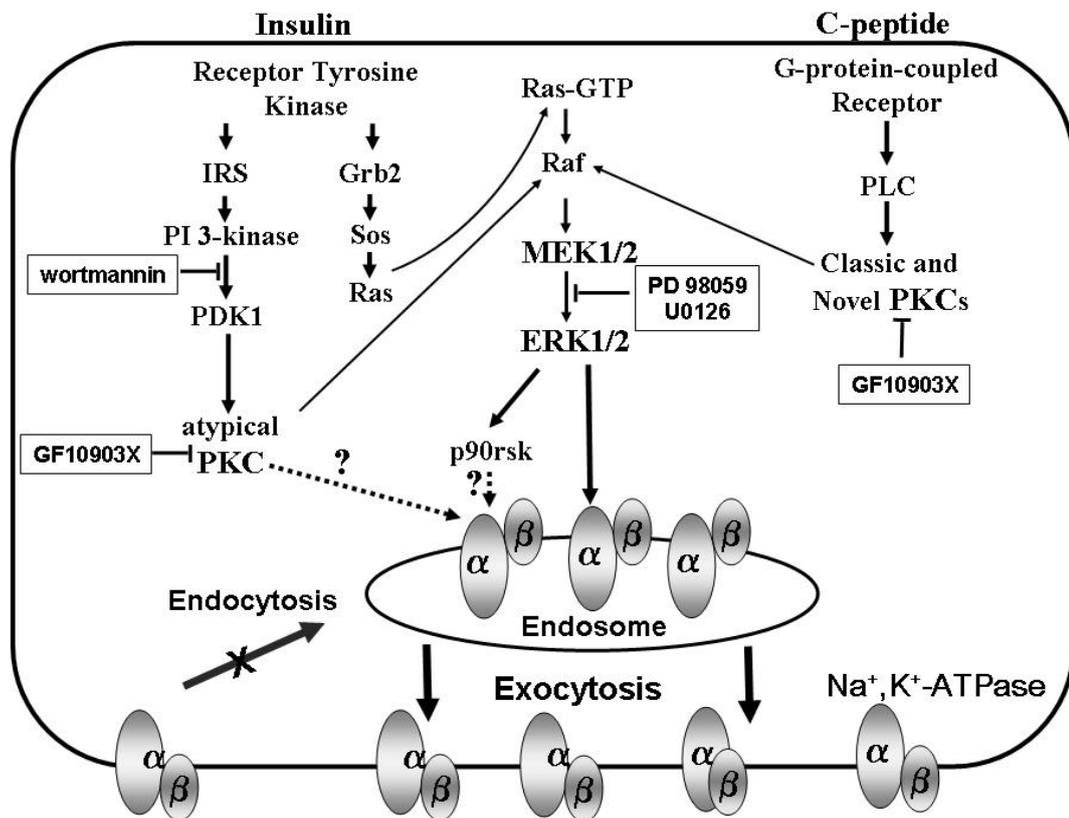
Figure 4. Proposed scheme of insulin and C-peptide signaling leading to activation of Na^+,K^+-ATPase.

Since renal tubular cells are polarized, ion transport is directed from apical to basolateral side of the cells. Na^+,K^+-ATPase is mostly expressed on basolateral membrane (BLMs) (Feraille et al., 2001). Activity of Na-pump on BLM of kidney cells is known to be regulated by endo- and exocytosis (Chibalin et al., 1998b; Chibalin et al., 1999; Feraille et al., 2001). We therefore analyzed the abundance of Na^+,K^+-ATPase α_1- and β_1-subunits in HRTCs basolateral membranes. The increase in BLM abundance of Na^+,K^+-ATPase α_1- and β_1-subunits was accompanied by a depletion of α_1- and β_1-

subunits from cellular endosomal compartments (paper II, Fig. 3 A-D). This effect of C-peptide on Na-pump subunits translocation to BLM was abolished in the presence of the MEK 1/2 inhibitor, PD98059.

Thus, C-peptide stimulation of Na^+,K^+-ATPase activity is dependent on ERK1/2 in human renal tubular cells. Our results indicate that C-peptide signaling leads to the stimulation of Na-pump activity, phoshorylation of the α-subunits on Thr residues and translocation to BLM via MAP kinase-dependent pathway. ERK1/2 is essential for the effect of C-peptide on Na^+,K^+-ATPase activation (Fig. 4). In the broad perspective, these data provide evidence for the involvement of ERK1/2 MAPK as a universal trigger of Na^+,K^+-ATPase activation in response to different stimuli and in the different mammalian tissues.

4.1.3. Effect of exercise and in vitro contraction on translocation of the $α_1$- and $α_2$-subunits of Na^+, K^+-ATPase to the plasma membrane of rat epitrochlearis muscle

Acute exercise increases the plasma membrane content of the $α_1$- and $α_2$- subunits of Na^+,K^+-ATPase, as well as the $α_1$- and $β_2$- mRNA in rat skeletal muscle (Tsakiridis et al., 1996). The effect of exercise on Na^+,K^+-ATPase activity was mostly exhibited in oxidative fibers. The molecular mechanism for the Na-pump regulation in response to exercise requires further elucidation. The aim of this study was to examine the mechanism of translocation of the Na^+,K^+-ATPase α-subunits to plasma membrane in response to exercise.

