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CANONICAL AND NONCANONICAL TRANSDUCERS FOR CALCIUM SIGNALING:

Role of Na,K-ATPase and angiotensin receptor

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To my family: Ali and Sam

ABSTRACT

Calcium (Ca^{2+}) is the most universal and versatile signal in the cell. This versatility is based on the speed, amplitude and spatio-temporal patterning of the Ca^{2+} events. The inositol 1,4,5-trisphosphate receptor (IP_3R) is one of the main Ca^{2+} channels responsible for the Ca^{2+} release from the intracellular stores. In a response to various stimuli, IP_3R is able to generate variety of Ca^{2+} signals, ranging from Ca^{2+} transients to Ca^{2+} oscillations of different frequencies.

In this thesis we studied regulation of canonical and non-canonical signaling pathways that activate IP_3R-mediated Ca^{2+} signaling. The canonical pathway is represented in this thesis by angiotensin II type 1 receptor (AT1R) $G\alpha q_{/11}$ protein-coupled signaling, which triggers IP_3R-mediated Ca^{2+} signals by stimulation of IP_3 production. The non-canonical pathway is represented by Na,K-ATPase, which activates IP_3R independently on the presence of IP_3 through allosteric effect.

AT1R, together with dopamine D1-like receptor (D1R), represent a counter-regulatory system that controls sodium uptake in renal proximal tubules. We have shown that AT1R and D1R form a heterodimer. We have demonstrated that the stimulation of either of the receptors induce heterologous desensitization of the other receptor. Activation of D1R resulted in rapid and reversible uncoupling of AT1R from its G protein-coupled signaling pathway followed by internalization of the receptor and *vice versa;* stimulation of AT1R abolished D1R mediated cAMP production and triggered D1R internalization.

Na,K-ATPase, in addition to its function as an ion pump, serves also as a signal transducer. Ouabain, a highly specific Na,K-ATPase ligand, was shown to trigger slow Ca^{2+} oscillations through the Na,K-ATPase/IP_3R signaling complex. We have described that the cytoskeleton associated protein, ankyrin B, stabilizes the Na,K-ATPase and IP_3R interaction. Down-regulation of ankyrin B in COS7 cells using siRNA, resulted in reduction and dysregulation of ouabain-triggered Ca^{2+} oscillations, and abolishment of NF-kB activation.

In 2006, Hilgenberg et al. (Cell 2006 Apr 125:359) reported that 20-kD C-terminal fragment of agrin (agrin C20) is a new ligand of Na,K-ATPase α3 that inhibits its pumping activity. The original purpose of our study was to examine whether agrin C20 has also capacity to induce signaling function of Na,K-ATPase α3. We have shown that the solubilized agrin C20, which we received from the authors of the Cell paper, is not selective for Na,K-ATPase α3, but has a capacity to trigger slow Ca^{2+} oscillations in COS7 cells *via* Na,K-ATPase α1 and also to inhibit the pumping activity of the Na,K-ATPase α1. These effects were dependent on the intact ouabain binding site. Solubilized agrin C20 was also found to trigger slow Ca^{2+} oscillations superimposed on the spontaneous fast frequency Ca^{2+} oscillations in rat primary hippocampal neurons. The naturally occurring 22-kD C-terminal fragment of agrin did not trigger Ca^{2+} signal in COS7 cells. Mass-spectrometry analysis revealed presence of 5-7 mM ouabain in the solubilized agrin C20 sample. Ouabain-free agrin C20 was not found to have any effect on the Na,K-ATPase activity in the mouse brain lysate.

In conclusion we have described new mechanisms regulating canonical and non-canonical activators of IP_3R Ca^{2+} signaling through protein-protein interaction and allosteric modulation.

LIST OF PUBLICATIONS

I. Xiao Liu*, **Zuzana Špicarová***, Susanna Rydholm, Juan Li, Hjalmar Brismar, Anita Aperia.
Ankyrin B modulates the function of Na,K-ATPase/Inositol 1,4,5-trisphosphate receptor signaling microdomain.
J Biol Chem 283:11461-11468, 2008

II. Farah Khan, **Zuzana Špicarová,** Sergey Zelenin, Ulla Holtbäck, Lena Scott, Anita Aperia.
Negative reciprocity between angiotensin II type 1 and dopamine D1 receptors in rat renal proximal tubule cells.
Am J Physiol Renal Physiol 295: F1110–F1116, 2008.

III. **Zuzana Khorshidi**, Xiao Liu, Alexander Bondar, Hjalmar Brismar, Anita Aperia.
Reported effects of agrin on the function of Na,K-ATPase may be explained by a contamination.
Manuscript.

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PUBLICATIONS NOT INCLUDED IN THIS THESIS

Blom H, Rönnlund D, Scott L, **Špicarová Z**, Rantanen V, Widengren J, Aperia A, Brismar H. Nearest neighbor analysis of dopamine D1 receptors and Na(+)-K(+)-ATPases in dendritic spines dissected by STED microscopy.Microsc Res Tech. 2012 Feb;75(2):220-8.

Blom H, Rönnlund D, Scott L, **Špicarová Z**, Widengren J, Bondar A, Aperia A, Brismar H. Spatial distribution of Na+-K+-ATPase in dendritic spines dissected by nanoscale superresolution STED microscopy.BMC Neurosci. 2011 Jan 27;12:16.

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LIST OF ABBREVIATIONS

AngII	Angiotensin II
Ank	Ankyrin
AM	Acetoxymethyl
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ATP	Adenosine trisphosphate
AT1R	Angiotensin II type 1 receptor
CaM	Calmoduline
cAMP	Cyclic adenosine monophosphate
CaMKII	Ca^{2+}/calmoduline-dependent kinase II
cGMP	Cyclic guanosine monophosphate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CICR	Calcium induced calcium release
CTS	Cardiotonic steroid
DAG	Diacylglycerol
D1R	Dopamine D1 receptor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FRAP	Fluorescence recovery after photobleaching
GAP	GTPase activating proteins
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GST	Glutathione S-transferase
GTP	Guanosine trisphosphate
IBMX	3-isobutyl-1-methylxanthine
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
MAPK	Mitogen activated protein kinase
MuSK	Muscle specific kinase
mGluR	Metabotropic glutamate receptor
NCX	Na^+/Ca^{2+}-exchanger
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
PM	Plasma membrane
PKA	cAMP dependent protein kinase
PKC	Protein kinase C
PKG	cGMP dependent protein kinase
PLC	Phospholipase C
RGS	Regulators of G protein signaling
RyR	Ryanodine receptor
siRNA	Short interfering RNA
SR	Sarcoplasmic reticulum
TCR	T-cell receptor
VGCC	Voltage gated calcium channel

1 INTRODUCTION

1.1 Calcium signaling

Calcium (Ca^{2+}) has been recognized as a universal second messenger that is involved in the variety of vital cellular functions and processes like proliferation, fertilization, differentiation, learning and memory, apoptosis, excretion, etc. The ability of Ca^{2+} signaling to regulate all these diverse processes is allowed by its high versatility, which depends on speed, amplitude and spatial-temporal patterning. Rapid highly localized Ca^{2+} spikes regulate fast responses, whereas slower responses are controlled by global Ca^{2+} transients and intracellular Ca^{2+} waves (Berridge et al., 2003).

In the resting state the cytosolic Ca^{2+} concentration is low, around 100 nM, whereas the extracellular and intracellular stores reach the Ca^{2+} concentration 1000 to 10 000 times higher (Uhlen and Fritz, 2010). This high gradient makes the cells capable to sense even small changes in the cytosolic Ca^{2+} level.

Every cell possesses a Ca^{2+} signaling toolkit containing mechanisms that trigger Ca^{2+} signal, mechanisms of Ca^{2+} entry into the cytoplasm, and mechanisms that restore the resting state of the cytosolic Ca^{2+}. The extensive variability of each component of the Ca^{2+} signaling and the possibility to assemble various combinations enables to create wide spectrum of different spatial and temporal profiles of the Ca^{2+} signal. The Ca^{2+} signaling toolkit includes cell surface receptors, Ca^{2+} channels, Ca^{2+} pumps and exchangers and Ca^{2+} sensors.

Ca^{2+} signal can be generated as a release from the intracellular stores as well as an influx from extracellular space across the plasma membrane. The main intracellular Ca^{2+} store is considered to be an endoplasmic reticulum (ER); however other cell compartments such as mitochondria, Golgi apparatus (GA), nuclear envelope, endosomes and lysosomes possess high Ca^{2+} concentration as well.

1.2 Ca^{2+} transport mechanisms

1.2.1 Plasma membrane

Ca^{2+} can enter cell from the extracellular space *via* variety of Ca^{2+} channels of different properties which use the electrochemical gradient across the plasma membrane as driving force for Ca^{2+} transport.

1.2.1.1 Voltage gated calcium channels (VGCCs)

VGCCs can be found mostly on the plasma membrane of excitable cells. They are activated by membrane depolarization and generate rapid Ca^{2+} fluxes that control processes such as contraction, secretion, neurotransmission, and gene expression. Their activity is essential to couple electrical signals in the cell surface to

physiological events in cells. Expression of the VGCCs has been also described on the plasma membrane of the non-excitable cells like renal tubular cells (where VGCCs play role for example in volume regulatory machinery, or apoptosis) (Bozic and Valdivielso, 2012), or T-cells (where VGCCs have been shown to shape the TCR Ca^{2+} response) (Badou et al., 2013).

1.2.1.2 Receptor-operated calcium channels

Receptor-operated Ca^{2+} channels are opened upon binding of their specific ligand. Typical example of the plasma membrane receptor operated Ca^{2+} channel is NMDA receptor that is activated by glutamate. NMDA receptor is not selective for Ca^{2+} but it is also permeable for Na^{+} and K^{+}. NMDA receptor is localized to the postsynaptic density and it is responsible for most of the Ca^{2+} influx in a response to the synaptic activity. NMDAR Ca^{2+} signal is crucial for induction of synaptic plasticity and for activating intracellular signaling pathways involved in learning and memory (Paoletti et al., 2013).

1.2.1.3 Store-operated calcium channels (SOCs)

SOCs are Ca^{2+} channels in the plasma membrane that are activated by depletion of the ER intracellular Ca^{2+} stores. SOCs are composed of the protein complex including plasma membrane protein Orai1 that forms a pore of the Ca^{2+} channel and STIM1 proteins that are located in the membrane of ER and can sense decrease of the luminal Ca^{2+} level. When the ER Ca^{2+} stores are depleted, STIM associates with Orai in the plasma membrane and activates store-operated Ca^{2+} entry and replenishing of ER Ca^{2+} stores (Berna-Erro et al., 2012).

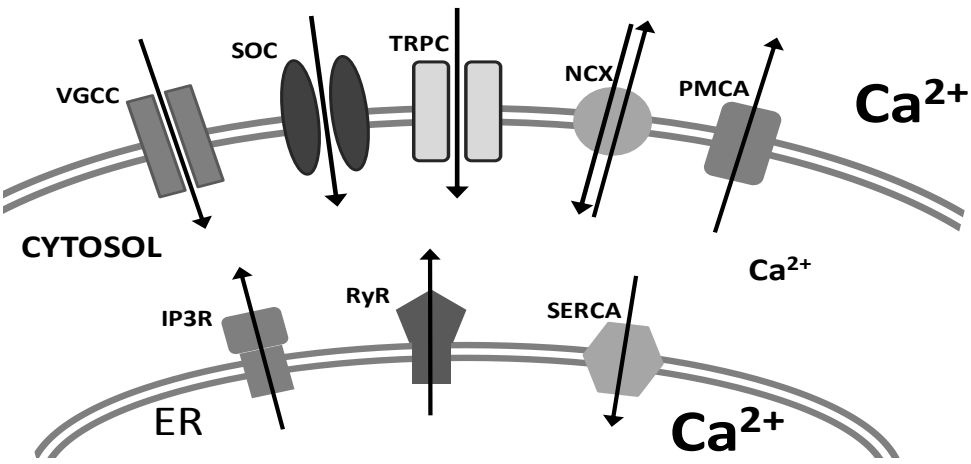

Fig 1.1 *Ca^{2+} transporters*

1.2.1.4 Na^{+}/Ca^{2+} exchanger (NCX)

NCX is an ion transport channel that transports Ca^{2+} across the plasma membrane against its electrochemical gradient utilizing energy stored in the

electrochemical gradient of Na^+ (mainly maintained by Na, K-ATPase). Typically it transports one Ca^{2+} in exchange for three Na^+. It is a reversible transporter and the direction of the Ca^{2+} transport depends on the Na^+, Ca^{2+}, and K^+ concentrations, membrane potential and the transport stoichiometry. In non-excitable cells it is one of the main mechanisms that extrude Ca^{2+} from the cell. In the conditions when intracellular Na^+ raises (for example after Na, K-ATPase inhibition) the NCX may work in forward direction and let Ca^{2+} flow in (Ruknudin and Lakatta, 2007).

1.2.1.5 Plasma membrane Ca^{2+} ATPase (PMCA)

PMCA is a transport protein that removes Ca^{2+} from the cell. To move Ca^{2+} ions across the plasma membrane against their electrochemical gradient it uses energy from the ATP hydrolysis. PMCA together with NCX are the main mechanisms of Ca^{2+} extrusion. Since there exists a great electrochemical gradient that forces entry of the Ca^{2+} into the cell, it is extremely important to keep the cytosolic Ca^{2+} levels low for proper cell signaling as well as to prevent Ca^{2+} induced cytotoxicity (Giacomello et al., 2013).

1.2.1.6 Transient receptor potential channels (TRPCs)

TRPCs are group of ion channels conducting Ca^{2+}, Na^+, or K^+, that are in the front line of the sensory systems, responding to the signals from the outer environment such as temperature, sound, touch, chemicals or light. Most of the TRPCs are not Ca^{2+} selective except TRPM4 and 5. Some of the TRPCs family members have been shown to be activated after Ca^{2+} depletion of the ER and contribute to the store-operated Ca^{2+} entry (Gees et al., 2012).

1.2.2 Endoplasmic reticulum

ER is the main store of the intracellular Ca^{2+}. Membrane of the ER contains a number of proteins, Ca^{2+} channels and pumps that regulate both Ca^{2+} release and Ca^{2+} re-uptake.

1.2.2.1 Inositol 1,4,5-trisphosphate receptor (IP_3R)

IP_3R is together with ryanodine receptor the major mechanism of the Ca^{2+} release from the ER. IP_3R is a ligand gated Ca^{2+} channel belonging to a super family of ion channels with six transmembrane domains. IP_3R mediated Ca^{2+} release is essential step in numerous signaling pathways of many vital cell function. IP_3R function can be regulated by high variety of mechanisms. This assures high versatility of the Ca^{2+} signal generated by IP_3R and its integration with multiple signaling pathways.

Classically, IP_3R is divided into three regions. N-terminal ligand binding region, regulatory and coupling region, and the C-terminal consisting of six transmembrane helices and short cytoplasmic tail. More recently the structure of IP_3R was divided into five functional domains (Fig 1.2). The IP_3 binding core domain is the minimum region necessary for recognition and binding of the IP_3. N-

terminal coupling domain physically interacts with IP_3 binding domain and functions as a suppressor of IP_3 binding. Deletion of this region leads to the massive enhancement of IP_3 binding. The signal for IP_3 binding is transferred *via* N-terminal and internal coupling domain to the gatekeeper domain which triggers conformational changes in the transmembrane/channel-forming domain that leads to opening of the channel (Mikoshiba, 2007).

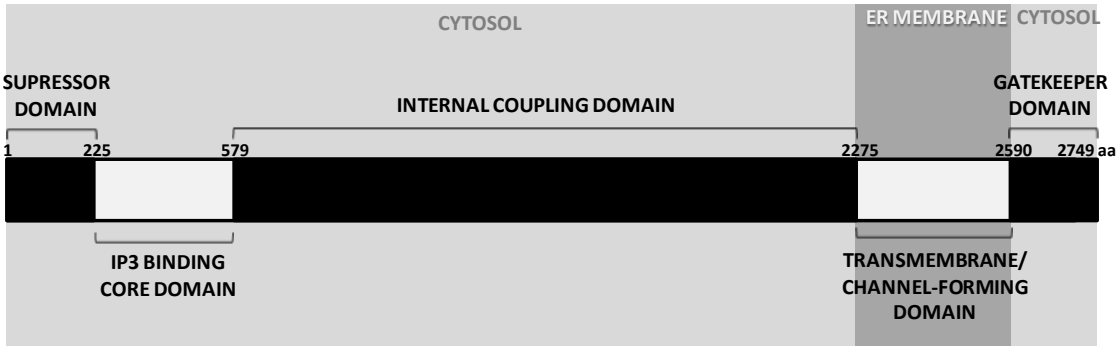

Fig 1.2 *IP3R structure*

Up to date there have been described three subtypes of IP_3R encoded by three genes: IP_3R type1, type2, and type3. Diversity of IP_3Rs is further increased by existence of splicing variants. Functional IP_3R Ca^{2+} channel is formed by four IP_3R molecules and there has been described both homo- and hetero-tetramers formation. IP_3R isoforms shares more than 65% homology in their sequence and differs in IP_3 sensitivity, susceptibility to various regulatory mechanisms, and spatial and temporal distribution. Most of the cell types express more than one IP_3R isoform but their expression patterns differ between cell types and tissues (Parys and De Smedt, 2012).

The IP_3R function is regulated by a number of factors:

***IP_3*:** IP_3 is a ligand and activator of IP_3R. IP_3 is a second messenger that is produced after stimulation of cell surface receptors by various signal molecules like hormones, growth factors, neurotransmitters, *etc.* Precursor of IP_3, phosphatidylinositol 4,5-bisphosphate, phospholipid present in the plasma membrane can be cleaved by phospholipase C (PLC) into IP_3 and 1,2-diacylglycerol (DAG), an activator of several effectors including protein kinase C (PKC). There have been described six families of PLCs and each of them consists of several isoforms and splicing variants. This assures the controlled production of IP_3 and DAG in different cells type triggered by different stimuli and receptors (Vines, 2012).