Figure 5. Changes in the abundance of the $α_1$- and $α_2$-subunits of Na^+,K^+-ATPase in response to insulin and exercise in membrane fractions isolated from rat gastrocnemius muscle. A. and C. Plasma membranes. B. and D. Intracellular membranes. Representative Western blots (upper panel) and quantitative data from 5 experiments are shown. Values are mean ± S.E, * p<0.05 insulin-stimulated vs basal value, † p<0.05 exercise-induced vs basal.
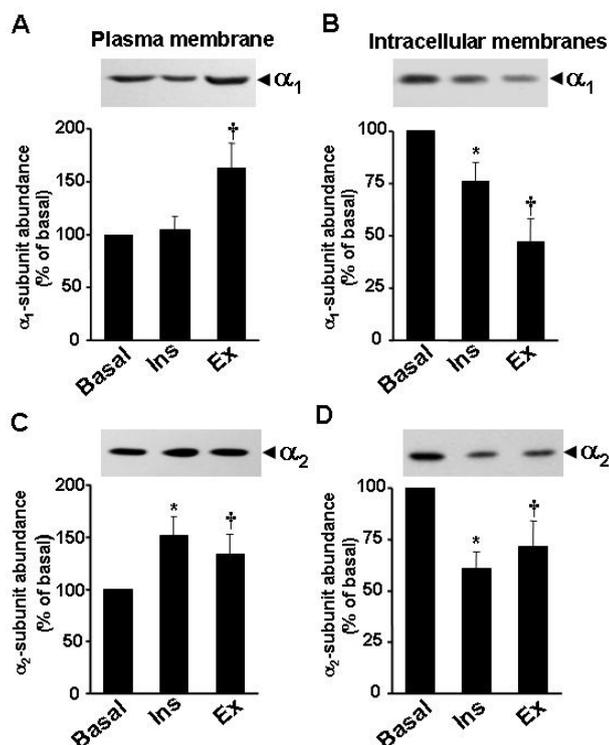
Wistar rats were subjected to 1 h swimming exercise or i.p. injection of insulin (7.5 U per kg). After the exercise, gastrocnemius muscles were isolated, homogenized and subjected to subcellular fractionation. SDS-PAGE and Western Blot analysis was used to assess the abundance of the Na-pump α-subunits on plasma membrane. In response to

exercise, an increase in the abundance of α_1- and α_2-subunits to plasma membrane was observed, while only the abundance of the α_2-subunit was increased in response to insulin. Insulin and exercise was associated with the depletion of α_1- and α_2-subunits from the intracellular membrane fraction (Fig. 5). Earlier in our laboratory we detected insulin-induced translocation of the α_2-subunit to plasma membrane using method of subcellular fractionation, and translocation of α_1- and α_2- subunits using method of cell surface biotinylation (Al-Khalili et al., 2003b). Taken together, our results provide evidence to suggest that exercise and insulin may cause a translocation of sodium pump to different plasma membrane subdomains that can be differentially recovered during subcellular fractionation.
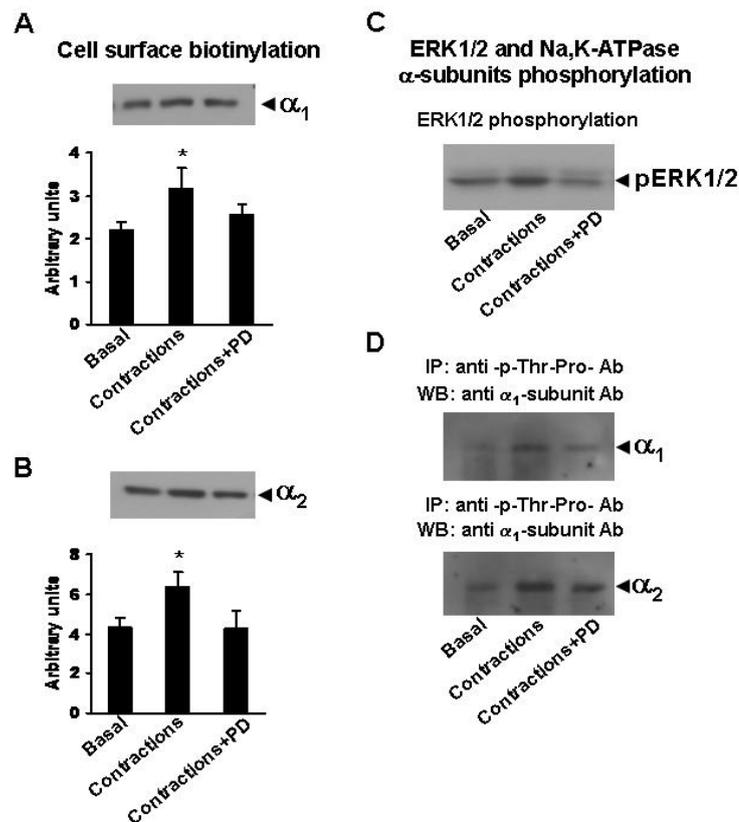
Figure 6. Translocation of Na$^+$,K$^+$-ATPase α_1- and α_2-subunits to cell surface in response to muscle contraction. Wistar rat epitrochlearis muscles were subjected to in vitro contraction, and thereafter pre-incubated in KRB or KRB, containing 20 μM PD98059. Cell surface Na$^+$,K$^+$-ATPase α-subunits abundance was determined by biotinylation with EZ-link Sulfo-NHS-SS-biotin and streptavidin-precipitation as described in "Material and Methods". (A, B). Na$^+$,K$^+$-ATPase α_1- and α_2-subunits cell surface abundance. Representative Western blots (upper panel) and quantitative data from 4 experiments (mean ± SE) (lower panel) are shown. *p<0.05 vs. basal. (C) ERK 1/2 and (D) Na$^+$,K$^+$-ATPase α-subunits phosphorylation in response to contraction, in the presence or absence of PD98059. After *in vitro* contraction in the presence or absence of PD98059 muscles were homogenized and subjected to immunoprecipitation with antibodies to p-Thr-Pro motif, with subsequent Western Blot analysis. Representative blots of ERK1/2 phosphorylation and α_1- and α_2-subunits phosphorylated on Thr-Pro motif from 4 independent experiments are shown.

We have also determined the effect of *in vitro* contraction in rat epitrochlearis muscles, caused by electrical stimulation of the muscle, on Na^+,K^+-ATPase α-subunit translocation to plasma membrane. Contraction caused a significant increase in the plasma membrane abundance of Na^+,K^+-ATPase α_1- and α_2-subunits (Fig. 6 A and B), as assessed using the cell surface biotinylation method. This effect was completely abolished by the MEK 1/2 inhibitor, PD98059 (20 µM). Contraction stimulated ERK1/2 phosphorylation as well, and this effect was abolished by PD98059 (Fig. 6 C). Immunoprecipitation of muscle lysates with antibodies to p-Thr-Pro motif, specific ERK phosphorylation site, with subsequent Western Blot analysis for α_1- and α_2-subunits of Na-pump revealed that contraction stimulated phosphorylation of α_1- and α_2-subunits on Thr-Pro amino acid motifs, which was diminished in the presence of PD98059 (Fig. 6 D).