***Ca^{2+}*:** The IP_3R function is regulated by Ca^{2+}. The IP_3R has bell shaped Ca^{2+} dependence. Low concentrations of Ca^{2+} (100-300 nM) have stimulatory effect on the opening of IP_3R channel whereas higher Ca^{2+} concentrations lead to the closing of the channel. In the absence of Ca^{2+}, IP_3 is not able to trigger Ca^{2+} release from ER.

Binding of Ca^{2+} also increases sensitivity of IP_3R to IP_3 (Patterson et al., 2004). There have been described seven sites for Ca^{2+} binding. Ca^{2+} has been also suggested to play a role in triggering IP_3R ubiquitination (Alzayady and Wojcikiewicz, 2005). Beside the regulation of IP_3R function by direct binding of Ca^{2+} there has been proposed an indirect effect through Ca^{2+} binding proteins like calmoduline. IP_3R is not only sensitive to the Ca^{2+} concentration in the cytoplasm but is also regulated by the concentration in the lumen of the ER. The low Ca^{2+} concentration in the ER lead to IP_3R inhibition to avoid excessive Ca^{2+} depletion of the ER (Caroppo et al., 2003).

***Phosphorylation*:** IP_3R is phosphorylated by multiple kinases. Phosphorylation of IP_3R by c-AMP dependent kinase (PKA) increases its responsiveness to IP_3. This is particularly interesting because it provides the cross-talk between signaling of two main second messengers, cAMP and Ca^{2+}. The state of PKA phosphorylation can be very dynamically changed since IP_3R is in the complex with both PKA and protein phosphatase 1 and 2A. cGMP dependent kinase (PKG) phosphorylates IP_3R at the same sites as PKA. Ca^{2+} activated protein kinase C (PKC) and Ca^{2+}/calmoduline protein kinase II (CAMK-II) phosphorylation of IP_3R increases its sensitivity to IP_3-mediated Ca^{2+} release and thus provides positive feedback loop. IP_3R can be further phosphorylated by the tyrosine kinases from Src family, various serine/threonine kinases, cell cycle- dependent kinases, and others (Patterson et al., 2004).

***Protein-protein interaction*:** To date there have been identified up to 50 proteins that can interact with IP_3R and regulate its localization and function. Together with previously described regulatory mechanisms, interaction with various proteins contributes to an accurate and precise control of IP_3R signaling in response to different extracellular stimuli. One group of IP_3R interacting proteins are the cytoskeleton associated proteins and scaffolding proteins which promote stable subcellular localization of IP_3R. One example are the ankyrins, a family of adaptor proteins that can interact both with proteins in the membrane of ER and the plasma membrane and thus can help to form microdomains between the two membranes (Mohler et al., 2005). In neuronal cells, another scaffolding protein, Homer, assembles IP_3R with mGluR and provides regulation of the mGluR/ IP_3R signaling at the excitatory synapse (Tu et al., 1998). Since increased cytosolic Ca^{2+} level leads to cell death by apoptosis, it is not surprising that the IP_3R is in complex with many apoptotic proteins. During apoptosis, IP_3R can be cleaved by apoptotic proteases caspase 3 and calpain. Soon after cell receives apoptotic stimuli, *cytochrome c* is translocated to the proximity of ER, where it interacts with C-terminus of IP_3R and inhibits Ca^{2+}-induced closing of the IP_3R. Subsequent increase of the cytosolic Ca^{2+} concentration causes release of more *cytochrome c* (Boehning et al., 2003). Recently there has been described another IP_3R binding protein, called IRBIT, which serves as the IP_3R pseudo-ligand. It binds to the IP_3 binding site of IP_3R. In low IP_3 concentration, IRBIT binds to the IP_3R and decreases its sensitivity to IP_3, while in high IP_3 concentrations IRBIT is released and IP_3R can be activated (Ando et al.,

2006; Ando et al., 2003). Study by Zhang et al. (2006) has shown that N-terminus of the IP_3R can directly interact with N-terminus of Na,K-ATPase, and that ouabain, Na,K-ATPase ligand, triggers conformational changes that lead to IP_3R activation independently on the presence of IP_3.

1.2.2.2 Ryanodine receptor (RyR)

Ryanodine receptors are localized in the plasma membrane of sarco-/endoplasmic reticulum (SR/ER). They are known mostly for their involvement in excitation-contraction coupling, releasing of Ca^{2+} from the sarcoplasmic reticulum and thus driving muscle contraction. The activation of RyR is controlled by the concentration of the cytosolic Ca^{2+}. Small elevation of intracellular Ca^{2+} induces opening of RyR and release of more Ca^{2+}. RyRs are mainly responsible for Ca^{2+}-induced Ca^{2+} release (CICR) which is crucial for excitation-contraction coupling in cardiac muscle (Fabiato, 1983) as well as it is important mechanism of Ca^{2+} signaling in many cells. The high intracellular Ca^{2+} concentration on the other hand causes closing of RyR channels. RyRs are also capable of sensing the Ca^{2+} concentration inside the SR/ER. In the condition when SR/ER is overloaded, RyR opens and releases Ca^{2+} into the cytosol. This process is called store overload-induced Ca^{2+} release (SOICR) (Palade et al., 1983). RyR opening is not entirely dependent on the presence of Ca^{2+}. The skeletal muscle isoform of RyR is believed to interact with dihydropyridine receptor and to be allosterically activated after the voltage changes across the plasma membrane even in the absence of the extracellular Ca^{2+} (Proenza et al., 2002). RyRs function can be modulated by protein-protein interactions (e.g. with calmoduline, homer), and phosphorylation (e.g. PKA, PKG, CaMK II) (Ozawa, 2010).

1.2.2.3 Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA)

SERCA is a transporter that actively translocates Ca^{2+} from the cytosol to the internal stores of SR/ER. SERCA plays major role in the muscle contraction and its dysfunction is connected with many pathophysiological conditions, such as heart failure (Frank et al., 2002). Overall, the Ca^{2+} homeostasis that SERCA pump contributes to is important for Ca^{2+} signaling. Nevertheless, more regulated function of SERCA has been implicated in specific Ca^{2+} signaling events such as generation and propagation of Ca^{2+} waves (Li et al., 2012; Maxwell and Blatter, 2012).

1.3 Ca^{2+} oscillations

Ca^{2+} signal is a result of the interplay between activation and inactivation of Ca^{2+} channels that transport Ca^{2+} from extracellular space as well as from internal stores. It is well known that a robust and prolonged increase of the cytosolic Ca^{2+} level leads to cell death both by apoptosis and necrosis. By utilizing brief pulses of Ca^{2+}, instead of tonic rises, cells avoid the deleterious effects of sustained cytosolic

Ca^{2+} levels. Furthermore, signaling with Ca^{2+} oscillations is beneficial for the cell since the combination of the duration, amplitude and frequency of the Ca^{2+} peaks can hypothetically give rise to an infinite number of signals.

With the development of methods enabling to detect Ca^{2+} levels in the living cells, Ca^{2+} oscillations have been shown to regulate numerous physiological processes through the life, including fertilization, cell maturation and differentiation, cell cycle progression, mitochondrial respiration, chemotaxis, secretion and gene transcription (Thul et al., 2008).

1.3.1 Generation of cytosolic Ca^{2+} oscillations

It is generally accepted that Ca^{2+} oscillations are generated as a periodical release of the Ca^{2+} from the internal stores through the IP_3R. In excitable cells the initial Ca^{2+} entry might be due to an activation of the plasma membrane channels, followed by the amplification of the signal by release from the internal stores.

The enormous complexity of the Ca^{2+} signaling makes it difficult to fully understand the mechanism behind the generation of Ca^{2+} oscillations. Data resulting from more than 20 years of extensive studies together with mathematical modeling proposed a model based on the biphasic regulation of IP_3R and phenomena of Ca^{2+} induced Ca^{2+} release (CICR).

In simplification, when the intracellular Ca^{2+} level increases, in most cases due to IP_3 induced IP_3R activation, IP_3R is sensitized by the Ca^{2+} which leads to amplification of the initial signal and further increase of the cytosolic Ca^{2+} level. However the high Ca^{2+} level has a negative feedback on the IP_3R, which closes allowing Ca^{2+} level to decrease (Fig 1.3). In this model, Ca^{2+} oscillations can be generated under the constant concentrations of IP_3 (Dupont et al., 2011).

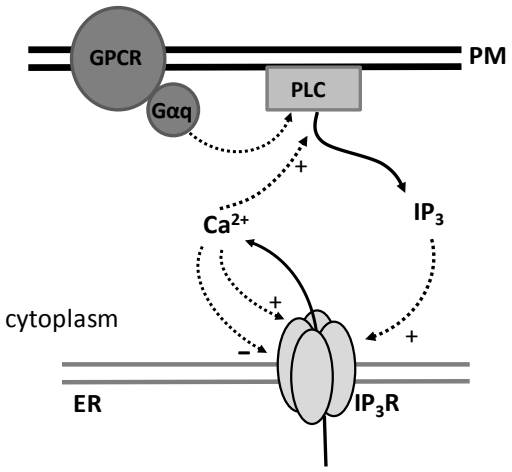

Fig 1.3 *Ca^{2+} induced Ca^{2+} release*

Although this model is applicable to most of the IP_3 stimulated Ca^{2+} oscillations, it can´t be considered to be universal. Experimental data have shown that in some systems IP_3R generated Ca^{2+} oscillations can´t be explained by CICR. For example in MDCK cells (Hirose et al., 1999) and Chinese hamster ovary cells (Young et al., 2003) each Ca^{2+} peak follows elevation of IP_3. In this model, cross-talk between second messengers IP_3 and Ca^{2+} plays a crucial role for a generation of the Ca^{2+} oscillations. IP_3 induced Ca^{2+} release increases sensitivity of IP_3R for IP_3. Furthermore, Ca^{2+} has been shown to be able to activate some of the PLC isoforms, which leads to production of more IP_3. Another model of Ca^{2+} oscillations that are not dependent on CICR are the oscillations induced by activation of mGluR5. mGluR5 is believed to undergo a rapid cycle of PKC phosphorylation and dephosphorylation mediated desensitization and resensitization (Kim et al., 2005; Nash et al., 2001).

1.3.2 Regulation of Ca^{2+} oscillations pattern

The pattern of Ca^{2+} oscillations is dependent on the timing of the Ca^{2+} channel opening, Ca^{2+} clearance, and Ca^{2+} stores reloading. It can be regulated on different levels and each component of the signaling machinery contributes to the shaping of the Ca^{2+} response.

1.3.2.1 Agonist concentration control of Ca^{2+} oscillations

Ca^{2+} oscillations are triggered by the physiological range of agonist concentration, while saturating concentrations often lead to a single and broad Ca^{2+} transient. Increase of the agonist concentrations within the physiological range often result in increase of Ca^{2+} oscillation frequency (Berridge, 1993; Woods et al., 1986).

1.3.2.2 The role of IP_3R in the regulation of Ca^{2+} oscillations

Regulation of the IP_3R properties, such as IP_3 or Ca^{2+} affinity, has an effect on the Ca^{2+} signaling pattern. IP_3R isoforms differs in their affinity for IP_3 and Ca^{2+} and it has been shown that activation of specific IP_3R subtypes results in generation of Ca^{2+} signaling with different characteristics. Miyakawa et al. (1999) has shown that in genetically modified B-cells expressing either one of the IP_3R isoforms or their combination, the most IP_3 sensitive IP_3R type 2 generates robust and highly regular Ca^{2+} oscillations, IP_3R type 1 generates less regular Ca^{2+} oscillations, and IP_3R type 3, which is the least IP_3 and Ca^{2+} sensitive gives rise only to a Ca^{2+} transient. In vascular myocytes expressing IP_3R1 and IP_3R2, acetylcholine fails to induce Ca^{2+} oscillations when IP_3R2 function is compromised (Morel et al., 2003). Hattori et al. (2004) has shown that wild type HeLa and COS7 cells, that express both IP_3R1 and IP_3R3, respond to ATP stimulation with robust Ca^{2+} transient, while downregulation of IP_3R3 isoform using siRNA leads to oscillatory response to the same stimuli. This suggests that IP_3R3 has in anti- Ca^{2+} oscillatory function in these cells.

Phosphorylation of the IP_3R in most cases changes its affinity for IP_3 and Ca^{2+} and therefore, it may play role in shaping of the Ca^{2+} oscillations. LeBeau et al. (1999) demonstrated that the phosphorylation of IP_3R in the pancreatic acinar cells

determines the properties of IP_3R generated Ca^{2+} oscillations. While acetylcholine or carbachol induce high frequency oscillations superimposed on elevated basal level of Ca^{2+}, cholecystokinin triggers slow baseline Ca^{2+} spikes. Both stimuli induce activation of PLC and release of Ca^{2+} through IP_3R, but only cholecystokinin induces rapid phosphorylation of the IP_3R. Lee et al. (2006) has shown that inhibition of MAPK/ERK2 mediated phosphorylation of IP_3R1 lead to inability of the IP_3R to generate Ca^{2+} oscillations during mouse oocyte maturation. In DT-40 lymphoma B-cells phosphorylation of the IP_3R1 by PKA decreases threshold for induction of Ca^{2+} oscillations (Wagner et al., 2004). The role of CaMKII activity in histamine induced Ca^{2+} oscillations in HeLa cells were demonstrated as the treatment with CaMKII specific inhibitor during oscillations caused one large Ca^{2+} spike before the oscillations were terminated (Zhu et al., 1996).

As mentioned earlier, IP_3R can interact with number of proteins. These interactions often contribute to shape the IP_3R generated Ca^{2+} signal. IP_3R has been shown to interact with the Bcl-2 family of anti-apoptotic proteins. There have been reported bi-directional regulation of the IP_3R generated Ca^{2+} signaling by Bcl-2. In T-cell cell line, strong activation of TCR receptor leads to massive increase of intracellular Ca^{2+} that is inhibited in the presence of Bcl-2. On the other hand, when the TCR receptor is activated in the moderate level, it triggers Ca^{2+} oscillations; which´s frequency and duration are promoted by the expression of Bcl-2 (Zhong et al., 2006). In MCF-7 cells, Ca^{2+} transient rather than Ca^{2+} oscillations was triggered by ATP in the cells overexpressing Bcl-2 (Palmer et al., 2004). IRBIT has been described as an IP_3R pseudoligand that decreases IP_3R sensitivity to IP_3. Depletion of IRBIT by siRNA in HeLa cells increased the number of HeLa cells responding to ATP treatment with Ca^{2+} oscillations (Ando et al., 2006). Mutations in presenilins are associated with development of the Alzheimer´s disease. Presenilins interact with IP_3R and the expression of Alzheimer associated presenilin mutants in DT-40 cells resulted in an increase of frequency of Ca^{2+} oscillations triggered by α-IgM (Cheung et al., 2008).

1.3.2.3 The role of GPCRs in the regulation of Ca^{2+} oscillations

One of the most common triggers that stimulates PLC activity and IP_3 production is *through* activation of G protein-coupled receptors (GPCRs). Many studies have reported the role of regulation of the GPCR function in the Ca^{2+} oscillation pattern formation. One of the mechanisms that regulate frequency of Ca^{2+} oscillations is controlled by the activity of regulators of G protein signaling proteins (RGS) that are responsible for inactivation of the G protein by GTP hydrolysis. Luo et al. (2001) have demonstrated that disruption of the RGS activity resulted in two-times increase of frequency of cholecystokinin (CCK)-induced Ca^{2+} oscillations compare to control (Shin et al., 2003). The mGluR5 triggered Ca^{2+} signaling has been shown to be modified by Norbin, factor involved in long-term potentiation (LTP), synaptic plasticity, learning and memory. Activation of mGluR5 transfected into HEK293 cells induced Ca^{2+} oscillations that assigned longer duration and higher

number of peaks in the presence of Norbin. Authors have shown that deletion of Norbin leads to decrease of the amount of mGluR5 in the plasma membrane (Wang et al., 2009).

1.3.2.4 Ryanodine receptors (RyRs)

Another way of Ca^{2+} release from internal stores is through ryanodine receptors. RyRs are activated by Ca^{2+} and thus they significantly contribute to the CICR. The RyR function is most studied in the sarcoplasmic reticulum of muscle cells, where it cooperates with the IP_3R to generate Ca^{2+} oscillatory signal.

1.4 Decoding of Ca^{2+} signal

Ca^{2+} activates a number of Ca^{2+} binding proteins that either trigger downstream effects or take part on the positive/negative feedback of the Ca^{2+} signaling machinery itself. Ca^{2+} can bind directly to an effector molecule that is responsible for the biological effect of the Ca^{2+} signal (like troponins responsible for muscle contraction) or it can activate other signaling molecules. In general, decoding of the Ca^{2+} signal depends on the kinetics of the Ca^{2+} binding and dissociation and the dynamics and timing of the activation and inactivation of the target molecule.

The key players responsible for Ca^{2+} oscillations frequency decoding are calmodulin (CaM) and protein kinase C (PKC). CaM is a promiscuous regulator with several hundreds of targets known to date, including kinases, phosphatases, cytoskeletal proteins, synaptic proteins, cell cycle proteins, ion channels, and the buffer proteins that regulate intracellular Ca^{2+} stores. Each CaM molecule possesses four Ca^{2+} binding sites that differ in Ca^{2+} binding affinity and kinetics. Moreover Ca^{2+} needs to compete with Mg^{2+} for CaM binding. Binding of Ca^{2+} results in conformational changes of CaM and binding to target molecule.