Overall, our results provide evidence to suggest that activation of MAPK signaling pathway constitutes a universally important mechanism of regulation and stimulation of the Na^+,K^+-ATPase.

4.1.4. Binding of adaptor protein 2 and cofilin to Na^+,K^+-ATPase α-subunit in human skeletal muscle cells is MAPK- dependent

Detailed molecular mechanisms of ERK1/2-dependent regulation of Na^+,K^+-ATPase are unclear. Recently, an ERK- and PKC- dependent phosphorylation of rat Na^+,K^+-ATPase α_1- subunit at Ser^{16} residue site has been implicated in clathrin-mediated endocytosis of Na- pump in response to parathyroid hormone (PTH) in opossum kidney (OK) cells, transfected with rat α_1-subunit of Na-pump (Khundmiri et al., 2004). However, the authors did not investigate ERK-dependent Thr phosphorylation of the pump subunits. In our studies, insulin and C-peptide, as well as acute exercise and *in vitro* muscle contraction, was associated with an increase in Na^+,K^+-ATPase activity through a mechanism involving α-subunit phosphorylation on specific -Thr-Pro rich motifs, and an increased cell surface abundance of α-subunits. Prolin-rich motifs have been shown to be involved in the regulation of receptor-mediated endocytosis of Na^+,K^+-ATPase (Yudowski et al., 2000). PI 3-kinase binding to Na-pump is essential, which in turn is necessary for recruitment of adaptor protein 2 and clathrin molecules, formation of clathrin-coated vesicles, and endocytosis of the whole complex. Conversely, PI 3-kinase is involved in actin cytoskeleton remodeling (Fraley et al., 2005; Torok et al., 2004). We therefore, investigated whether binding of Na^+,K^+-ATPase to adaptor protein 2, as well as to cofilin, ubiquitous actin-binding factor (Huang et al., 2006; Paavilainen et al., 2004), occurs in response to insulin, in the presence or absence of MEK1/2 inhibitor PD98059 in human skeletal muscle cells (Fig. 7).

Our data indicate that Na-pump interaction with adaptor protein 2 is decreased in response to insulin, and this effect is abolished in the presence of PD98059 (Fig. 7 A). Insulin had opposite effect on binding of cofilin to Na-pump. Cofilin binding was increased in response to insulin, while PD98059 abolished this effect (Fig. 7 B).

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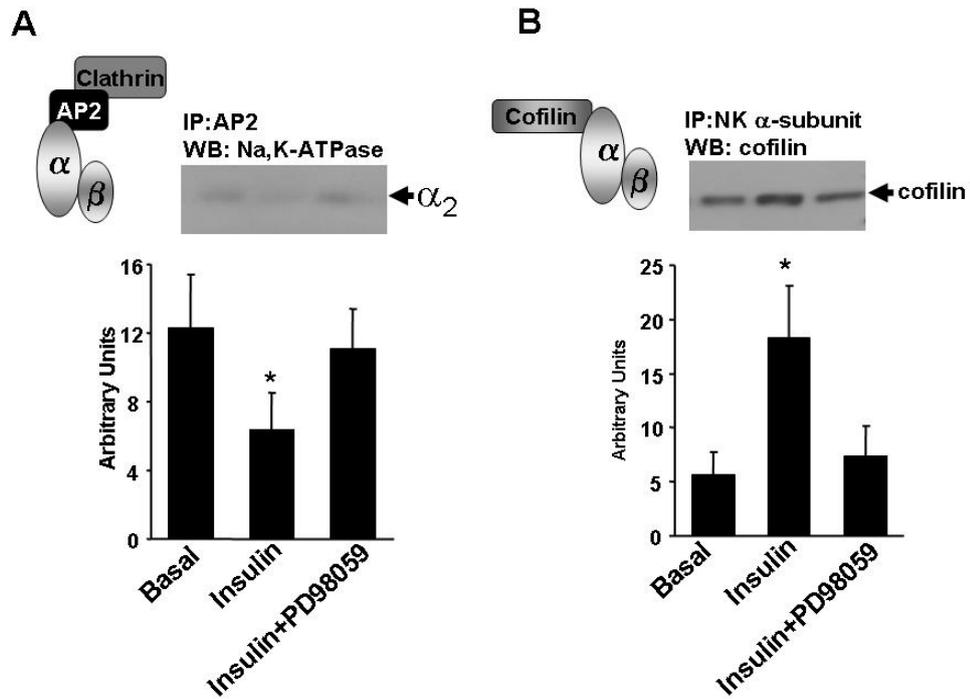
Figure 7. Binding of the adaptor protein 2 and cofilin to the Na,K-ATPase α-subunit in human skeletal muscle cells is MAPK-dependent. Human differentiated myotubes were incubated with or without 120 nM insulin in the presence or absence of 20 µM PD98059. Myotubes were lysed, and equal amounts (700 µg) of protein were immunoprecipitated with either (A) anti-AP2 antibody, or (B) anti-NK1 antibody with subsequent Western Blot analysis. Representative blots of Na^+,K^+-ATPase α_2-subunit (A) and cofilin (B) from 3 independent experiments are shown.
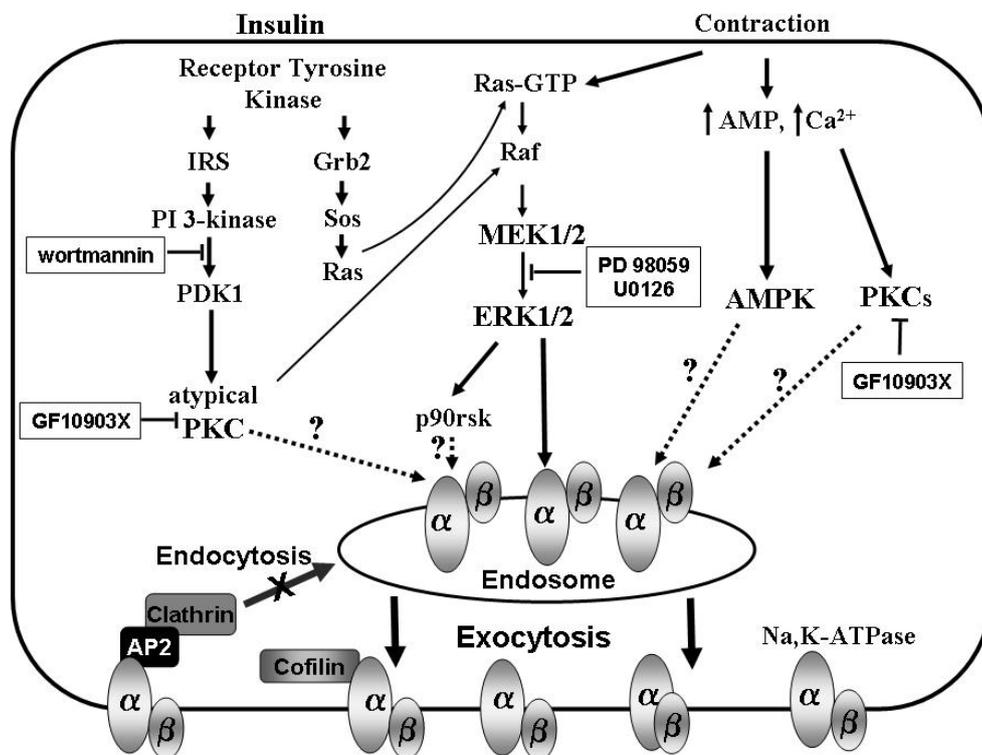

Figure 8. Proposed scheme of insulin- and contraction-induced signaling in skeletal muscle, leading to activation of Na^+,K^+-ATPase.

Therefore, we show that in human skeletal muscle cells, MAPK pathway is involved in the regulation of molecular trafficking of Na^+,K^+-ATPase (Fig. 8). We hypothesize that phosphorylation of Na-pump α-subunits by ERK arrests the formation of an endocytotic complex, due to decreased interaction of Na-pump with AP2, while the process of exocytosis is maintained and may even be enhanced, since interaction with cofilin is increased. As a direct consequence, the plasma membrane α-subunits abundance increases in response to insulin.

4.2. Changes in expression of Na^+,K^+-ATPase in rat skeletal muscle: effects of exercise and high fat diet

The aim of this study was to examine Na^+,K^+-ATPase subunit expression and insulin- stimulated translocation in skeletal muscle after high-fat diet (HFD) and short-term moderate exercise training (ET). Animals receiving high fat diet (55% of calories coming from fat) serve as the model of insulin resistance. The expression of Na^+,K^+-ATPase isoforms was determined in skeletal muscle from Wistar rats after 4 or 12 weeks chow diet (CHD) or HFD, before and after 5 days of swimming ET. After 4 weeks of HFD, Na^+,K^+-ATPase α_1-subunit protein expression increased 1.6 fold (p<0.05), whereas α_2-subunit decreased 2-fold (p<0.01) in *gastrocnemius* muscles. α-subunits expression was restored to control level after ET. β_1-subunit protein expression decreased 2.2 fold (p<0.01) in muscles from HFD rats and was restored after ET. β_2-subunit expression was unaltered by HFD, but expression decreased 2 fold (p<0.05) after ET regardless of diet (Fig. 9 A-D). Changes in Na^+,K^+-ATPase subunit expression were persistent after 12 weeks of HFD, when muscle insulin resistance developed.
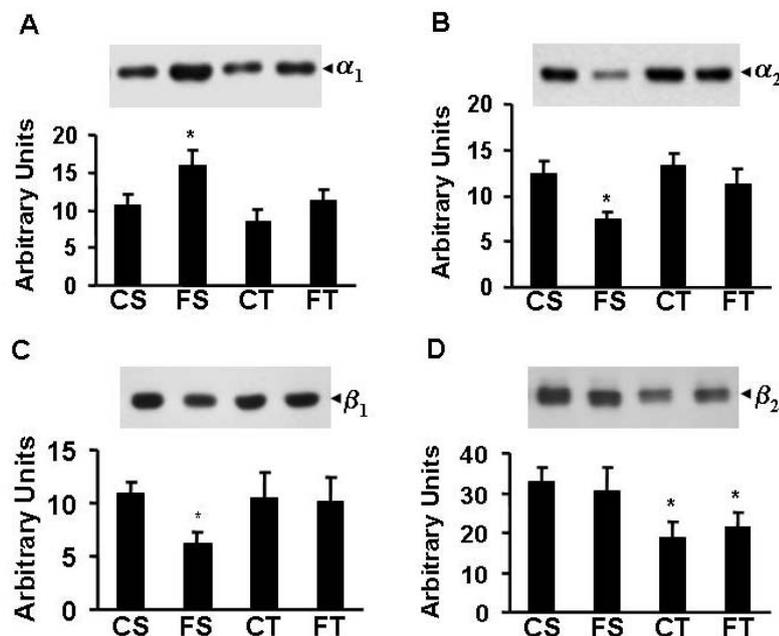
Figure 9 (A-D). Changes of Na^+,K^+-ATPase subunit expression in gastrocnemius muscle of Wistar rats after high fat diet and exercise training. CS – chow sedentary, FS – fat sedentary, CT – chow trained, FT – fat trained. Representative Western blots and bar graphs. Values are mean ± S.E. n=7-8, * p<0.05 FS basal CS, † p<0.05 CT and FT vs CS and FT, respectively.

The plasma membrane abundance of α_2-subunit under basal and insulin-stimulated conditions was impaired in skeletal muscles from animals on HFD, while the abundance of α_1- subunit remains unchanged. After exercise training, α_2-subunit content was restored, under basal and insulin-stimulated conditions; and α_1- subunit expression was unaffected (Fig. 10 A and B).