One of the most widely studied CaM target is the family of Ca^{2+}/calmodulin-dependent kinases (CaMK). The most common CaMKII can be activated by high frequency (in a range of Hz) Ca^{2+} spikes. CaMKII is an octamer or decamer and each of the subunit is independently regulated by Ca^{2+}-bound CaM. One single Ca^{2+} peak is not enough to fully activate the kinase. Instead cumulative activation during repetitive Ca^{2+} elevations leads to the kinase activation. If the time between two Ca^{2+} peaks is longer than a time needed for CaM dissociation, CaMKII activity remains low (De Koninck and Schulman, 1998; Dupont et al., 2003). Calcineurin is a Ca^{2+}/CaM activated serine/threonine phosphatase that beside many other targets dephosphorylate CaMKII and thus decreases its activity. Unlike CaMKII, calcineurin is optimally activated by Ca^{2+} oscillations of frequency around 10 m Hz. The properties of calcineurin allow it to be activated with the high Ca^{2+} oscillations frequency as well. However, calcineurin is competing with CaMKII for active CaM binding and at higher frequencies, the activation is shifted towards CaMKII (Li et al., 2012).

Conventional protein kinase C (PKC) is activated by binding of Ca^{2+} and DAG. Upon Ca^{2+} stimulation, PKC is translocated to the proximity of the plasma

membrane, where it interacts with DAG and is fully activated. The low frequency of the Ca^{2+} oscillations leads to the fluctuations of the PKC activity, which always returns to the basal level between two Ca^{2+} peaks. On the other hand higher frequencies or persistent Ca^{2+} increase lead to higher and stable amplitude of the PKC activity (Oancea and Meyer, 1998).

Ca^{2+} oscillation frequency has been shown to dictate which of the Ca^{2+} dependent transcription factors NF-AT (Dolmetsch et al., 1998; Li et al., 1998), NF-κB (Dolmetsch et al., 1998), and Oct/OAP (Dolmetsch et al., 1998) will be activated. While high frequencies are able to efficiently activate function of all three transcription factors, when the periodicity of Ca^{2+} spikes declines to less than 400s only NF-κB is activated (Dolmetsch et al., 1998). The difference in the frequency requirements among these factors lies in their stability in the nucleus. NF-κB has been shown to persist in the nucleus up till 16 min, while NF-AT is rapidly rephosphorylated and exits the nucleus (Dolmetsch et al., 1997).

MAPK/ERK signaling cascade is activated by many stimuli and integrates different signaling pathways. Ca^{2+} oscillations frequency has been shown to modulate activity of Ras protein, an upstream component of the MAPK/ERK pathway. It has been shown that Ras is more efficiently stimulated by Ca^{2+} oscillations than by sustained Ca^{2+} increase (Kupzig et al., 2005). Guanine nucleotide exchange factors (GRFs) and RasGTPase activating proteins (RasGAPs), the main regulators of Ras activity, have been shown to be regulated by direct binding of the Ca^{2+} (Feig, 2011; Walker et al., 2004).

1.5 Na,K-ATPase

Na,K-ATPase is an integral plasma membrane enzyme expressed in virtually every cell. Its main function is to maintain high gradient of Na^+ and K^+ across the plasma membrane. It is a main determinant of intracellular Na^+ concentration. It uses energy from the ATP hydrolysis to transport 3 Na^+ out and 2 K^+ in the cell (Fig 1.4). Due to this 3 Na^+/2 K^+ stoichiometry the transport is electrogenic, thereby generating electric potential difference across the plasma membrane. The energy saved in the electrochemical gradient of Na^+ is used by many symport and antiport transporters such as Na^+/Ca^{2+}-exchanger, Na^+/H^+-exchanger, $Na^+/K^+/2Cl$, Na^+/glucose, Na^+/amino acids co-transporters. This way Na^+ controls directly or indirectly many essential cell functions like transport of various compounds, regulation of cell volume, intracellular pH, and cytosolic Ca^{2+} concentration. The turnover of the Na,K-ATPase is responsible for approximately 30% of the total oxygen consumption and in some organs such as brain and kidney more than 50% of the energy released is used for the active Na^+/K^+ transport (Clausen et al., 1991).

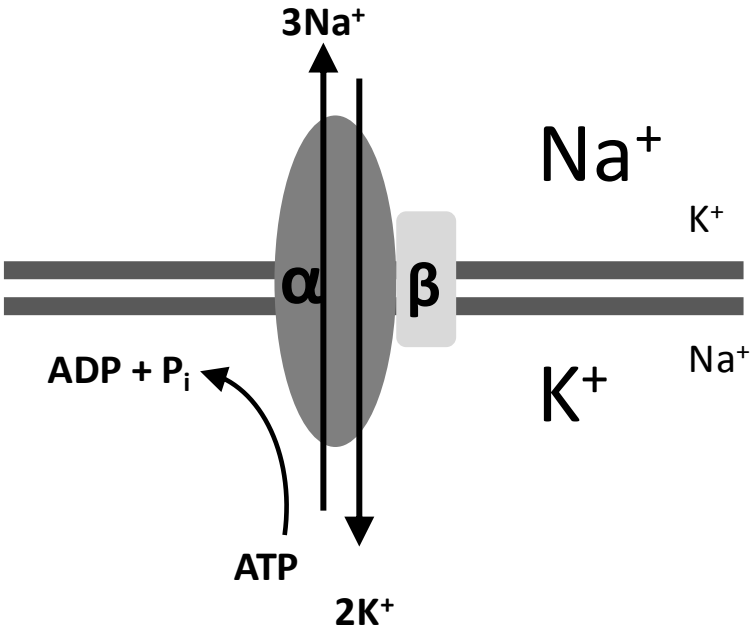

Fig 1.4 *Na,K-ATPase ion transport*

1.5.1 Na,K-ATPase structure

Na,K-ATPase is composed of three subunits in equimolar ratios. The catalytic α subunit together with the β subunit forms functional enzyme regulated by third subunit that belongs to the FXYD protein family.

The catalytic α subunit is a large polypeptide (≈110 kD) consisting of ten transmembrane domains, cytosolic N- and C-termini and four cytoplasmic loops. The α subunit is responsible for the ion transport activity. It contains ATP binding site, Na^+ and K^+ binding sites, regulatory phosphorylation sites and binding sites for cardiac glycosides (Lingrel and Kuntzweiler, 1994).

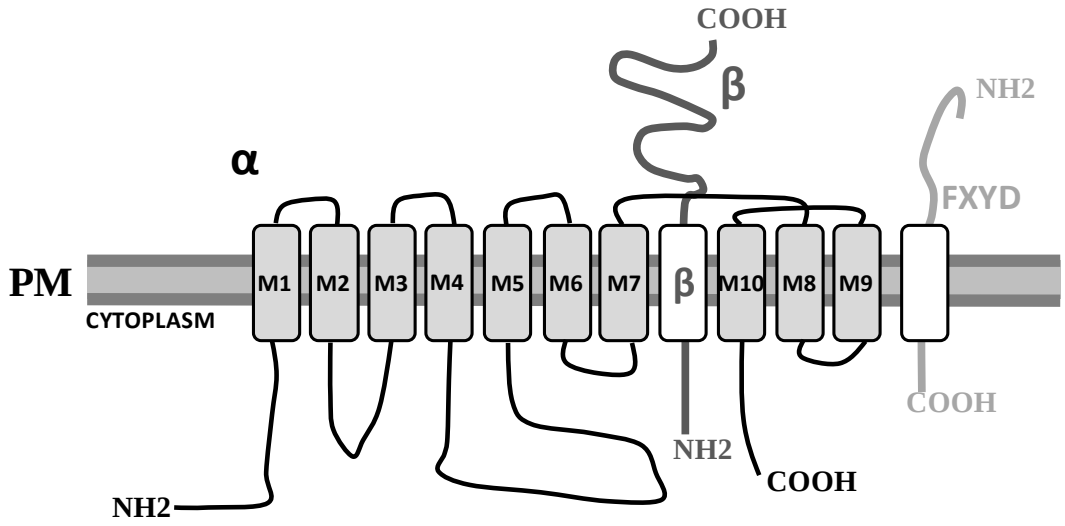

Fig 1.5 *Structure of the Na,K-ATPase*

The β subunit is an accessory subunit which is important for the maturation and transport of the enzyme to the plasma membrane (Geering, 1991) and is involved in the occlusion of K^+ and the modulation of the K^+ and Na^+ affinity of the enzyme (Geering, 2001; Lutsenko and Kaplan, 1993). It crosses plasma membrane only once and the small N-terminal end is located into the cytoplasm, while long C-terminus is extracellular. Last regulatory subunit represented by FXYD proteins is not essential for Na,K-ATPase catalytic function. It spans plasma membrane once having short intracellular C-terminus and extracellular N-terminus. They have a regulatory function modulating kinetics of the Na^+ and K^+ transport (Garty and Karlish, 2006; Therien and Blostein, 2000). Crystal structure of the Na,K-ATPase heterotrimer has been recently resolved (Kanai et al., 2013; Morth et al., 2007; Nyblom et al., 2013; Shinoda et al., 2009).

Four isoforms of the α subunit, α1, α2, α3, and α4; three isoforms of the β subunit, β1, β2, and β3; and 7 FXYD proteins have been identified in mammals. (Geering, 2006). Alpha isoforms are expressed in a tissue-specific and developmentally regulated manner. The α1 is expressed ubiquitously. High levels of the α2 isoform expression can be found in skeletal muscle, heart, glia cells, adipocytes, vascular smooth muscle, and eye. The α3 isoform is neuron specific but can be also found in ovaries, embryonic cardiomyocytes, and white blood cells of some species. The α4 isoform is expressed in sperms, where it is required for sperm motility (Lingrel, 2010; Orlowski and Lingrel, 1988; Woo et al., 2002). Alpha isoforms differ in their kinetics and substrate affinities (Dobretsov and Stimers, 2005; Munzer et al., 1994).

1.5.2 The catalytic cycle of Na,K-ATPase

During the catalytic cycle of the Na,K-ATPase three Na^+ ions are transported out of the cell and two K^+ ions are countertransported out. The Post-Alberts model describes the enzymatic kinetics of Na,K-ATPase (Albers, 1967; Post et al., 1972). There are two major conformational states of Na,K-ATPase, E1 and E2. The pump with bound ATP binds three intracellular Na^+ ions (E1-ATP $3Na^+$). ATP is hydrolyzed leading to the phosphorylation of the pump (E1P-ADP $3Na^+$). Release of ADP and one Na^+ ion leads to conformational change of the pump towards E2P state (E2P $2Na^+$), release of the two remaining Na^+ and binding of two extracellular K^+ (E2P $2K^+$). Binding of K^+ leads to dephosphorylation of the pump (E2-$2K^+$). Dissociation of the liberated phosphate and ATP binding (E2-ATP $2K^+$) stimulates K^+ release and changing the enzyme conformation from E2 to the E1 state.

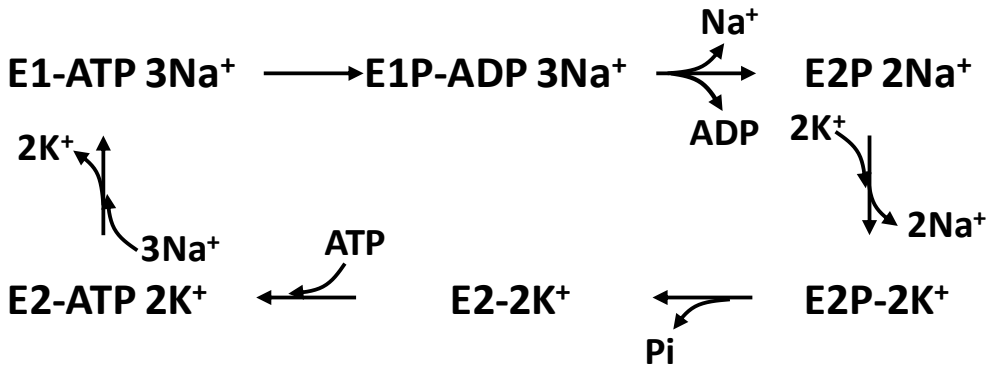

Fig 1.6 *Simplified Post-Albers model for ion transport by Na,K-ATPase*

Analysis of the crystal structure of Na,K-ATPase in different states helped to understand the mechanisms of ion transport through the plasma membrane. Ion binding sites and ion pathways have been described (Kanai et al., 2013; Morth et al., 2007; Nyblom et al., 2013; Ogawa et al., 2009). The identification of the critical amino acid residues for the catalytic function of Na,K-ATPase helps to explain or predict the functional consequences of the Na,K-ATPase disease-related mutations.

1.5.3 Regulation of the Na,K-ATPase function

The role of the Na,K-ATPase as a determinant of the electrochemical gradient across the plasma membrane is pivotal for the cell survival. Cell is a very dynamic system, therefore Na,K-ATPase activity has to be controlled in a precise way in response to the changes of the ionic environment. Na,K-ATPase activity is regulated by a number of mechanisms.

1.5.3.1 Substrate concentration

The most straightforward mechanism of the Na,K-ATPase activity regulation is through substrate availability and concentration. Na,K-ATPase is activated by Na^+

and ATP in the cytosolic side and K^+ in the extracellular space. Since the cytosolic concentration of Na^+ is under normal conditions low, even a small change in the intracellular Na^+ level has significant effect on Na,K-ATPase activity (Therien and Blostein, 2000).

1.5.3.2 Phosphorylation

Activity of the Na,K-ATPase is regulated by a number of hormones such as dopamine (Aperia et al., 1987), parathyroid hormone (Ribeiro et al., 1994), prolactin (Ibarra et al., 2005), angiotensin II (Gutman et al., 1972), epinephrine, norepinephrin (Aperia et al., 1992), or insulin (Gavryck et al., 1975). The main mechanism of hormone control of the Na,K-ATPase activity is through its phosphorylation and dephosphorylation. Na,K-ATPase contains phosphorylation sites for PKA and PKC kinases (Therien and Blostein, 2000). PKC site is localized to the N-terminus of the α1 subunit (Beguin et al., 1994). PKA phosphorylation site is located in the last cytoplasmic loop of α subunit and is conserved among all isoforms (Cheng et al., 1997; Fisone et al., 1994). Cheng et al. (1999) have demonstrated that the intracellular Ca^{2+} concentration is important determinant of the effect of PKA and PKC phosphorylation on the Na,K-ATPase activity. Dynamic simulation based on the crystal structure of the Na,K-ATPase revealed that the PKA phosphorylation site can modulate pumping activity by changing the accessibility of the ion-binding site (Poulsen et al., 2012). Na,K-ATPase is dephosphorylated by phosphatases like PP1, PP2B, or calcineurin (Therien and Blostein, 2000). The phosphorylation and dephosphorylation represent short-term regulation mechanism of Na,K-ATPase activity. Hormones such as corticosteroids (Verrey et al., 1996) or thyroid hormone (Cheng et al., 2010) control long term regulation of Na,K-ATPase by affecting its synthesis and degradation.

1.5.3.3 Plasma membrane lipid composition

The lipid composition of the plasma membrane has been suggested to modulate the Na,K-ATPase activity. Crystal structure of the Na,K-ATPase showed binding of a cholesterol molecule (Morth et al., 2007) and the level of cholesterol in the plasma membrane has been shown to have effect on the Na,K-ATPase activity (Cornelius, 1995; Cornelius et al., 2001).

1.5.3.4 Association with cytoskeletal proteins

Association of Na,K-ATPase with cytoskeletal proteins regulate trafficking and subcellular localization of Na,K-ATPase and thus indirectly affect its activity (Devarajan et al., 1994). Na,K-ATPase activity has been also shown to be affected directly by interaction with ankyrin and adducin (Ferrandi et al., 1999). Ankyrins are a family of adaptor proteins responsible for the localization of membrane and cytosolic proteins into specialized membrane domains. Vertebrate ankyrins fall into three classes: ankyrins-R (Ank-R), ankyrins-B (Ank-B), and ankyrins-G (Ank-G) (Mohler et al., 2002). Ank-B has been shown to tether Na,K-ATPase, NCX, and IP_3 receptor into microdomains localized in the T-tubules of cardiomyocytes (Mohler et

al., 2005). Mutations in Ank-B lead to cardiac arrhythmia and long-QT syndrome (Mohler et al., 2003). Ank-G has been also shown to interact with Na,K-ATPase and to regulate its trafficking from ER to Golgi apparatus in polarized cells (Stabach et al., 2008).

1.5.3.5 Ligands of Na,K-ATPase

Na,K-ATPase activity is dose dependently inhibited by its specific ligands, cardiotonic steroids (CTS). To the CTS family belongs for example plant derived digitalis which is used for centuries as a cardiac stimulant. In the venom of toads structurally related bufadienolides have been found. Another digitalis-like compound, widely used in the research, is ouabain. Endogenous ouabain (Hamlyn et al., 1991) and bufadienolides (Hilton et al., 1996) have been identified in human body. Recently a crystal structure of ouabain/Na,K-ATPase complex has been resolved (Laursen et al., 2013; Ogawa et al., 2009). The affinity of the Na,K-ATPase α subunit to CTS differs between isoforms and species. While for example primates express all α isoforms with high ouabain sensitivity, in rodents the ubiquitous α1 isoform is about 1000 times less ouabain sensitive compare to α2 and α3 isoforms (O'Brien et al., 1994).

In 2006, Hilgenberg et al. reported that Na,K-ATPase α3 is a neuron specific ligand for agrin. Agrin is a proteoglycan abundantly expressed in neuromuscular junctions where it induces acethlycholine receptor clustering *via* signaling through MuSK/LRP4 receptor complex. Authors described 20kD C-terminus of agrin to bind selectively to α3 isoform of Na,K-ATPase and inhibit its activity.

In a small-diameter neurons in the dorsal root ganglion, Na,K-ATPase α1 has been shown to interact with secreted protein follistatin 1 (Li et al., 2011). Follistatin 1 has been reported to function as a Na,K-ATPase agonist as it increases the Na,K-ATPase activity by direct binding from the extracellular side.

1.5.4 Na,K-ATPase as a signal transducer

Beside its vital role as an ion pump, Na,K-ATPase has an additional role as a signal transducer. In many studies the modulatory effect of non-inhibitory doses of ouabain on processes like proliferation, apoptosis, cell motility, and cell to cell contact have been demonstrated. To date there are described two main signaling pathways triggered by ouabain: Ca^{2+} signaling and Src kinase activation.

1.5.4.1 Na,K-ATPase and Ca^{2+} signaling

Our group has described an unique and novel mechanism of IP_3R activation. In 2001, Aizman et al. reported that low doses of ouabain trigger slow Ca^{2+} oscillations in the rat proximal tubule cells. These oscillations originated from release of the Ca^{2+} from ER through the IP_3R. Further studies of this phenomenon in COS7 cells led to an interesting finding that the ouabain-induced IP_3R activation is IP_3 independent (Miyakawa-Naito et al., 2003). C-terminal portion of the IP_3R that

includes the IP_3 binding domain has been shown to directly interact with N-terminal portion of Na,K-ATPase. Binding of ouabain to Na,K-ATPase results in stabilization of this binding and opening of the IP_3R channel probably via conformational changes. A motif of 35 Leu-Lys-Lys 37 amino acids residues at Na,K-ATPase N-terminus has been identified to be critical for Na,K-ATPase/IP_3R interaction (Zhang et al., 2006). Upon ouabain stimulation, NF-κB, a Ca^{2+} dependent transcription factor, was shown to be activated and induce cell proliferation and protects cells from apoptosis (Aizman et al., 2001; Burlaka et al., 2013; Li et al., 2006). Recently other cardiotonic steroids digoxin and marinobufagenin have been shown to be able to trigger slow Ca^{2+} oscillations in COS7 cells (Fontana et al., 2013).

1.5.4.2 Na,K-ATPase and Src signaling pathway

Na,K-ATPase has been shown to interact with the members of Src tyrosine kinase family. The binding of ouabain triggers release of the Src kinase resulting in Src phosphorylation and activation. Src is known to phosphorylate multiple targets. One of the downstream effects of ouabain-triggered Src activation is Ca^{2+} release *via* IP_3R. Yuan et al. (2005) demonstrated, that Na,K-ATPase is in the complex with Src, IP_3R and PLC in porcine proximal tubular cells (LLC-PK) and that ouabain binding induces Ca^{2+} transient through the IP_3R activation in Src and PLC dependent manner. In cardiac myocytes and vascular smooth muscle cells (A7r5), ouabain-induced Src activity was shown to be responsible for ouabain-triggered phosphorylation and transactivation of epidermal growth factor receptor (EGFR) and subsequent activation of Ras/MAPK signaling cascade (Haas et al., 2002).

Recently a new concept suggesting cross-talk between ouabain-triggered Ca^{2+} oscillatory signaling and Src dependent pathway has evolved. Inhibition of the Src activity resulted in a decrease of a number of COS7 cells that responded to ouabain with Ca^{2+} oscillations (Fontana et al., 2013).

1.5.4.3 Physiological and pathophysiological effects of the Na,K-ATPase signaling

The Na,K-ATPase signaling triggered by low non-inhibitory doses of cardiotonic steroids has been implicated in many physiological and pathological processes. Low frequency Ca^{2+} oscillations triggered by ouabain in renal epithelial cells activates NF-κB transcriptional factor, which in this system has pro-proliferative and anti-apoptotic effects. Ouabain has been shown to protect proximal tubule cells from apoptosis induced by serum deprivation (Li et al., 2006) and shigatoxin (Burlaka et al., 2013) through regulation of expression of pro- and anti-apoptotic factors Bax and Bcl-xL. Ouabain-induced NF-κB activation has been also shown to protect embryonic kidneys from defective development triggered by malnutrition (Li et al., 2010).

Cardiotonic steroid-induced Src and ERK signaling regulate cell growth and differentiation (Lee et al., 2011), apoptosis, and hypertrophic growth (Ferrandi et al., 2004; Liu et al., 2007) in a cell type specific manner. Ouabain-triggered ERK

signaling was implicated in a pathogenesis of autosomal dominant polycystic kidney disease as it stimulates cell proliferation and cyst growth (Jansson et al., 2012). Na,K-ATPase is considered to be a new target for anticancer therapy since cardiotonic steroids assign anti-proliferative and pro-apoptotic effects on various types of cancer cells (Kometiani et al., 2005; Kulikov et al., 2007; Xu et al., 2011). Ouabain has been also shown to modulate cell migration (Barwe et al., 2005; Pongrakhananon et al., 2013) and cell-cell contact (Larre et al., 2010), processes that are known to be dysregulated in cancer cell invasion and metastasis.

Cardiotonic steroid induced signaling *via* Na,K-ATPase, Src and ERK was shown to promote glycogen synthesis in skeletal muscle cells (Kotova et al., 2006).

1.5.5 Na,K-ATPase associated genetic diseases

In last decade a new interest in Na,K-ATPase has aroused as mutations of α2 and α3 Na,K-ATPase were found to be associated with familial neurological diseases. Twenty-three missense mutations of the Na,K-ATPase α2 have been identified in the patients with familial hemiplegic migraine type 2 (FHM2), a rare autosomal dominant subtype of migraine with aura. These patients suffer from very severe migraine symptoms including motor weakness or paralysis, in some cases signs of diffuse encephalopathy, coma, prolonged hemiplegia lasting several days, and in few cases seizures (Pietrobon, 2007).

Nine mutations in α3 Na,K-ATPase have been found to associate with rapid-onset dystonia parkinsonism (RDP). RDP is a distinct form of dystonia in which patients experience sudden, often stress induced, onset of parkinsonian symptoms that are unresponsive to standard dopaminergic treatments and not associated with typical Parkinson´s brain pathology. Symptoms, which evolve over hours to days, usually start in late adolescence or early adulthood. Patients have limb and cranial dystonia with dysarthria and dysphagia accompanied by bradykinesia, slow gait, and posture instability. Seizures and psychiatric symptoms have been observed in a few patients (Geyer et al., 2011).

In 2012 *de novo* mutations of Na,K-ATPase α3 were identified in the patients with alternating hemiplagia of childhood (ACH) (Heinzen et al., 2012; Rosewich et al., 2012). ACH usually occurs in the age of six months and attack frequency ranges from several times a month to several times a day. Symptoms are characterized by transient episodes of hemiplagia, dystonia, nystagmus, autonomic disturbances, seizures, and severe intellectual disability and developmental delay.

Although in most of the cases the disease associated mutations of Na,K-ATPase α2 and α3 lead to severe impairment of the pump function, some of the mutated Na,K-ATPase still possess satisfactory ion transporting capacity. For example Phe785Leu and Thr618Met mutants of Na,K-ATPase α3 associated with RDP are functional but assign decreased affinity to Na^+ (Rodacker et al., 2006). This demonstrate the essential role of Na,K-ATPase in the brain physiology since even

small disturbances of Na,K-ATPase function have a great consequences resulting in severe neurological symptoms.

1.6 G protein-coupled receptors

One of the largest and most diverse membrane protein families is the G protein-coupled receptors (GPCRs), which are encoded by more than 800 genes in the human genome (Fredriksson et al., 2003). GPCRs are activated by a wide spectrum of extracellular signals, including photons, ions, small organic molecules and entire proteins. GPCR malfunction is associated with many diseases, and it is estimated that more than 50% of pharmaceuticals target GPCRs (Lundstrom, 2009). The physiological function of a large fraction of GPCRs remains still unknown; these receptors are referred to as orphan GPCRs.

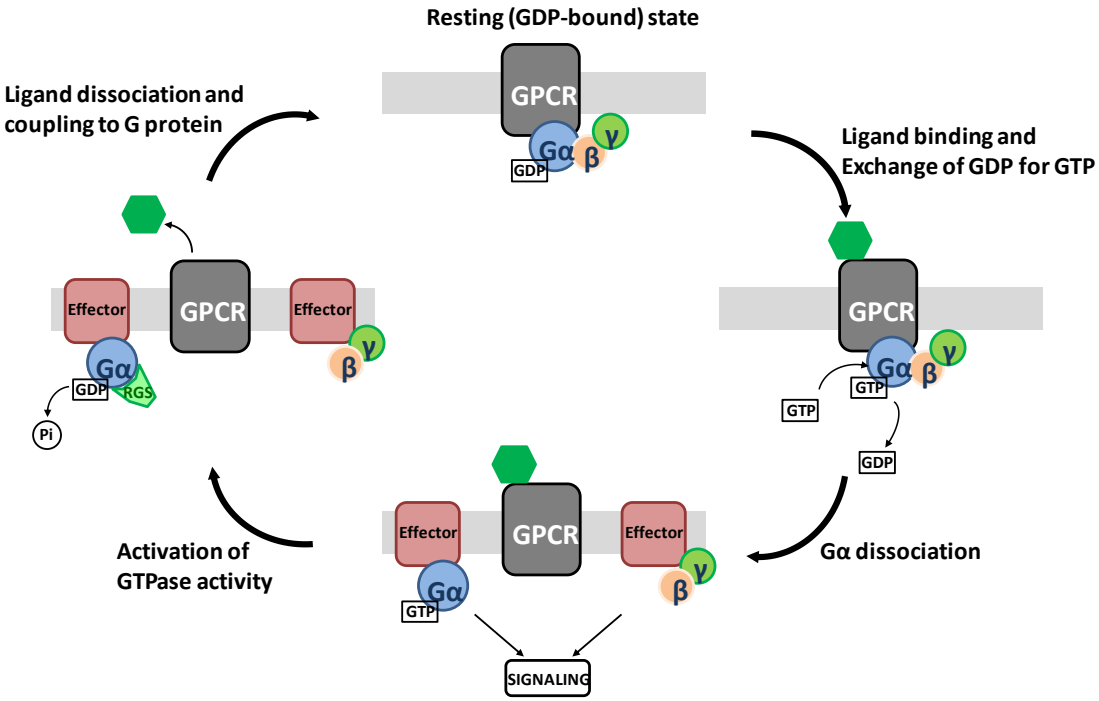

Fig 1.7 *G protein-coupled receptor activation*

GPCRs share a common structural characteristic of seven transmembrane domains, with an extracellular N-terminus and an intracellular C-terminus. After ligand binding, GPCRs undergo conformational changes, causing the activation of canonical signaling through G proteins. However, some GPCRs signal through a G

protein independent, non-canonical, signaling pathway. G protein is a trimer of α, β, and γ subunits (known as Gα, Gβ, and Gγ), which is rendered to be in an inactive state when bound to GDP. When the GPCR is activated by a ligand it undergoes a conformational change which stimulates a replacement of GDP with GTP at the Gα protein. In the GTP-bound state the Gα-GTP subunit dissociates from Gβγ and is free to modulate activity of its target proteins. Hydrolysis of GTP to GDP allows re-association of Gα with Gβγ and return to the resting state (Fig 1.7).

There are different classes of Gα proteins which have similar mechanism of activation but show differences in recognition and regulation of the effector. Gαs directly stimulates adenylyl cyclase and thus induces cAMP production which activates protein kinase A (PKA). Gαi opposites the Gαs effect by inhibiting adenylyl cyclase. $G\alpha q/_{11}$ stimulates phospholipase C (PLC) which cleaves PIP_2 into second messengers IP_3 and DAG, which in turn induce Ca^{2+} release *via* IP_3R and activate protein kinase C (PKC) respectively. $G\alpha_{12/13}$ regulates Rho GTPase signaling and control cytoskeleton remodeling and cell migration (Suzuki et al., 2009).

The Gβγ subunit has in some systems an active role as well. It has been shown to couple to and inhibit voltage gated Ca^{2+} channels (Zamponi and Currie, 2013), binds to and activate G protein gated potassium channels (Kurachi and Ishii, 2004), or activate PLC-epsilon (Wing et al., 2001).

GPCRs can also trigger a G protein independent signaling pathway through β-arrestins. After binding of an agonist, the GPCR is phosphorylated and binds β-arrestin protein. This is part of the desensitization process that is described in detail in next section. Beside desensitization and internalization of the GPCR, β-arrestins have been shown to scaffold signaling molecules to agonist activated GPCRs. β-arrestins are able to scaffold members of c-Src tyrosine kinase family, Akt, PI3 kinase, players of MAPK signaling pathway including ERK1/2, p38,and JNK3 (Lefkowitz and Shenoy, 2005). In addition there is evidence that β-arrestins can also regulate nuclear processes such as transcription of new RNA. β-arrestin was identified as a binding partner of IκB. Beta-arrestin can sequester IκB –NFκB complex in the cytosol and thus attenuate NFκB-dependent transcription (DeWire et al., 2007).

1.6.1 Regulation of GPCR activity

GPCR activity is regulated through different mechanisms including interaction with regulatory proteins, desensitization, endocytosis, recycling, and trafficking.

Regulators of G protein signaling (RGS) proteins comprise a group of proteins that modulate GPCR signaling by acting as GTPase activating proteins (GAPs). RGS binds to the Gα subunit and accelerate its GTPase activity and thus terminating the GPCR signaling after agonist stimulation. This action causes reduced amplitude and duration of the G protein mediated signaling. Up to date there have been described 20 RGS proteins that differ in their specificity towards Gα isoforms and in their spatial and temporal expression. The potential importance of RGS, as GPCR

regulators, in disease has driven effort to pharmaceutically target RGS protein function (Kach et al., 2012; Sjogren, 2011).

Activation of a GPCR by its ligand initiates the process of receptor desensitization, an adaptive response used by cells to arrest G protein signaling, therefore preventing the potentially harmful effects that can result from persistent receptor stimulation. Agonist activated GPCR is phosphorylated by G protein-activated serine/threonine kinases (GRKs). Phosphorylation promotes high affinity binding of the β -arrestin, which physically prevents further coupling of the receptor to the G protein. Beta -arrestins also scaffold enzymes, such as phosphodiesterases and diacylglycerol kinases, which degrade second messengers generated by G protein coupling, thus providing additional mechanisms for efficient dampening of signaling (Perry et al., 2002; Shenoy and Lefkowitz, 2011). Phosphorylation of GPCRs by several other kinases, such as PKA, PKC, and c-Src can result in receptor desensitization. This process of desensitization involves a feedback mechanism in which the second messenger generated by the agonist-stimulated GPCR activates a kinase that decreases the activity of the receptor and ultimately attenuates production of the second messenger (Kohout and Lefkowitz, 2003).

Beta -arrestins bind several molecules involved in the machinery for receptor sequestration, including AP-2, clathrin, and *N*-ethylmaleimide-sensitive fusion protein. Thus, β -arrestins serve as adaptor molecules that target the GPCR to clathrin-coated pits followed by their internalization. GPCRs exhibit different patterns of agonist-induced β-arrestin interaction, which allow the receptors to be grouped into two distinct classes. Class A receptors (including the β2 and α1B adrenergic, μ opioid, endothelin A and dopamine D1A receptors) bind to β-arrestin 2 with higher affinity than to β-arrestin 1. They transiently recruit β-arrestin, traffic with it to clathrin-coated pits and then dissociate. Class A receptors then internalize without β-arrestin and are generally rapidly recycled. Class B receptors, represented by the angiotensin AT1a, neurotensin 1, vasopressin 2, thyrotropin-releasing hormone or neurokinin NK-1 receptors, bind to β-arrestin 1 and β-arrestin 2 with equal affinity. These receptors form stable complexes with β -arrestin, such that the receptor-β-arrestin complex internalizes as a unit that is targeted to endosomes (Reiter and Lefkowitz, 2006). Resensitization of the GPCR requires its dephosphorylation and dissociation from its ligand. Several lines of evidence support the hypothesis that receptor internalization is required for resensitization of many GPCRs (Luttrell and Lefkowitz, 2002).

1.6.2 Angiotensin receptors

Angiotensin II (AngII) is an octapeptide hormone which is an important part of the rennin-angiotensin system (RAS), the major regulator of blood pressure, electrolyte balance and renal, neuronal as well as endocrine functions related to cardiovascular control. AngII actions are mediated through binding to AngII receptors. There exist two AngII receptor subtypes, angiotensin II type 1 receptor

(AT1R) and type 2 receptor (AT2R). Although both receptors have similar affinity to AngII and belong to the same family of GPCRs, their function and AngII triggered signaling is different.

The AT1R is the most elucidated of angiotensin receptors and mediates the majority of all known physiological actions of AngII. Human genome contains a unique gene coding AT1R. In contrast, there are two isoforms of the AT1R in rodents, $AT1_A$ and $AT1_B$ receptors.

AT1R is one of the most promiscuous receptors of the GPCR family that activates large number of G protein dependent and independent signaling pathways. Several signaling pathways triggered by AngII through AT1R have been described. The most studied is the signaling through coupling to the $G\alpha q_{/11}$ protein activating Ca^{2+} signaling and PKC mediated pathway. The effects of $G\alpha q_{/11}$ protein mediated AT1R signaling varies in different tissues, including vasoconstriction, aldosterone release, renal Na^{+} reabsorption, adrenergic facilitation, vascular smooth muscle cells hypertrophy, and cardiac myocyte hyperplasia. *Via* coupling to $G\alpha_{11/12}$, AT1R activates PLC, phospholipase D, L-type Ca^{2+} channels, and Rho kinase (Hunyady and Catt, 2006). AT1R also couples to $G\alpha i_{/}o$ protein that inhibits adenylyl cyclase in some tissues, and in adrenocortical cells contributes to the regulation of voltage gated T- and L- type Ca^{2+} channels (Lu et al., 1996). AT1R was also shown to couple to $G\alpha_{12/13}$ which mediates AngII induced ROS production (Fujii et al., 2005).