Figure 10. Effect of high fat diet and training on translocation of α_1 and α_2 subunit isoforms of Na^+,K^+-ATPase under basal and insulin-stimulated conditions. CS – chow sedentary, FS – fat sedentary, CT – chow trained, FT – fat trained. Representative blots and bar graphs. Values are mean ± S.E. n=7-8, * p<0.05 insulin-stimulated vs basal value, # and † p<0.05 FS vs CS in basal and insulin-stimulated conditions, respectively.
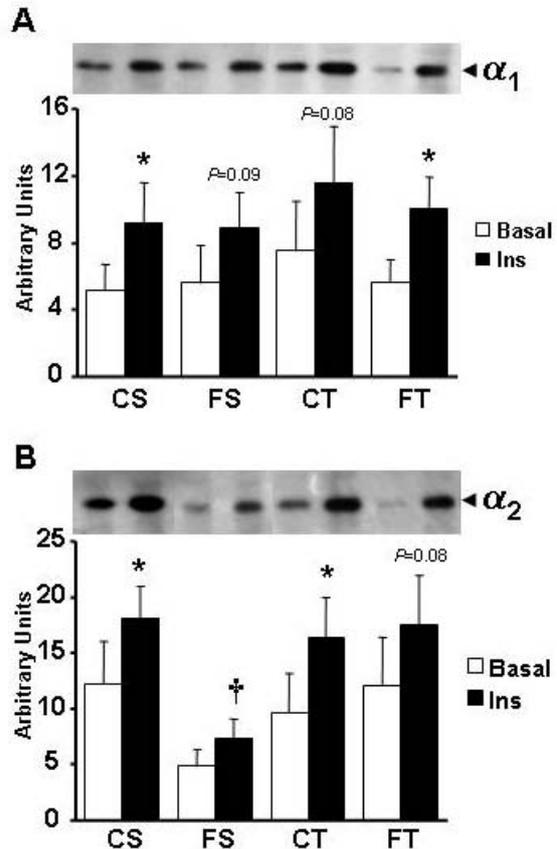
Our findings identify an altered Na^+,K^+-ATPase regulation pattern under conditions of high fat feeding. This may also play a role in the development of impaired K^+ fluxes, which is often associated with the features of the metabolic syndrome including obesity and insulin resistance. An increased Na^+/K^+-ratio in skeletal muscle has been observed in insulin-resistant patients (Landin et al., 1989). In type 2 diabetic patients, number and activity of Na^+,K^+-ATPase is decreased in erythrocytes (Mimura et al., 1994). The sodium pump content was decreased in identical twins, discordant for type 2 diabetes (Djurhuus et al., 2001). In rats, SZD-induced diabetes leads to a 24-48% decrease in skeletal muscle Na^+,K^+-ATPase content (Kjeldsen et al., 1987). In humans, insulin treatment leads to an increase in Na^+,K^+-ATPase content in skeletal muscle from type 1 and type 2 diabetic patients (Schmidt et al., 1994). Impaired insulin action on K^+ fluxes has been reported in obesity (DeFronzo, 1988) and young people with type 1 diabetes (Arslanian et al., 1991). Insulin-treated diabetic patients have an improved capacity for extra-renal clearance of an acute K^+ load (Smoller et al., 1988).

Thus, our results indicate that skeletal muscle Na^+,K^+-ATPase content is decreased in response to high fat diet and even precedes the development of insulin resistance. Exercise restores sodium pump content and activity. We also suggest that α_2-subunit of Na^+,K^+-ATPase may play a specific role in the regulation of K^+ homeostasis.

4.3. Na$^+$, K$^+$-ATPase as a signal transducer

4.3.1. Cardiotonic steroids stimulate glycogen synthesis in skeletal muscle via Src- and ERK1/2-dependent mechanism (papers III and IV)

Research over the past decade provides evidence that the Na-pump can also act as a receptor of ouabain-like substances (cardiotonic steroids). Most of this research has been performed on cardiac myocytes, though growing number of reports appear on other tissues. The effect of ouabain to increase in glycogen synthesis in oxidative rat skeletal muscle (specimen of diaphragm) has been demonstrated by Clausen (Clausen, 1966). However, ion and nutrient leakage from cut specimens of diaphragm may lead to alteration in muscle metabolism. Thus, it was of interest to study the effect of cardiotonic steroids in intact muscle or in isolated muscle cells, with respect to the fact that skeletal muscle is the most abundant tissue in mammalian body.

Therefore, we have studied the role Na$^+$,K$^+$-ATPase as the receptor of ouabain-like substances and signal transducer in skeletal muscle. The aims of these studies were to investigate the effects of cardiotonic steroids on glucose utilization in human skeletal muscle cells (HSMC) and rat *soleus* muscle, and the mechanisms of cardiotonic steroids signal transduction.

4.3.1.1. Ouabain stimulates glucose incorporation into glycogen without affecting glucose uptake

In HSMC, ouabain increased glycogen synthesis in concentration-dependent manner reaching the maximum at 100 nM (paper IV, Fig. 1A). The effect of ouabain was additive to the effect of insulin (paper IV, Fig. 4A), was independent of the PI 3-kinase inhibitor LY294002 (Fig. 3A), but was abolished in the presence of the MEK 1/2 inhibitor (PD98059) and the c-Src inhibitor (PP2). Exposure of muscle to 100 nM ouabain caused a 16% inhibition of total ouabain-sensitive ^{86}Rb$^+$ uptake (Fig. 1B) in human myotubes.

In isolated rat *soleus* muscle (paper III) [U-^{14}C]-glucose incorporation into glycogen was significantly stimulated by 200 μM ouabain under basal or insulin-stimulated conditions (p<0.05). Much higher concentrations of ouabain, in comparison with HSMC, was required because rodent Na$^+$,K$^+$-ATPase is ouabain- resistant. The effect was additive to insulin (paper III, Fig. 2A) and was abolished by inhibitors PD98059 and PP2.

The ouabain-induced increase in glycogen synthesis was accompanied by a decrease in glucose oxidation under basal and insulin-stimulated conditions (paper III, Fig. 2B) (p<0.05). The decrease was restored to control levels in the presence of c-Src inhibitor PP2 (Fig. 2B).