AngII can also stimulate G protein-independent signaling pathways. Beta-arrestins scaffold and mediate AngII induced activation of ERK and JNK3. AT1R was further shown to activate Cdc42, Src, Jak/STAT, and transactivate EGFR in G protein independent way (Hunyady and Catt, 2006).

AT1R signaling activity is modulated by formation of homo- and hetero-dimers. In COS7 cells, AT1R was demonstrated to form constitutive homo-oligomers (Hunyady et al., 2002). AT1R dimerization appears to promote AT1R signaling and desensitization (AbdAlla et al., 2004). Heterodimerization of AT1R with the B2 bradykinin receptor enhances AT1R signaling response (AbdAlla et al., 2000). AT1R interaction with β-adrenergic receptor leads to formation of molecular complex in which blockade of the AT1R can inhibit signaling of the β-adrenergic receptor and *vice versa.* Association of AT1R with AT2R on the other hand inhibits its signaling activity and function (Hunyady and Catt, 2006).

AT2R is an atypical GPCR. It was shown to couple with Gαs in the absence of Gβγ. AT2R has been shown to activate protein phosphatases such as MAPK phosphatase 1 (MKP-1) and SH2 domain-containing phosphatase 1 (SHP-1). AT2R further stimulates nitric oxide synthase and nitric oxide production, and increases cyclic guanosine monophosphate synthesis. AT2R signaling has been demonstrated to promote apoptosis, inhibit AT1R mediated cell proliferation, lower blood pressure, and inhibits proinflamatory events (Berk, 2003).

1.6.3 Dopamine receptors

Dopamine is an important endogenous catecholamine which exerts widespread effects both in neuronal (as a neurotransmitter) and non-neuronal tissues (as an autocrine or paracrine agent). Within the central nervous system, dopamine binds to specific membrane receptors presented by neurons and it plays the key role in the control of locomotion, learning, working memory, cognition, and emotion (Beaulieu and Gainetdinov, 2011). Outside the central nervous system, dopamine receptors have been described in kidney, liver, heart (Dearry et al., 1990), or pancreas (Rubi et al., 2005). Renal dopamine is now recognized as an important regulator of Na^+ metabolism and electrolyte balance (Aperia, 2000).

Dopamine receptors, members of the GPCR family, are based on their structure and pharmacology divided into two subtypes, D1- like and D2-like receptors. D1-like receptors are further classified into D1 and D5, $D1_A$ and $D1_B$ in rodents, respectively. These are linked to the Gαs, which stimulates the activity of adenylyl cyclase and increase production of cAMP, which in turn activates PKA. By activating PKA, D1R can induce many important effects such as phosphorylation of dopamine and cAMP-regulated phosphoprotein of 32 kD (DARPP-32), which is a potent inhibitor of protein phosphatase-1 (Svenningsson et al., 2004). D1 receptors in addition couple to Gαq and activate phopholipase C and generate intracellular Ca^{2+} release (Beaulieu and Gainetdinov, 2011). In striatum, D1R has been shown to couple to the $G\alpha_{olf}$, which also stimulates adenylyl cyclase (Corvol et al., 2001).

D2-like receptors are subdivided into D2, D3, and D4 receptors, and are typically coupled to Gαi/Gαo that inhibits adenylyl cyclase activity. D1 and D2 receptors were shown to form heterooligomers coupled to Gαq, which on activation of both receptors activate phopholipase C and generate intracellular Ca^{2+} release (Beaulieu and Gainetdinov, 2011).

1.7 Dopamine and angiotensin control of natriuresis

Renal Na^+ metabolism, a major determinant of blood pressure, is regulated with great precision by an interaction between anti-natriuretic and natriuretic factors. Counter-regulatory dopaminergic and rennin-angiotensin systems belong to the key regulators of renal Na^+ balance. AngII is a potent regulator of Na^+, HCO_3^-, and water reabsorption in proximal tubule. AngII controls proximal tubular transport mainly through regulation of Na^+, H^+ exchanger (Banday and Lokhandwala, 2011), Na,K-ATPase (Aperia et al., 1994), and Na^+,HCO_3^- cotransporter (Geibel et al., 1990). AngII exerts biphasic effect on the Na^+ reabsorption that is partly mediated *via* regulation of Na,K-ATPase activity. In low pico- to nanomolar concentrations AngII stimulates, whereas in micromolar concentrations inhibits the activity of Na,K-ATPase (Aperia et al., 1994). The mechanism behind the biphasic effect of AngII on

Na,K-ATPase activity is unclear; however the stimulatory effect is traditionally attributed to the activation of PKC, and/or the decrease of the intracellular cAMP level (Bharatula et al., 1998; Efendiev and Pedemonte, 2006).

Dopamine opposes the antinatriuretic effects of AngII. Dopamine regulates the activity of various Na^+ transporters, including Na,K-ATPase and Na^+, H^+ exchanger, and promotes natriuresis. Dopamine is produced in renal tubular cells. The natriuretic effects of dopamine are mediated mainly through D1-like receptors. The downstream signaling includes production of cAMP, activation of PKA, and phosphorylation of cAMP-regulated phosphoprotein DARPP32, a potent inhibitor of PP1A. Dopamine activation of PLC and PKC was also demonstrated in renal proximal tubules. PKA and PKC phosphorylate Na,K-ATPase and modulate its activity. The effect of PKA and PKC on Na,K-ATPase activity is highly dependent on the intracellular Ca^{2+} concentrations (Cheng et al., 1999).

The dopamine and angiotensin systems are closely connected. Dopamine inhibits AngII induced Na^+ retention, while AngII decreases dopamine uptake in kidney (Choi et al., 2006). Long-term dopamine exposure is known to decrease AT_1 receptors (AT1R) in renal proximal tubular cells (Cheng et al., 1996). Furthermore, studies by (Zeng et al., 2005) have shown that long-term stimulation of AT1R results in an upregulation of D1-like receptors (D1R). AT1R and D1R have been shown to form heterodimers and decrease the expression of each other after long term stimulation of either of them with agonist (Zeng et al., 2003).

2 SPECIFIC AIMS

This thesis examined a role of protein-protein interaction on the function of the canonical and non-canonical signaling pathways that trigger IP_3R mediated Ca^{2+} signaling. The specific aims of this thesis were:

- To explore the role of AT1R/D1R heterodimerization on the short-term regulation of AT1R and D1R G protein-coupled signaling

- To investigate the role of cytoskeleton associated protein, ankyrin B, in ouabain-triggered Ca^{2+} signaling through Na,K-ATPase/IP_3R signaling complex.

- To study the role of 20 kD C-terminal fragment of agrin as a potential activator of Na,K-ATPase signaling.

3 METHODOLOGICAL CONSIDERATIONS

3.1 RNA interference

In 2006 Andrew Fire and Craig C Mello were awarded with the Nobel Prize in Physiology and Medicine for describing a novel regulation of gene expression in *C.elegans*, called RNA interference (RNAi). Since this phenomenon was described in 1998, the use of small RNAs became a powerful and widely used method for down-regulation of gene expression. There are two main types of the RNA molecules that can mediate gene silencing, micro RNA (miRNA) and small interfering RNA (siRNA). The miRNA and siRNA differ in the mechanism of gene expression regulation. While miRNA binds to the target mRNA and inhibits its translation, siRNA recognition of target mRNA leads to mRNA degradation. In this thesis siRNA technique has been used for silencing of the ankyrin B gene in COS7 cells.

Small interfering RNA is a short double stranded RNA. It originates from the double stranded RNA (dsRNA) that is recognized by the ribonucleotide protein Dicer which binds and cleaves dsRNA into 20-25 nucleotide fragments of double stranded siRNA. siRNA is recognized by and incorporated in a protein complex RNA-induced silencing complex (RISC). siRNA serves as a guide for RISC to identify the sequence of the target mRNA. Upon perfect base-pair match, RISC, which contains endonucelases, cleaves the target mRNA in the middle of the duplex formed with the siRNA.

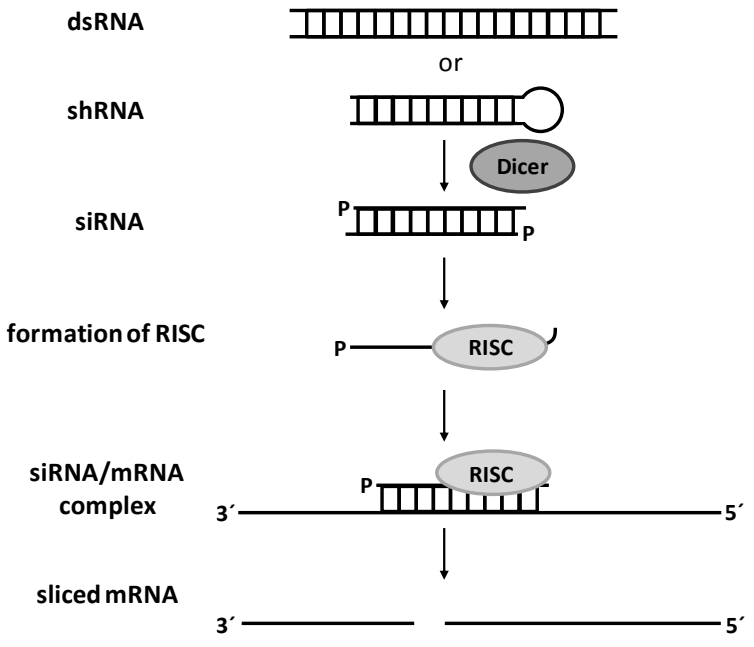

Fig 3.1 *Mechanism of RNA interference*

siRNA can be used for down-regulation of gene expression both *in vivo* and *in vitro.* siRNA can be introduced to the cells either directly in the form of double stranded siRNA or as a plasmid DNA that encodes double stranded hairpin RNA (shRNA), which can be recognized and cleaved by DICER. In our study we choose to use siRNA that was transfected to the COS7 cells. The advantages of siRNA in this system are a higher transfection efficacy and better control over the siRNA concentration. Although siRNA is considered to be highly specific, a variety of controls need to be followed. In 2003, Nature Cell Biology published a list of recommended controls for RNAi experiments (2003). In our work we followed three out of five suggested controls. Both mismatch and scrambled control siRNAs were used. The mismatch and target siRNA sequences differed in three nucleotides. Besides that, siRNA targeting rat ankyrin B, but not monkey ankyrin B, was used as a scrambled siRNA control. We examined the ankyrin B expression both on protein and mRNA level. This control ensured that the ankyrin B downregulation occurs *via* degradation of mRNA and not through inhibition of protein translation. As siRNA can be often effective already at minimal concentrations, titration of the siRNA is recommended to reduce the chance of unspecific effects. We tested the minimal concentration of siRNA that would give the maximal effect of the ankyrin B down-regulation. Further it is recommended to use functional "rescue" control by expression of the target gene in a form refractory to siRNA. In ideal situation the expression of the rescue gene should be in a physiological range. To enhance a confidence in RNAi data a similar effect of two or more siRNAs can be demonstrated.

3.2 Ca^{2+} imaging

The development of the fluorescent Ca^{2+} indicator dyes has to a great extent contributed to our understanding of the importance and mechanisms of Ca^{2+} signaling in the cell. Ca^{2+} indicators change their spectral properties when bound to free Ca^{2+}. There exists a number of Ca^{2+} specific dyes and genetically encoded Ca^{2+} indicators that differ in their excitation and emission spectra, Ca^{2+} sensitivity, kinetics of Ca^{2+} binding, as well as subcellular localization (e.g. cytosolic, mitochondrial, nuclear).

The carboxylic acid of the Ca^{2+} specific dyes can be modified with acetoxymethyl (AM) ester group that enables the dye to permeate the cell plasma membrane. Once inside the cell, the lipophilic groups are cleaved off by non-specific esterases, resulting in a charged molecule that is less prone to leak out of the cell. The AM modified dyes enable easy and non-destructive cell loading, providing sufficient intracellular dye concentrations to measure the changes of cytosolic Ca^{2+} in a time course up to several hours. When imaging changes in intracellular Ca^{2+}, several considerations must be taken. The Ca^{2+} sensitivity of the dye must be high enough to detect Ca^{2+} changes and low enough to avoid saturation. Since Ca^{2+} dyes

function as Ca^{2+} buffers, the concentration of the dye should be optimized to not buffer all free Ca^{2+}. The frequency of the image acquisition should be high enough to record all the Ca^{2+} changes but at the same time it should be as low as possible to reduce phototoxicity.

In this thesis fura-2 AM and fluo-4 AM Ca^{2+} dyes were used to record cytosolic Ca^{2+} changes. Fura-2 AM is a ratiometric dye that requires UV light excitation. In the Ca^{2+} bound state it is excited at the wavelength of 340 nm and in the Ca^{2+} unbound state at the wavelength of 380 nm. The emission wavelength maximum is at 510 nm. The ratiometric properties make fura-2 measurements less sensitive to focus shifts and bleaching than single-excitation dyes. The UV excitation spectrum enables the combination of fura-2 with a variety of other dyes or fluorescent proteins which are excited with visual light. On the other hand, high levels of UV light are cytotoxic and therefore using other Ca^{2+} sensitive dye with visual light excitation spectrum should be considered when more sensitive cells are imaged or when cells are exposed to high levels of UV light for long periods of time. Fura-2 AM was used to monitor ouabain and AngII-induced Ca^{2+} signaling in COS7 cells and primary cultures of renal proximal tubules. In both models the sensitivity of fura-2 was adequate to the Ca^{2+} response. Since primary hippocampal neurons display high frequency spontaneous Ca^{2+} activity, requiring high acquisition rate, we used fluo-4 AM instead of fura-2 to avoid exposure of the neurons to UV light. Fluo-4 excitation maximum is at the wavelength of 488 nm and the emission maximum is at the wavelength of 510 nm. Line scanning confocal microscopy, which enables high speed image acquisition, was used to image fluo-4 in neurons. The primary neuronal culture contains a heterogeneous cell population consisting of neuronal and non-neuronal cells. To functionally distinguish between neuronal and non-neuronal cells, the ability of the neuronal cells to respond to treatment with NMDA with elevated Ca^{2+} level was examined in the end of each experiment.

3.3 Ca^{2+} data analysis

Ca^{2+} signaling can be very complex, encoded both in amplitude and frequency and Ca^{2+} oscillations can be formed by a sum of individual frequency components. Power spectrum analysis is used to extract these individual frequency components. Spectral analysis based on the Fourier transform was used to analyze Ca^{2+} oscillations triggered by ouabain and agrin in COS7 cells. The Ca^{2+} oscillations observed in neurons consisted of two frequency components superimposed on each other. Since the difference between the slow and fast frequency component was big, the moving average filter was applied on the data to isolate the slow frequency component. The high frequency component was then isolated by subtracting the slow component from the raw data. The slow frequency component was then analyzed using spectral analysis.

3.4 Na,K-ATPase activity assays

The activity of the Na,K-ATPase can be assessed using variety of methods. The most common assays, which were used in this thesis, are measurement of the rubidium uptake and the ATP hydrolysis measurement.

In rubidium uptake assay the amount of radioactive isotope $^{86}Rb^+$, which refers to the amount of K^+, taken up by the cells is measured. The ouabain sensitive Rb^+ uptake is measured in order to isolate Rb^+ uptake mediated by Na,K-ATPase from the Rb^+ uptake mediated by other ion channels. The great advantages of this assay are its sensitivity and the fact that it is performed on intact cells. The obvious drawback of this method is use of radioactive isotope that is source of both β and γ radiation. This can be bypassed by detection of non- radioactive Rb^+ uptake using flame photometry. However the sensitivity of the method would be significantly decreased in this case. Another limitation of this method is that it assesses the bulk activity of the Na,K-ATPase, giving no information about the differences between individual cells. Therefore it is impossible to determine the Na,K-ATPase activity in specific cell type in the heterogenic cell population using this method.

The measurement of the ATP hydrolysis is the most commonly used method to determine Na,K-ATPase activity. The production of inorganic phosphate Pi from ATP is measured in the permeabilized cells or on membrane preparations. To distinguish between ATPase activity of Na,K-ATPase and another ATPases, ouabain is used. This approach is optimal to measure the kinetic properties of the Na,K-ATPase in different conditions, such as different ion concentrations. The drawback of this method is that it does not measure ATP hydrolysis in intact cells and that it does not distinguish between the ATPase activity of the plasma membrane and the intracellular Na,K-ATPase. The concentration of inorganic phosphate (Pi) as a product of ATP hydrolysis is measured to quantify the ATPase activity. Pi can be either detected with colorimetric reaction and quantified using spectrophotometer, as we did in our study, or concentration of radioactive [^{32}P], as a product of [^{32}P]-ATP hydrolysis, can be measured with liquid scintillation counter.

Another method that can be used to indicate the changes of Na,K-ATPase activity is the measurement of intracellular Na^+ using Na^+- sensitive fluorescent dyes. This method was not used in this thesis; however it is discussed in the third study. Since the Na,K-ATPase is the main determinant of the intracellular Na^+ level, the changes of its activity can be reflected in the changes of the basal intracellular Na^+. This method enables monitoring of the intracellular Na^+ level on the single cell, or even subcellular level. This is very beneficial when highly differentiated cells, such as neurons, are studied. The activity of Na,K-ATPase is however assessed indirectly since there are many other factors that influence the changes of intracellular Na^+ level.

3.5 Identification of the protein-protein interaction

In studies presented in this thesis, the protein-protein interaction plays an essential role in the regulation of function of both Na,K-ATPase and angiotensin

receptor. There exists a big variety of biochemical, imaging, and biophysical methods that help to identify proteins forming a protein complex. In this thesis a combination co-immunoprecipitation and GST (glutathione S-transferase) pull down assay was used to identify the interacting proteins and to describe their binding regions.

Co-immunoprecipitation (co-IP) belongs to the golden standard assay for the study of the protein-protein interaction. It is considered to be very specific method, especially when endogenous proteins (not overexpressed) are studied. One of the limitations of this method is the availability of specific antibodies. Not all antibodies that show high specificity in other immuno-assays are suitable for co-IP. Moreover some of the antibodies might interfere with the protein binding site. The co-IP assay can be used only to verify a predicted interaction between two proteins. In order to screen for new protein partners, co-IP can be combined with mass-spectrometry. Some of the protein-protein interactions are transient or weak and can be disrupted during the co-IP protocol. The protein-protein interaction can be stabilized by chemical cross-linking that introduces the covalent bindings between two proteins in close proximity. Co-IP gives information about the composition of the protein complex, but can´t confirm direct interaction. The binding of proteins that co-immunoprecipitate together might be mediated by another intermediate protein, and thus co-IP assay can´t confirm whether two proteins interact directly.

GST pull down assay uses the affinity between glutathione S-transferase (GST) and glutathione. The most common application for GST pull down is to map the protein regions that are responsible for protein-protein interaction. GST-fused protein fragments are produced in bacteria and purified using glutathione covered beads. These beads with bound GST-proteins can be incubated with cell or tissue lysate containing protein of interest. In this case GST pull down does not give information whether the GST-protein and protein from the lysate interact directly. To address this question, GST-pull down assay can be modified to so called *in vitro* binding assay to examine the binding between purified GST-protein fragment and purified protein of interest, or purified protein fragments. GST-pull down assay is useful for the screening of the binding regions; however the 3D structure of the protein is often important for the effective binding, often several protein regions that do not directly adjoin form a binding pocket. This must be considered when the results from the GST-pull down are interpreted to avoid false-negative or false-positive conclusions.

In both assays the use of proper controls assures specificity of the obtained results. In co-IP assay the unspecific IgG of the same species origin and the same isotype as a specific antibody against the protein of interest is used. In GST-pull down, beads covered with GST protein are used as a control. The use of the GST-protein fragment with the mutated binding site gives higher confidence that the observed binding is specific.

3.6 NF-κB activation

The downstream effect of the ouabain-induced slow Ca^{2+} oscillations is activation of NF-κB transcription factor. There exist several assays monitoring NF-κB activity. In present thesis we used immunocytochemistry to detect the translocation of NF-κB to the nucleus and ELISA assay detecting NF-κB binding to its target DNA sequence. We used the immunocytochemistry labeling of p65 subunit of NF-κB for qualitative monitoring of p65 subcellular localization. Measurement of intensity ratio between cytoplasm and nucleus can be used for data quantification. In this thesis we used ELISA assay to quantify the p65 activity in control and ankyrin B knock-down cells. The nuclear extracts were incubated with the oligonucleotides containing p65 consensus sequence immobilized to the 96-well plate. The bound p65 was detected using horse radish conjugated antibodies and chemiluminescence. Since we have used homogenous COS7 cell population, the quantification of NF-κB activity using ELISA assay, provided very sensitive and reproducible way to measure ouabain-induced NF-κB activity.

3.7 cAMP production assay

cAMP is a second messenger produced from ATP by adenylyl cyclase. The cAMP production measurement is used to monitor the signaling activity of Gαs and Gαi coupled GPCRs. In our study, cAMP production was measured to determine the signaling capacity of D1R. We used an ELISA based commercial available assay which enables detection of cAMP synthesis in cells as well as tissue. During the treatment of the cells it is possible to include IBMX, phosphodiesterase inhibitor, to avoid degradation of the cAMP. The cells are lysed in 0.1 HCl which itself inhibits phosphodiesterase activity. This assay is sensitive; however it measures only cumulative production of the cAMP. The information about the cAMP production dynamics would be rather difficult to obtain using this method.

Recently, fluorescent protein indicators of cAMP have been developed enabling real time cAMP detection in the living cells. This approach is particularly interesting when combined with indicators of other second messengers, such as Ca^{2+}, to study the cross-talk between different signaling pathways.

3.8 Transfection and over-expression of the proteins

To study the changes of signaling and pumping function of the Na,K-ATPase α3 and of the ouabain insensitive mutant of Na,K-ATPase α1, we used transient transfection and over-expression of exogenous Na,K-ATPase in COS7 cells. The over-expression system is considered to be artificial, since the expression level of the exogenous protein is several times higher than the expression of endogenous protein. This can affect the protein distribution and function. However the amount of the plasma membrane Na,K-ATPase is limited by the expression of its β subunit, which targets Na,K-ATPase to the plasma membrane. Competition between the overexpressed exogenous and endogenous α subunits for binding to the

β subunit assures, that the Na,K-ATPase will be expressed in the plasma membrane in a physiological level.

To specifically inhibit the signaling function of Na,K-ATPase with minimal effect on its pumping function, the overexpression of dominant negative peptide to disrupt Na,K-ATPase/IP_3R interaction was used. This strategy was used previously (Zhang et al., 2006), however in this work we improved the design of the dominant negative peptide to make it more specific. We used the whole length N-terminus of Na,K-ATPase and we fused it to the transmembrane domain of syntaxin. This ensured that this N-terminus of Na,K-ATPase is localized in the plasma membrane and mimics the endogenous protein localization. The overexpression of the fluorescent proteins usually results in overloading of the cellular expression system that yields a large amount of the exogenous protein in the intracellular compartments. That makes it difficult to distinguish between the plasma membrane and intracellular fluorescent signal in flat cells, such as COS7 cells. Therefore we attached pHluorin fluorescent protein to the extracellular tail of syntaxin. pHluorin fluorescence is eclipsed in a low pH, and therefore it is invisible in the intracellular compartments. On the other hand the pHluorin facing the extracellular space is fluorescent and thus the plasma membrane localization of pHluorin fused protein is easy to monitor.

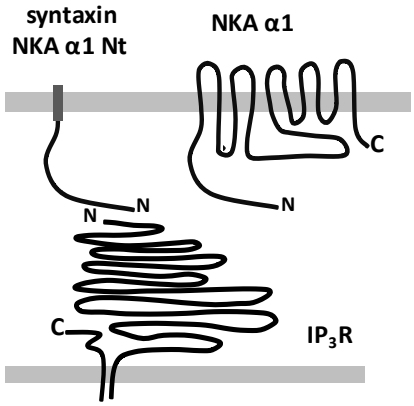

Fig 3.2 *Competition between syntaxin fused N-Terminus of Na,K-ATPase α1 and endogenous Na,K-ATPase α1for binding to the IP_3R*

3.9 Fluorescence recovery after photobleaching (FRAP)

FRAP is an imaging method that measures the mobility of fluorescently labeled proteins. It is widely used to measure the lateral diffusion of proteins in the plasma membrane. In principle the fluorescent signal in the defined region of the plasma membrane is bleached with a high power laser beam and the fluorescence recovery is monitored over time. One of the parameter that characterize protein mobility is the

recovery half time, which represents the time of recovery of the fluorescence intensity to the 50% level of the intensity before bleaching. FRAP can also determine the mobile pool of the diffusing protein, which represents the percentage of the maximal fluorescence recovery compared to fluorescence intensity before bleaching. The mobile pool of the protein is dependent on the amount of fluorescent protein in the plasma membrane that is not anchored and can enter the bleached area by lateral diffusion. In this thesis FRAP was used to examine if down-regulation of ankyrin B effects the size of the Na,K-ATPase mobile pool in the plasma membrane. We transiently transfected COS7 cells with GFP-fused Na,K-ATPase α1. However, since the overexpressed fluorescent protein filled the intracellular compartments it was difficult to differentiate between plasma membrane and intracellular fluorescent signal. To bypass this problem, we detached the cells from the culture plate surface in order to reach the spherical shape of the cell. This improved the ratio between the fluorescent signal in plasma membrane and cytoplasm.

3.10 Cell surface biotinylation

Internalization and trafficking is one of the mechanisms of the regulation of the plasma membrane receptor function. Biotinylation of the cell surface can be used to monitor the amount of the protein in the plasma membrane. Biotin can be covalently conjugated to the proteins of the cell surface of non-permeabilized cells. The biotin labeled proteins can be purified using streptavidin, which has high affinity to biotin. The bond between biotin and proteins is resistant to heat, pH, and proteolysis which enables to capture of biotinylated molecules in a wide variety of environments (Hermanson et al., 1994). Cell surface biotinylation will only provide information about the amount of the protein in the plasma membrane in the whole population of the cells. When there is question about changes of protein content in specialized plasma membrane compartments, another approach must be taken. In this thesis cell surface biotinylation was used to examine if ankyrin B downregulation affects the amount of plasma membrane Na,K-ATPase and to study the effects of AT1R and D1R activation on their internalization.

4 RESULTS AND DISCUSSION

4.1 Reciprocal interaction between AT1R andD1R

4.1.1 Results and comments

Angiotensin and dopamine represents counterregulatory system controlling blood pressure. AT1R and D1R have been shown to form a heterodimer in immortalized proximal tubule cells. Effects of chronic activation of D1R on the AT1R expression have been described previously (Zeng et al., 2005). However, since the Na^+ excretion must be regulated over a short time frame, we examined in this study the role of AT1R-D1R heterodimerization in the short-term regulation of AT1R and D1R function.

Co-immunoprecipitation study showed that the D1R and AT1R form a protein complex in the outer layer of the kidney cortex that is mainly composed of proximal tubules. GST fused C-terminal region of the D1R containing S417-T446, pulled down AT1R from the renal cortical lysate. Substitution of serines S397 and S398 with alanines significantly reduced the binding of AT1R.

To test whether the activation of either of the receptors affects AT1R-D1R interaction, outer renal cortical slices were treated for 15 min either with AngII or SKF 81297, an agonist of D1R. Co-immunoprecipitation assay demonstrated that exposure to either AT1R or D1R agonist resulted in a reduction of AT1R-D1R interaction. Unlike in the previous study by Zheng et al. (2003), the total amount of the AT1R and D1R protein was not affected. Short term exposure to AT1R or D1R agonists led to the changes in the subcellular distribution of the receptors. AngII treatment led to D1R internalization and exposure to the SKF 81297 resulted in the AT1R internalization.

Next we examined the functional significance of AT1R-D1R interaction. AT1R is typically coupled to Gαq. Activation of the AT1R induces PLC activity, production of IP_3 and DAG, and activation of the IP_3R, which results in Ca^{2+} release from ER in a form of a Ca^{2+} transient. Activation of the D1R with SKF 81297 rapidly and reversibly disabled AT1R capacity to induce Ca^{2+} signaling in proximal tubule cells. The signaling capacity of the D1R was determined by measurement of cAMP production, as D1R couples to the Gαs which activates adenylyl cyclase. Pretreatment of renal cortical slices with AngII on the other hand abolished SKF 81297 induced cAMP production.

The dopamine D1-like receptor family includes D1 and D5 receptors. It is not possible to distinguish between these two receptors using pharmacological approaches. Both D1R and D5R are present in proximal tubules and the function of

both receptors is implied in the blood pressure control. The lack of D5R leads to an increase of the blood pressure (Asico et al., 2011). Activation of D5R promotes AT1R degradation (Gildea et al., 2008; Li et al., 2008). In present study we have shown that the C-terminus of D1R interacts with AT1R. We however did not examine whether D5R is able to interact with AT1R as well. In the follow up study, Li et al. (2012) demonstrated that the signaling function and plasma membrane distribution of D1R, co-transfected into the HEK293 cells with AT1R, is affected by losartan, the AT1R specific inhibitor. This supports the notion that the effects observed in our study were mediated by D1R. However, the contribution of the D5R signaling on the regulation of the AT1R can´t be excluded.

Mechanism underlying the heterologous desensitization of the AT1R and D1R remains elusive. The initial rapid and reversible desensitization of AT1R is unlikely to be explained by the receptor internalization. Classical mechanism of the GPCR desensitization is mediated through phosphorylation of activated receptor by GRKs, binding to the β-arrestin proteins that physically prevent coupling of the receptor to the G protein, and subsequent receptor internalization. The time course of the AT1R desensitization is very rapid (in a range of minutes) and would correspond to the time scale observed in the AT1R-D1R heterologous desensitization. AT1R belongs to the group B of GPCRs that is characterized by stable coupling of activated receptor to the β-arrestin, leading to internalization and degradation rather than recycling back to the plasma membrane (Reiter and Lefkowitz, 2006; Shenoy and Lefkowitz, 2011). We have observed that the SKF 81297 treatment completely abolished AngII induced Ca^{2+} signaling which was recovered as soon as the SKF was washed out. The kinetics of the AT1R signaling recovery after complete desensitization by D1R activation is much faster than the recovery after AngII induced desensitization (partial recovery of AngII induced Ca^{2+} signaling in rat primary proximal tubule cells was observed after 30 min, unpublished observation) . Therefore it is not likely that the mechanism behind AT1R homologous and heterologous desensitization is the same.

A number of protein kinases that phosphorylate and desensitize dopamine D1 receptor, including PKC (Rankin and Sibley, 2010), GRK4, PKA, ERK1/2, has been described (Xu et al., 2013). It is now well documented that GRK4 is responsible for the renal D1R phosphorylation and desensitization. Polymorphisms in GRK4 gene that lead to increased GRK4 expression are associated with hypertension. AngII treatment has been shown to induce c-myc dependent increase of GRK4 expression (Gildea et al., 2013). The role of GRK4 in the short-term regulation of D1R function by AngII is however elusive.

The homologous and heterologous desensitization might be mediated by a different set of protein kinases acting on different phosphorylation sites. It is of interest that the serines 397 and 398 on the D1R C-terminus, which we demonstrated to be of importance for interaction between D1R and AT1R, are PKC phosphorylation sites that contribute to D1R desensitization (Rankin and Sibley,

2010). Since AT1R activation induces PKC activity, we can´t exclude that the phosphorylation of D1R at PKC sites contributes to AngII induced D1R desensitization. D1R has been shown to be constitutively phosphorylated (Rankin et al., 2006). It has been demonstrated that D1R is hyperphosphorylated in proximal tubules of hypertensive rats (Yu et al., 2006). Thus we can speculate that nonphosphorylated D1R binds inactive AT1R. AT1R activation might induce a signaling cascade that leads to D1R phosphorylation, resulting in a potential uncoupling of D1R from its signaling pathway, and its internalization. The role of the phosphorylation in AngII-induced D1R desensitization needs to be examined.

GPCRs are natural allosteric proteins because agonist mediated signaling requires a conformational change that is transmitted between two topographically distinct binding sites, one for the agonist and another for the G protein. There has been recently described a number of allosteric modulators that alter binding and signaling properties of GPCRs. Study by Li and co-workers (2012) suggests that the binding of AT1R has allosteric effect on D1R signaling. Losartan, a highly specific agonist of AT1R, increased the AT1R/D1R binding, induced recruitment of D1R into the plasma membrane and most importantly induced D1R dependent cAMP production in the absence of D1R agonist. Losartan was however not able to transactivate D1R mutated at S397 and S398. Interestingly, losartan was able to induce maximal D1R activity, as co-treatment with SKF did not exert any additive effect on cAMP production. These findings support the hypothesis that the mechanism of the reciprocal regulation between AT1R and D1R is dependent on the conformational changes induced by binding of the receptor agonist and/or antagonist.

4.1.2 Future perspectives

The interaction between AT1R and D1R seems to be critical for the reciprocal regulation between the receptors. It is however not clear whether the regulation is purely mediated by conformational changes after agonist binding or the activation of the signaling of one of the receptor is required to inhibit the signaling function of the other one.

AT1R blockers are nowadays effectively used to treat patients with hypertension. AT1R signaling *via* β-arrestin has been associated with cellular effects such as protection from apoptosis, cell proliferation and migration (Violin and Lefkowitz, 2007). Angiotensin induced β-arrestin signaling promotes cell survival during acute cardiac injury (Kim et al., 2012). Therefore there is a big effort to develop biased ligands of AT1R, which activates β-arrestin mediated signaling and uncouple AT1R from G protein-coupled signaling, combining the properties of AT1R blockers with the beneficial β-arrestin signaling. The activation of D1R leads to the uncoupling of AT1R from the G protein coupled signaling pathway. It would be of interest to examine whether the β-arrestin signaling pathway remains intact.

Li et al. (2012) showed that antihypertensive effect of losartan is attenuated when administered together with D1R antagonist. These results demonstrate the functional importance of the allosteric interaction between AT1R and D1R *in vivo*

and imply that attempts should be made to develop a low-dose combination therapy of AT1R antagonists and D1R agonists in the treatment of hypertension.

4.2 The role of ankyrin B in the Na,K-ATPase/IP3R signaling

4.2.1 Results and comments

In this study we have shown that ankyrin B forms complex with Na,K-ATPase and IP_3R in COS7 cells. We have described new ankyrin B binding regions for both Na,K-ATPase and IP_3R. Second and third cytoplasmic domains of Na,K-ATPase have been previously reported to bind ankyrin R and ankyrin G isoforms (Devarajan et al., 1994; Jordan et al., 1995). However the ankyrin B binding region have had not been described previously. Since the N-terminus of Na,K-ATPase binds directly to the IP_3R we choose to examine whether the same region binds also to the ankyrin B. We have found that the GST-fused N-terminus of Na,K-ATPase is able to pull down purified ankyrin B. Bourguignon and Jin (1995) reported a portion of C- terminus of the IP_3R to be an ankyrin binding site. However this motif is located close to the IP_3R ion channel pore and therefore it is unlikely to be available for protein interaction. We found that the N-terminus portion of IP_3R, consisting of the suppressor domain and IP_3 binding domain, directly interacts with purified ankyrin B. Our finding was later confirmed by Kline et al. (2008).