We have also examined effect of ouabain on glucose uptake by *soleus* muscle. Ouabain exposure (200 μM) for 1 hour decreased basal [^{3}H]-3-O-methylglucose uptake 27%. Insulin-stimulated glucose uptake (0.6 and 12 nM) was unaffected by ouabain (paper III, Fig. 1A). Pre-incubation of rat *soleus* muscle with 2 mM ouabain reduced basal glucose uptake (2.32 ± 0.464 vs 1.174 ± 0.238 μM /ml/h, p < 0.05) and did not affect insulin-stimulated glucose uptake (8.32 ± 1.206 vs 8.5 ± 1.169 μM /ml/h).

Incubation of HSMC with 100 nM ouabain did not affect glucose uptake, either under basal (7.03 ± 0.83 vs 6.79 ± 1.0 pmol x mg of protein^{-1} x min^{-1}) or insulin-stimulated (13.61 ± 1.08 vs 13.04 ± 0.96 pmol x mg of protein^{-1} x min^{-1}) conditions.

Cell surface content of glucose transporters in *soleus* muscle reflect glucose uptake. The photolabeling technique using Bio-LC-ATB-BMPA revealed a decrease in the basal cell-surface GLUT1 content without changes in cell-surface GLUT4 content (paper III, Fig. 1B).

Experimental evidence for a membrane receptor from cardiac glycosides that differs from Na^+,K^+-ATPase is lacking. Effects, caused by ouabain in skeletal muscle, are most likely not secondary to Na-pump inhibition, but may occur as a consequence of ouabain binding to a fraction of skeletal muscle Na-pumps and initiation of signaling cascade. Indeed, even in the condition of low occupancy, receptors effectively transmit signals inside a cell. Alternatively, low concentration of ouabain may target a "high affinity" binding site that is present only in the signaling complex formed by Na^+,K^+-ATPase and specific proteins and is functionally different from a well characterized "low affinity" inhibitory binding site (Ferrandi et al., 2004).

4.3.1.2. Does ouabain cause cellular stress?

We have evaluated the phosphorylation of p38 and c-Jun MAP kinases, in order to assess whether incubation of skeletal muscles with ouabain causes any intramuscular stress. Phosphorylation of these kinases was unchanged following ouabain exposure (paper III, Fig. 4E and F). Importantly, incubation of HSMC in K^+- free media, which inhibits Na-pump activity, did not increase ERK1/2 and GSK3 phosphorylation.

Since ouabain is the specific inhibitor of Na-pump, we considered that incubation of skeletal muscle with ouabain may lead to a rise in the intramuscular Na^+ concentration and ATP levels. Therefore, we analyzed intramuscular levels of nucleotides in the presence and absence of ouabain in rat *soleus* muscle. However, the levels of ATP, ADP, AMP and IMP were unaltered after 2 hours incubation of *soleus* with 200 μM ouabain (paper III, Fig. 3A).

Exposure of muscle to 200 μM ouabain had no effect on AMPK phosphorylation, however ACC phosphorylation was reduced under basal and insulin-stimulated conditions (paper III, Fig. 3B).

4.3.1.3. Ouabain activates Src-ERK1/2-p90rsk-GSK3 signaling cascade in skeletal muscle

Considering the data on ouabain signaling as reported in literature, we evaluated phosphorylation of various protein kinases in human myotubes and rat *soleus* muscle. Src kinase has been implicated as a key molecule in ouabain signaling pathway in a variety of reports (Aydemir-Koksoy et al., 2001a; Haas et al., 2002; Kometiani et al., 2005; Xie et al., 2003b). The interaction of Na^+,K^+-ATPase with Src-homology domain containing proteins has been reported previously (Chibalin et al., 1998b; Isenovic et al., 2004).

We found that ouabain increased Src-dependent tyrosine phosphorylation of the α_1- and α_2-subunits of Na^+,K^+-ATPase and promoted the interaction of α_1- and α_2-subunits with c-Src, as assessed by co-immunoprecipitation with c-Src (paper IV, Fig. 2 A-D and paper III, Fig. 5 B,C). These data emphasize the significance of c-Src activation in ouabain signaling and formation of Na^+,K^+-ATPase signalosome complex. Phosphorylation of c-Src, ERK1/2, p90rsk and GSK $3\alpha/\beta$ was increased in response to 100 nM ouabain in HSMC (paper IV, Fig. 1C) and in response to 200 μM ouabain in rat *soleus* muscle (paper III, Fig. 5A, 6A and 7A), and this effect was abolished in the presence of PD98059 and PP2 (paper IV, Fig. 4 A-C and paper III, Fig. 6 B and 7 B, C).

Akt/PKB signaling pathway is one of the main pathways regulating glycogen synthesis in skeletal muscle. Glycogen synthase kinase 3 (GSK3) is a gatekeeper of glycogen synthesis, and down-stream target of Akt/PKB. However, in rat *soleus* muscle insulin- stimulated phosphorylation of Akt/PKB was unaffected by ouabain, and basal Akt/PKB phosphorylation was reduced 2.4-fold in the presence of 200 μM ouabain ($p<0.05$) (paper III, Fig. 4A). In HSMC basal and insulin-stimulated phosphorylation of Akt was unaffected by 100 nM ouabain (paper IV, Fig. 3 B).

Ouabain increased PKC isoform phosphorylation and activity in cardiac myocytes (Xie et al., 2002). In our study, in rat soleus muscle, ouabain reduced phosphorylation of PKC α/β and δ isoforms under basal and insulin-stimulated conditions (paper III, Fig. 4C and D) ($p<0.05$), whereas phosphorylation of PKC ζ was unchanged (paper III, Fig. 4B).