To study the role of ankyrin B in the Na,K-ATPase/IP_3R signaling, expression of ankyrin B was down-regulated using siRNA. The 80% reduction of ankyrin B protein expression, reached with siRNA, resulted in decrease of Na,K-ATPase/IP_3R interaction.

To test whether the reduced interaction between Na,K-ATPase and IP_3R will be reflected on the capacity of ouabain to activate IP_3R, the changes of the intracellular Ca^{2+} in response to ouabain were recorded in ankyrin B down-regulated COS7 cells. The number of ankyrin B silenced cells that responded to ouabain with Ca^{2+} oscillations was reduced and the remaining oscillations assigned lower mean frequency and regularity compare to control cells. The power spectrum analysis revealed that while Ca^{2+} oscillations in control cell were highly regular with one frequency, the Ca^{2+} oscillations in ankyrin B down-regulated cells consisted of several frequency components (Fig 4.1).

Although the ouabain-induced Ca^{2+} signaling in ankyrin B down-regulated cells was not completely abolished, there was virtually no detectable change in the NF-κB activity in a response to ouabain treatment.

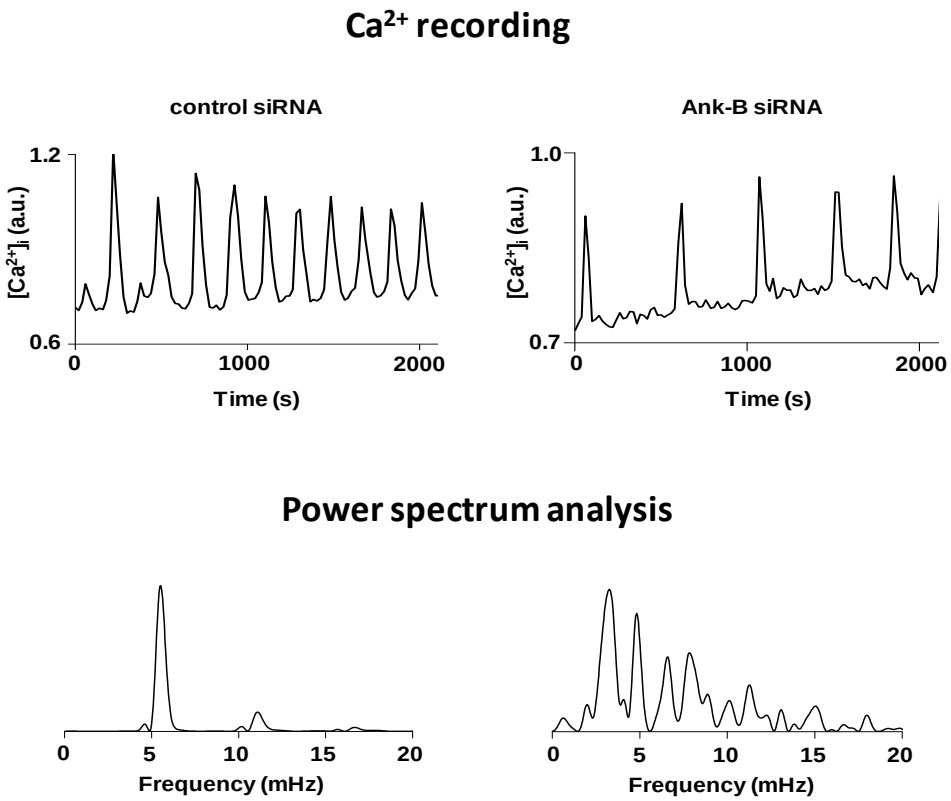

Fig 4.1 *The ouabain-triggered Ca^{2+} signaling is deregulated in COS7 cells with down-regulated ankyrin B expression.* Representative traces of ouabain (20 pM) induced Ca^{2+} oscillations and corresponding power spectral analysis in cells transfected with control or ankyrin B targeting siRNA

It is clear that ankyrin B plays an essential role in the formation of the functional ouabain/Na,K-ATPase/IP_3R signaling complex. The exact mechanism how ankyrin B exerts this function is elusive. Miyakawa-Naito et al. (2003) has shown that a portion of Na,K-ATPase and IP_3R constitutively interact even in the absence of ouabain. This interaction is however weak or transient since it does not result in IP_3R activation. Ouabain increases the formation of a complex between Na,K-ATPase and IP_3R (Miyakawa-Naito et al., 2003) and we have demonstrated that this increase is dependent on the presence of ankyrin B. Ankyrin B can thus provide a scaffolding function that stabilizes the ouabain-induced conformational changes of Na,K-ATPase and IP_3R and physically strengthen the binding between Na,K-ATPase and IP_3R.

Ankyrin B is cytoskeleton associated protein that has been shown to tether Na,K-ATPase and IP_3R into the specialized functional microdomains. In the lymphocytes it tethers IP_3R into the lipid rafts and thus promotes Ca^{2+} signaling (Singleton and Bourguignon, 2004). A pool of Na,K-ATPase has been shown to be located in the caveolae lipid rafts. Ankyrin B can thus tether and stabilize Na,K-

ATPase and IP_3R in caveolae and thus enables their interaction and promotes ouabain-induced signaling.

The work by Feldmann et al. (2011) suggests the role of NCX in the cardiotonic steroid-induced slow Ca^{2+} oscillations in the COS7 cells. Since ankyrin B has been reported to tether Na,K-ATPase, IP_3R, and NCX into the microdomain in the cardiomyocytes (Mohler et al., 2005), it can´t be excluded that NCX forms a complex with Na,K-ATPase, IP_3R, and ankyrin B in COS7 cells. The potential loss of NCX from the close proximity of Na,K-ATPase and IP_3R in ankyrin B down-regulated cells may contribute to the dysregulation of the ouabain-induced Ca^{2+} signaling.

The pattern of the Ca^{2+} oscillations can be modulated by the interaction of IP_3R with other proteins. Therefore the binding of ankyrin B to the IP_3R might directly affect some of the IP_3R properties, such as Ca^{2+} affinity, and this way contribute to the typical pattern of the Ca^{2+} oscillatory signal induced by ouabain.

Ankyrin G is known to bind to the Na,K-ATPase, promote its basolateral distribution in polarized epithelial cells, and regulate its vesicular trafficking (Morrow et al., 1989; Stabach et al., 2008). We have not observed any implications of such a role of the ankyrin B in COS7 cells as we did not observe any changes in the plasma membrane distribution, no sign of increased internalization, or the changes in the pumping activity of the Na,K-ATPase in the ankyrin B down-regulated cells.

4.2.2 Future perspectives

Our group has shown that the IP_3R can be activated in the absence of IP_3 through the interaction with Na,K-ATPase and conformational changes induced by binding of ouabain. In a number of studies, ouabain has been shown to stimulate also tyrosine kinase activity. Recent study by Fontana et al. (2013) suggests the role of Src kinase in the ouabain-induced Ca^{2+} oscillations in COS7 cells. The application of the Src kinase inhibitor resulted in the failure of ouabain to trigger Ca^{2+} oscillations in COS7 cells. There is a time gap between application of ouabain and initiation of the Ca^{2+} oscillations. Activation of the Src kinase might be therefore an initial step in the ouabain-induced Ca^{2+} oscillations. Further studies need to be performed to elucidate the connection between Src activity and ouabain-induced Ca^{2+} oscillations.

Ouabain is an endogenous hormone that is produced in adrenal glands (Laredo et al., 1994). The circulating concentrations of the endogenous ouabain are in the pico- to nanomolar range (Dvela et al., 2012; Vakkuri et al., 2001). At these concentrations, ouabain would have minimal effect on the Na,K-ATPase pumping function. This leads to the hypothesis that the main physiological function of ouabain is the activation of the Na,K-ATPase signaling. Significantly higher levels of the endogenous ouabain have been observed in many patients with congestive heart failure, essential hypertension, renal failure and some cancers. The levels of endogenous ouabain are elevated in the conditions requiring extensive cell growth

and differentiation. Higher circulating levels of ouabain were identified in pregnancy (Vakkuri et al., 2000) and in newborns (Gooz et al., 2004). The tissue protective effects of ouabain-induced signaling in kidney have been described in several pathological conditions *in vitro* as well as *in vivo* (Li et.al, 2010, Burlaka et al.,2013). The role of Na,K-ATPase signaling in the physiological conditions remains elusive. Our group has demonstrated that ouabain protects cultured embryonic kidneys from serum starvation-induced apoptosis. Application of ouabain to the pregnant rat subjected to a low protein diet rescued the development of the embryonic kidneys. To dissect the importance of the physiological role of Na,K-ATPase signaling *in vivo* the animal model needs to be studied. The generation of the transgenic mouse expressing Na,K-ATPase α1 with inserted mutation in the IP_3R binding motif is in the progress.

Although there have been many reports describing the signaling function of Na,K-ATPase, there are many open questions concerning the dual function of Na,K-ATPase. In 2007 Liang et al. described a pool of non-pumping Na,K-ATPase in LLC-PK1 cells. Authors showed that this non-pumping pool that is located in the caveolae possesses signaling function. Furthermore, by disruption of the caveolae structures it was possible to convert the signaling pool of Na,K-ATPase to pumping pool. This finding was opposed by (Liu et al., 2011), showing that the alveolar fraction of Na,K-ATPase keeps the pumping function. In any case, there are many indications that the interaction of Na,K-ATPase with other membrane or cytoplasmic proteins is crucial determinant of the Na,K-ATPase function.

4.3 The role of agrin in regulation of Na,K-ATPase function

4.3.1 Results and comments

In 2006 Hilgenberg et al. published a study describing the proteoglycan agrin as a new endogenous ligand of Na,K-ATPase α3. Since cardiotonic steroids were to date the only known Na,K-ATPase ligands, this finding was rather sensational and attracted a lot of attention. Hilgenberg et al. showed that a 20 kD C-terminal fragment of agrin cross-linked specifically to the α3 isoform of Na,K-ATPase and inhibited its ion transporting capacity. Many effects of agrin in neurons were described, including signaling events such as Ca^{2+} release from intracellular stores, c-foes activation, CREB activation. It seems unlikely that these effects can be explained simply by inhibition of Na,K-ATPase pumping function. Therefore we decided to test the possibility that agrin activates the signaling function of Na,K-ATPase α3.

We established the collaboration with the group of Martin Smith and obtained expression plasmid and protocol to purify 20 kD agrin fragment (agrin C20) in bacteria. Our attempt to purify agrin C20 was not successful since the agrin C20 was

expressed in insoluble form in inclusion bodies. The following solubilization using urea and dialysis led to loss of the recombinant protein. Since we faced difficulties to purify agrin C20, we welcomed the offer of Martin Smith's group to supply us with solubilized agrin C20.

To study the capacity of agrin to trigger Na,K-ATPase signaling we chose the model of COS7 cells. These cells are, in our lab, a well established model to study ouabain-triggered signaling and were used to describe the mechanism of ouabain-triggered slow Ca^{2+} oscillations.

First we examined whether solubilized agrin C20 activates the signaling function of Na,K-ATPase α3. COS7 cells were transfected with GFP-fused human Na,K-ATPase α3 and exposed to solubilized agrin C20. Recordings of intracellular Ca^{2+} showed that solubilized agrin C20 triggered slow Ca^{2+} oscillations similar to ouabain-induced Ca^{2+} oscillations. Non-transfected COS7 cells, expressing only α1 isoform of Na,K-ATPase, were used as a control. Unexpectedly, solubilized agrin C20-induced slow Ca^{2+} oscillations also in the untransfected COS7 cells. We have showed, using over-expression of Na,K-ATPase α1 N-terminus, which serves as dominant negative peptide competing with endogenous Na,K-ATPase for binding to IP_3R (Zhang et al., 2006), that solubilized agrin C20-triggered Ca^{2+} signaling is mediated by Na,K-ATPase α1. Furthermore, the Rb^{+} uptake assay showed that solubilized agrin C20 dose-dependently inhibits the pumping function of Na,K-ATPase α1 in COS7 cells.

At this point we presented our findings to Martin Smith and Lutz Hilgenberg. They expressed doubts about the direct effect of solubilized agrin C20 on Na,K-ATPase α1, as they were not able to cross-link agrin C20 with Na,K-ATPase α1 in COS7 cells.

To study whether solubilized agrin C20 directly affects Na,K-ATPase α1, we measured the ATPase activity of purified porcine Na,K-ATPase α1 in the presence of solubilized agrin C20. We found that the solubilized agrin C20 inhibited ATPase activity of purified Na,K-ATPase α1 to the same extent as 1 mM ouabain.

We attributed the differences in our and Hilgenberg's observations to the different species origin of the used cell models. The monkey and porcine Na,K-ATPase α1used in our study is ouabain sensitive, while rodent Na,K-ATPase α1 used by Hilgenberg et al. is ouabain insensitive. This led us to the hypothesis that ouabain and agrin binding sites overlap. Indeed, solubilized agrin C20 was shown to compete with ouabain for binding to surface of COS7 cells. Furthermore, mutation of three amino acid residues important for ouabain binding, turned Na,K-ATPase α1 agrin insensitive. We presented this solid evidence of effect of solubilized agrin C20 on the Na,K-ATPase α1 to Martin Smith and Lutz Hilgenberg. As a response we received a news that Lutz Hilgenberg managed to cross-link agrin C20 to Na,K-ATPase α1 in COS7 cells.

Since there are many reports on agrin function in hippocampal neurons (Karasewski and Ferreira, 2003; Matsumoto-Miyai et al., 2009; McCroskery et al.,

2009), we decided to test whether solubilized agrin C20 triggers similar signaling pathway in primary hippocampal neurons as it does in COS7 cells. The neurons were derived from E18 rat hippocampus and cultured for two weeks. At this time synaptic connections were established, as demonstrated by the presence of spontaneous synchronized intracellular Ca^{2+} oscillations. Exposure of the hippocampal neurons to solubilized agrin C20 resulted in a fundamental change in the characteristics of the Ca^{2+} signaling. Superimposed on the spontaneous high frequency Ca^{2+} signal was an additional low frequency Ca^{2+} oscillation (Fig 4.2).

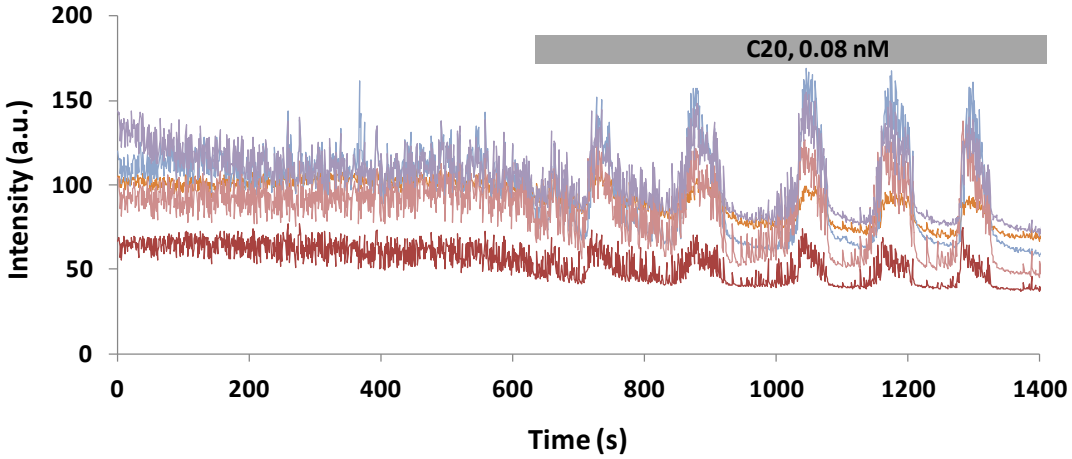

Fig.4.2 *Solubilized agrin C20 triggers slow Ca^{2+} oscillations in primary hippocampal neurons.* Representative recording of the intracellular Ca^{2+} in primary rat hippocampal neurons using the fluo-4 Ca^{2+} sensitive dye.

After isolating the low and high frequencies using a moving average filter (Fig2b), the frequency of the slower oscillation was found to be 7 mHz, and the fast spontaneous frequency component 124 mHz. These slow oscillations were synchronized across the neuronal network.