These data strengthen the role of c-Src-ERK1/2-p90rsk-GSK3 pathway in mediating the ouabain-induced effect on glycogen synthesis and allows us to propose the scheme of ouabain signal transduction in skeletal muscle (Fig. 11).
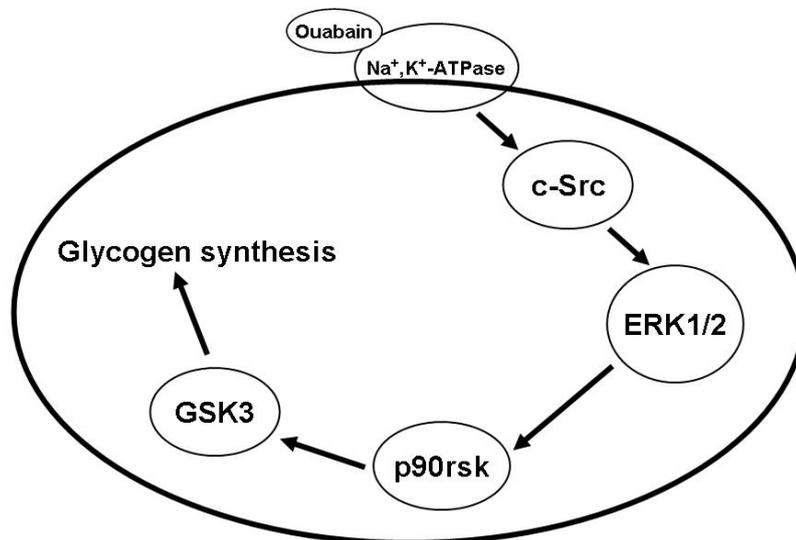
Figure 11. Proposed scheme of ouabain signal transduction in skeletal muscle.

Exercise and cellular stress has been demonstrated to cause an acute rise in the concentration of cardiotonic steroids (CTS) in the circulation (Bauer et al., 2005; Goto et al., 1995; Schoner et al., 2003). CTS release from the adrenal cortical cells is stimulated by angiotensin II and catecholamines (Bauer et al., 2005). In humans, the level of CTS peaked immediately after 15 min cycling exercise and returned to basal level 1 hour after exercise (Bauer et al., 2005). In rats subjected to acute stress by swimming, the level of CTS in the circulation peaked 40 min after stress, and return to basal level at 70 min. Although exercise in humans increases the level of CTS into 100 nmol x l^{-1} range (Bauer et al., 2005), results from animal studies indicate an increase in the CTS concentration in response to different stimuli within a modest low nanomolar range (Dostanic et al., 2005; Goto et al., 1995).Therefore, our data on stimulatory effect of ouabain on glycogen synthesis in skeletal muscle may have physiological relevance, and provides one of the

mechanisms of adaptation to exercise *in vivo*. Ouabain may activate glycogen synthesis immediately after exercise, when insulin levels are low.

4.3.1.4. Ouabain alters the molecular traffic of Na^+,K^+-ATPase

Considering our previous work on the involvement of ERK1/2 kinase in the regulation of Na-pump (papers I-II and unpublished studies on the effect of exercise and in vitro muscle contraction), we determined phosphorylation of α-subunits of Na-pump on ERK 1/2 phosphorylation site - Thr-Pro motif. As expected, incubation of HSMC with 100 nM ouabain increased the phosphorylation of α-subunits on -Thr-Pro motif (paper IV, Fig. 6A). Such phosphorylation can cause a feedback mechanism to regulate Na^+,K^+-ATPase activity. Therefore, the ouabain-induced signaling from Na^+,K^+-ATPase provides one more important piece of evidence of unique significance of MAPK signaling pathway in regulation of Na-pump.

Ouabain treatment decreased cell surface abundance of α_2- subunit, while the abundance of the α_1- subunit was unchanged (paper IV, Fig. 6B). Interestingly, the α_2-subunit was more sensitive to insulin stimulation in skeletal muscle, compare to α_1-subunit (Fig. 6 of the current "Results" section, paper I and Al-Khalili et al. (Al-Khalili et al., 2003b).

4.3.1.5. Endogenous cardiotonic steroid marinobufagenin acts similarly to ouabain in skeletal muscle

To further evaluate physiological significance of ouabain-induced stimulation of glycogen synthesis in HSMC, we compared the effect of ouabain with an effect of the endogenous ouabain-like substance marinobufagenin, which is synthesized in the adrenal glands and hypothalamus (Schoner, 2002a). Marinobufagenin stimulated glycogen synthesis at 10 nM (paper IV, Fig. 5A). This effect was similar to that induced by 100 nM ouabain. Exposure of muscle to MBG at 1 and 10 nM led to 15 and 21% of inhibition of total ouabain-sensitive $^{86}Rb^+$ uptake, respectively (Fig. 5B). This inhibition was similar to that caused by 100 nM ouabain. The phosphorylation of c-Src, ERK1/2, p90rsk and GSK $3\alpha/\beta$ was increased in response to marinobufagenin (paper IV, Fig. 5C).

In conclusion, our studies indicate that Na^+,K^+-ATPase inhibitors cardiotonic steroids, ouabain and marinobufagenin, increase glycogen synthesis additively to insulin in skeletal muscle. This increase in glycogen synthesis is mediated by activation of the c-Src- ERK1/2 - p90rsk-GSK3 signaling pathway. Taking into account the rapid increase in endogenous cardiotonic steroids in the circulation after acute exercise, an ouabain-induced increase in glycogen synthesis may have physiological significance.

Our results provide evidence that regulation of Na^+,K^+-ATPase via phosphorylation of the α-subunits by ERK1/2 MAPK is a universally important mechanism, providing feedback in the regulation of Na-pump activity during ouabain inhibition.

5 CONCLUSIONS

Based on the work in this thesis, we conclude that:

- In human skeletal muscle cells, insulin stimulates Na^+,K^+-ATPase activity via phosphorylation of catalytic α_1 and α_2-subunits by ERK1/2 MAPK-dependent mechanism and translocation of α-subunits to plasma membrane.

- In human renal tubular cells, C-peptide stimulates Na^+,K^+-ATPase activity via phosphorylation of catalytic α_1-subunits by ERK1/2 -dependent mechanism and translocation of α_1- , β_1- subunits to basolateral membrane from endosomal compartments.

- Exercise and insulin may cause translocation of the Na- pump to different plasma membrane subdomains.

- Exercise and *in vitro* contraction of rat skeletal muscle causes a significant increase in the plasma membrane abundance of Na-pump α_1- and α_2- subunits via ERK1/2 MAPK-dependent mechanism.

- Skeletal muscle Na^+,K^+-ATPase content is decreased in response to high fat diet and even precedes the development of insulin resistance. Exercise restores Na-pump content and activity. We suggest that the α_2-subunit of Na^+,K^+-ATPase may play a specific role in regulation of K^+ homeostasis.

- Na^+,K^+-ATPase inhibitors cardiotonic steroids, ouabain and marinobufagenin, increase glycogen synthesis additively to insulin in skeletal muscle. This effect is mediated by activation of c-Src- ERK1/2 - p90rsk-GSK3 signaling pathway. Taking into account the rapid increase in the level of endogenous cardiotonic steroids in the circulation after acute exercise, ouabain-induced increase in glycogen synthesis may have physiological significance.

- Activation of the MAPK signaling pathway constitutes a universally important mechanism of regulation and stimulation of Na^+,K^+-ATPase.

6   FUTURE PURSPECTIVES AND IMPLICATIONS

Phosphorylation of the Na-pump is a well known mechanism of its short-term regulation. The Na-pump can be phosphorylated by a variety of protein kinases on serine, threonine and tyrosine residues. Many studies have show Na^+,K^+-ATPase is regulated through phosphorylation of the catalytic α- subunit by PKC and PKA protein kinases. In our studies, we provide evidence of the unique importance of activation of the MAPK signaling cascade in the regulation of Na^+,K^+-ATPase. In this perspective, the role of downstream target of ERK1/2, p90rsk kinase, has been studied.

Bioinformatic assays predict the Ser^{943} site in the –KTRRNS- motif of the human α_1-subunit as an optimal p90rsk phosphorylation site. In rodents Ser^{943} is PKA phosphorylation site and it is phosphorylation by PKA, which leads to an increase in Na^+,K^+-ATPase activity. In proximal convoluted tubules, PKA activation by forskolin increases Na-pump activity via recruitment of pump molecules to plasma membrane (Carranza et al., 1998). If the Na-pump α-subunit is phosphorylated by p90rsk at the same residue, phosphorylation may have the same physiological effect.

Activation of Akt/PKB protein kinase is the important step in the insulin signaling cascade. Insulin is known to stimulate Na^+,K^+-ATPase activity. Interestingly, the α_2-subunit of Na-pump contains Thr^{414}, which is potential site for Akt phosphorylation. Therefore, the α-subunit can be potentially regulated through phosphorylation by Akt.

We have shown that the plasma membrane abundance of α_2- subunit of Na^+,K^+-ATPase under basal and insulin-stimulated conditions as well as α_2- protein expression were impaired in skeletal muscle from Wistar rats on a high-fat diet. The α_2- subunit expression was restored to control level after exercise training. Therefore, we suggest that the α_2-subunit of Na^+,K^+-ATPase may play a specific role in the regulation of K^+ homeostasis and may be linked to the metabolic syndrome, as well as being associated with obesity and insulin resistance. The effects of adipokines and inflammatory cytokines on Na^+,K^+-ATPase expression and regulation of its activity can be studied. While intensive acute exercise causes translocation of the Na- pump to the plasma membrane, it is of interest to investigate a possible involvement of AMPK in this process.

Detailed molecular mechanisms of the ERK1/2-dependent regulation of Na^+,K^+-ATPase requires further elucidation. We have shown that in human skeletal muscle cells, the MAPK pathway is directly involved in the regulation of molecular trafficking of Na^+,K^+-ATPase. Based on the study of the interaction of the Na-pump with the adaptor protein 2 and cofilin, we hypothesize that phosphorylation of the Na-pump α-subunits by ERK arrests the formation of an endocytotic complex, due to decreased interaction of Na-pump with AP2, while the process of exocytosis is maintained and may even be enhanced, since the interaction with cofilin is increased. Studies of kinetics of Na-pump trafficking can be performed utilizing a cell surface biotinilation technique with a cleaveable labeling reagent. We can evaluate the kinetics of internalization of labeled Na-pump and reappearance of labeled Na-pump on the cell surface under basal and insulin-stimulated conditions. Kinetics can shed more light into the question of the role of endo- and exocytosis in Na-pump regulation.

Cardiac glycosides have been used for about 200 years to treat patients with congestive heart failure. Cardiovascular disorders are serious complications of diabetes mellitus. The endogenous cardiotonic steroid marinobufagenin (MBG) is associated with development of diabetes mellitus. Renal excretion and plasma levels of MBG were

increased and erythrocyte Na$^+$,K$^+$-ATPase activity inhibited in rats with type 1 and type 2 diabetes (Bagrov et al., 2005). It can be very interesting to further evaluate the role of cardiotonic steroids in diabetes and to measure circulatory levels in patients with different degree of severity of type 1 and type 2 diabetes, as well first-degree relatives, and patients with impaired glucose tolerance and obesity. Such a study may reveal whether cardiotonic steroids can be used as a potential markers of diabetic state.

Moreover, exercise has a positive effect on the metabolic condition in diabetic patients. Circulatory levels of endogenous cardiotonic steroids rapidly rise after exercise. We have shown that ouabain stimulates glycogen synthesis in skeletal muscle additively to insulin and via activation of a MAPK signaling pathway. This effect of cardiotonic steroids may provide one of the mechanisms of adaptation to exercise *in vivo*. Cardiotonic steroids may activate glycogen synthesis when insulin levels are low immediately after exercise, or play some compensatory role in case of impaired insulin production or insulin resistance. MAPK pathway plays the important role in insulin signaling cascade as well. Therefore, it can be interesting to study the possible cross-talk between insulin and ouabain signaling in skeletal muscle.

Understanding of the molecular regulation of Na$^+$,K$^+$-ATPase may have important clinical implications for treatment and prevention of diabetic complications such as neuropathy, retinopathy, nephropathy and cardiovascular disorders. Elucidation of precise molecular processes involved in the regulation of the Na-pump by a MAPK signaling pathway may facilitate the development of novel therapeutic strategies for patients with complications of diabetes mellitus.

7 ACKNOWLEDGEMENTS

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