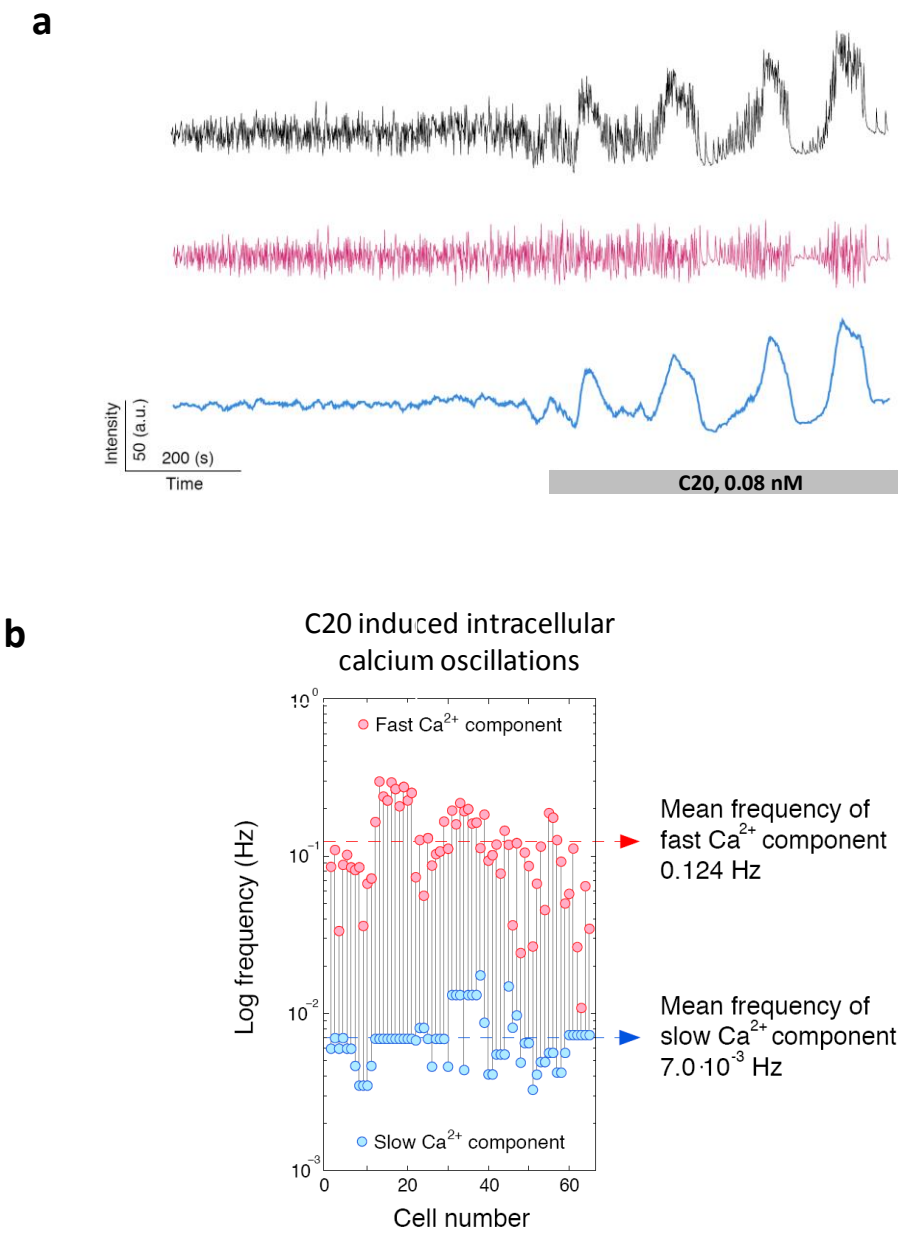

Fig 4.3 *Solubilized agrin C20 triggers slow Ca^{2+} oscillatory signal superimposed on fast frequency* Ca^{2+} *oscillations.* **(a)** Representative trace of intracellular Ca^{2+} recording in the primary hippocampal neuron (black trace). The fast frequency (pink trace) and the slow frequency (blue trace) components were isolated by applying a moving average filter. **(b)** The frequency of the slow oscillation was 7 mHz (n=65 cells, 13 experiments). The remaining fast frequency component was continuous throughout the recording, suggesting that two Ca^{2+} signaling systems were simultaneously active. Graph demonstrates the frequencies of the fast and slow Ca^{2+} oscillations components for each single cell.

To study the origin of the solubilized agrin C20-induced Ca^{2+} oscillations in hippocampal neurons we applied a variety of inhibitors. 2-APB, inhibitor of IP_3R and store operated Ca^{2+} channels, abolished the solubilized agrin C20-induced slow Ca^{2+} oscillations. D-AP5, an inhibitor of NMDAR, also inhibited agrin C20-induced Ca^{2+} signaling (Fig 4.4). Beside these inhibitors, tetrodotoxin (TTX) that inhibits neuronal activity by blocking voltage-gated Na^{+} channel, abolished all Ca^{2+} activity in the hippocampal neurons (data not shown). These results indicate that the solubilized agrin C20-induced Ca^{2+} signaling is complex and is generated both by intracellular Ca^{2+} store release as well as by Ca^{2+} influx from extracellular space.

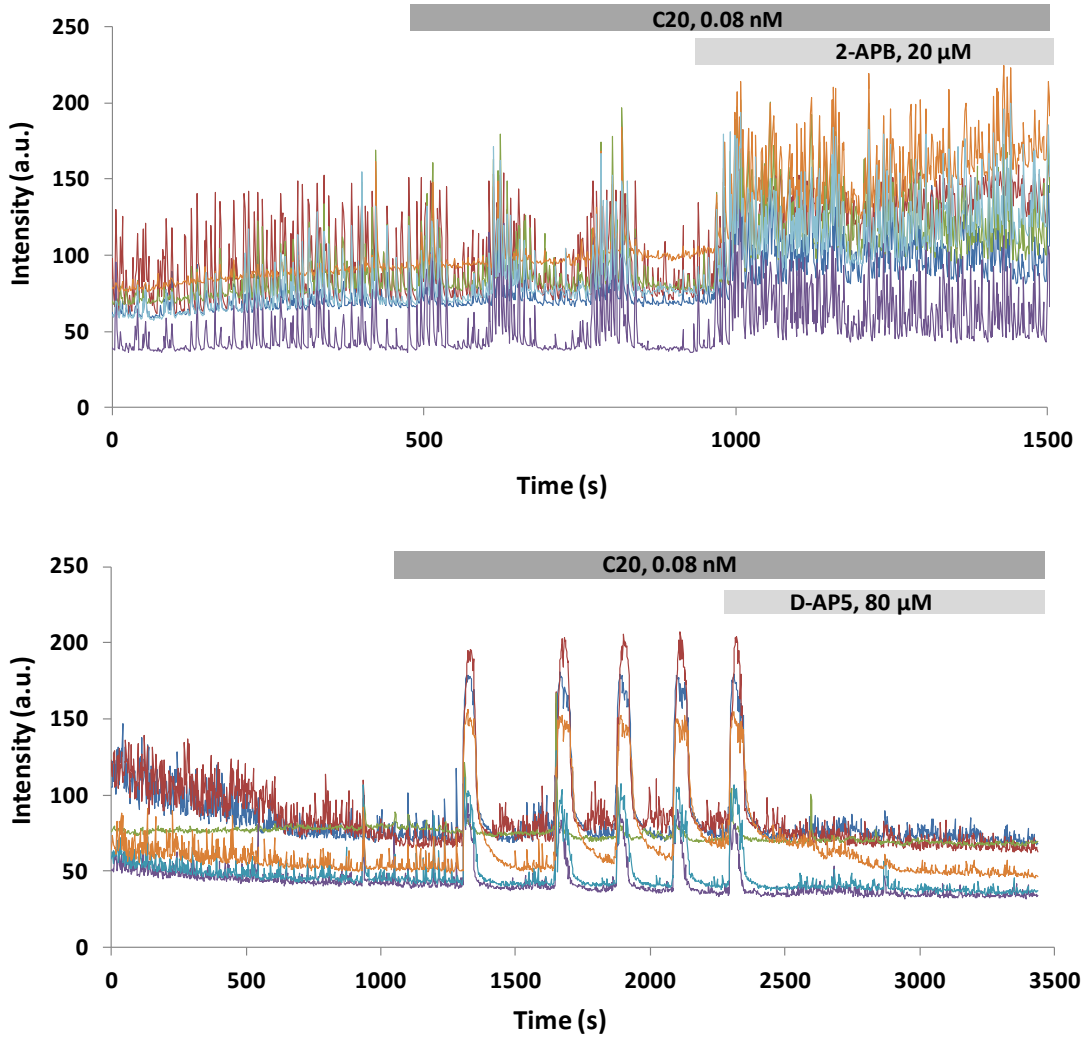

Fig 4.4 *2-APB and D-AP5 inhibits agrin C20-induced Ca^{2+} oscillations in primary hippocampal neurons.*

Hilgenberg et al. (2006) have shown that the 15 kD C-terminal agrin fragment competes with agrin C20 for binding to the Na,K-ATPase α3, but does not inhibit its pumping function. Application of agrin C15 to the primary hippocampal neurons

resulted in a complete and reversible inhibition of the spontaneous Ca^{2+} activity (Fig 4.5). This finding was in line with the observation of Hilgenberg et al. (2006) that agrin C15 blocks the spontaneous action potentials in cortical neurons. These agrin C15 effects were interpreted as a competition with the endogenous agrin C20.

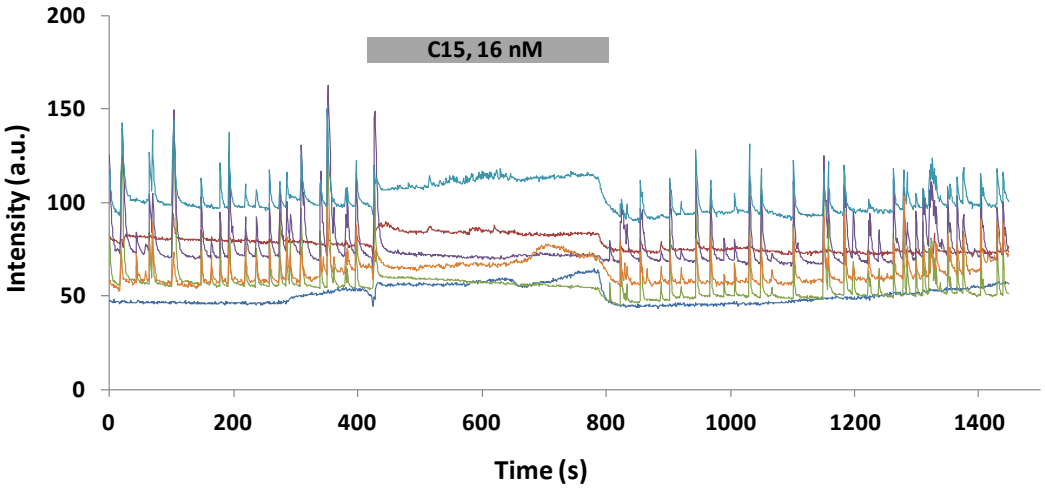

Fig 4.5 *Agrin C15 inhibits spontaneous Ca^{2+} activity in primary rat hippocampal neurons.* Representative recording of the intracellular Ca^{2+} in primary rat hippocampal neurons using the fluo-4 Ca^{2+} sensitive dye.

Since we did not use the full length agrin in our study, we were concerned about the physiological relevance of the effect of the C-terminal 20 kD agrin fragment. Therefore when in 2009 the group of Sonderegger reported that a 22 kD C-terminal fragment of agrin is endogenously produced on the synapse (Matsumoto-Miyai et al., 2009) we were anxious to test its effects. We obtained a purified human agrin C22 from the Swiss company Neurotune AB. Application of agrin C22 on COS7 cells however did not result in induction of Ca^{2+} signaling. We discussed this finding with our collaborators at Aarhus University in Denmark. The comparison of the crystal structure of the mouse agrin C22 resolved by Dr. Henning Tidow from Aarhus University in Denmark (unpublished data) with the published structure of the chicken agrin C20 (Stetefeld et al., 2004) did not reveal any major differences. We concluded that it is unlikely that the 2kD difference between agrin C20 and agrin C22 can be the reason for a loss of ability of agrin C22 to activate Na,K-ATPase signaling function.

This made us question if the solubilized agrin C20 might contain any contamination. SDS-PAGE followed by Coomassie Blue staining and Western blot analysis revealed presence of agrin C20 fragment and BSA as the two most abundant proteins in the solubilized agrin C20 sample. Non-protein fraction of the solubilized agrin C20, obtained by filtration using membrane with 3 kD cut-off, however exerted potent inhibitory activity on Rb^{+} uptake in COS7 cells. Mass-spectrometric analysis

of non-protein fraction of solubilized agrin C20 performed independently in the facility in Aarhus University in Denmark and in Science for Life Laboratory in Sweden, revealed the presence of ouabain in a 5-7 mM range of concentration. Agrin C15 on the other hand was shown to be ouabain free. However the presence of CNQX, the AMPA receptor inhibitor, was identified in the agrin C15 instead.

We contacted Martin Smith, but no explanation about the ouabain contamination in the solubilized agrin C20 samples that we received in 2008 and 2010 was provided. We obtained from Martin Smith´s group freshly prepared ouabain free solubilized agrin C20. This ouabain free solubilized agrin C20 was used in the assay measuring ATP hydrolysis in the murine brain lysate that contains abundance of α1, α2, and α3 isoforms of Na,K-ATPase. The high concentration of 20kD agrin did not affect ATPase activity in the brain lysate.

4.3.2 Review of studies identifying Na,K-ATPase α3 as a new agrin receptor

In the light of our findings we reviewed the Cell paper by Hilgenberg et al. (2006) and in retrospect many of the presented results raise some serious concerns.

The interaction between agrin and Na,K-ATPase α3 was identified by cross-linking of agrin C20 to the surface of neuronal culture followed by mass spectrometry analysis. It seems likely that mass-spectrometric analysis had revealed additional candidate proteins in complex with agrin, and a complete list of the proteins identified by mass-spectrometry would have been of interest for interpretation of the functional studies. Western blot analysis further confirmed that agrin C20 cross-links with Na,K-ATPase α3. Cross-linking between agrin C20 and Na,K-ATPase α1 and α2 was negative. The cross-linking assay followed by western blot analysis is generally considered to be a specific method to identify weak or transient protein-protein interactions. The inconsistencies in the ability of agrin C20 to cross-link to the Na,K-ATPase α1 in COS7 cells (personal communication) raises a concern about the specificity of the cross-linking protocol used in these studies. Methods such as co-immunoprecipitation or pull down assay, that would support these findings, were not performed.

There are inconsistencies in the presented immunostaining in Fig2. While Fig 2b shows a typical plasma membrane distribution of Na,K-ATPase α3, in Fig 2c there is no plasma membrane localization of Na,K-ATPase α3 and a strikingly high degree of co-localization with the pre-synaptic marker synaptophysin can be found. This could be either due to the use of primary antibodies from the same species (information about origin of synaptophysin antibody is missing) or due to problems with signal bleed through between synaptophysin and Na,K-ATPase α3 channel during imaging. Binding of the agrin C20 to the surface of non-neuronal GFAP-positive cells transfected with Na,K-ATPase α3 was also studied. In Fig4a confocal images of EGFP and fluorescein tagged GFAP are presented. Since their excitation and emission spectra overlap, no conclusions about their localization can be made.

The measurement of intracellular Na^+ level using SBFI Na^+ sensitive dye was performed as a functional experiment showing that agrin C20 inhibits the pumping function of Na,K-ATPase α3. The way the results are presented however makes it difficult to draw much conclusion from these studies. The intracellular Na^+ level recordings were performed without calibration. The responses of the individual cells were normalized to their maximal response when treated with the ionophore gramicidin. Since the SBFI intensity increase is not linear relative to the increase of Na^+ concentration, the data should not be subjected to quantification. The very rapid and robust increase of intracellular Na^+ level after agrin C20 application, in the presence of a cocktail of drugs inhibiting action potentials and neurotransmission, is somewhat surprising. This would suggest that the Na,K-ATPase α3 is the main determinant of basal level of intracellular Na^+ concentration although expression of Na,K-ATPase α1 in cortical neurons has been demonstrated (Zhang et al., 2009). The concentration of agrin used in this study is missing. Authors state that the concentration of agrin was assessed "based on their experience of ability of agrin to induce intracellular Ca^{2+} transient in cortical neurons" and refer to their earlier publication (Hilgenberg and Smith, 2004). The cited publication however does not contain any detail information that would clarify how the agrin C20 concentration was determined. The paper also lacks essential information about the experimental procedures and the origin of used antibodies.

The follow-up study Hilgenberg et al. (2009) performed on mouse cardiac ventricle tissue and cultured cardiomyocytes does not raise as many concerns. The Na,K-ATPase activity is assessed by measurement of ATP hydrolysis and by intracellular Na^+ measurements. Again the experiments using SBFI Na^+ sensitive dye were not calibrated. The increase of intracellular Na^+ induced by agrin C20 was 50% of the maximal response induced by gramicidin, while 5 mM ouabain induced 70% increase of intracellular Na^+ level compared to the maximal response to gramicidin. This would suggest that Na,K-ATPase α1 plays a minor role in the determination of the intracellular Na^+ level in cardiomyocytes. Whether the ouabain contaminated agrin C20 was used in this study is not clear.

In conclusion, the finding that agrin potentially binds to Na,K-ATPase α3 is very interesting, but the function of this interaction remains elusive. We feel that since the functional studies presented by Hilgenberg et al. are inconclusive and since we were not able to reproduce an inhibitory effect of solubilized agrin C20 on ATPase activity, the conclusions made by Hilgenberg and colleagues need to be revised and their data re-interpreted. In any case we have unintentionally reproduced our previous findings describing ouabain/Na,K-ATPase signaling mechanism in a novel kind of "double-blinded study."

4.3.3 Future perspectives

We have shown that ouabain-free agrin C20 has no effect on ATPase activity in brain lysate. It is however of importance to test the ability of agrin to inhibit Na,K-ATPase pumping function in other models. There are published studies where

authors suggest that the described agrin effects might be mediated through Na,K-ATPase α3. In none of these studies however was the activity of Na,K-ATPase in response to agrin examined. Burk et al. (2012) showed that agrin is necessary for morphological differentiation and survival of new olfactory bulb interneurons. Overexpression of dominant negative 15kD agrin C-terminus resulted in loss of mature interneurons. Since agrin C15 was reported by Hilgenberg et. al (2006) to compete with full length agrin for binding to Na,K-ATPase α3, the authors concluded that agrin acts *via* Na,K-ATPase in olfactory bulb interneurons. This is however indirect and insufficient evidence since agrin C15 might disrupt agrin binding with any putative receptor. Kirshenbaum et al. (2012) demonstrated that partial suppression of agrin expression in heterozygote agrin deficient mice reduces mania-like symptoms in mice with loss of function mutation in Na,K-ATPase α3. Authors admit that since the agrin suppression did not result in expected increase of Na,K-ATPase activity in the brain, agrin does not inhibit Na,K-ATPase activity directly and the mechanism behind the behavior changes in these mice is more complex. Zhang et al. (2009) has shown that agrin depolarizes the membrane in cortical neurons. The rapid membrane depolarization induced by agrin was in contrast to the slowly build increase of the membrane potential induced by 50 μM ouabain. This suggests different mechanisms of ouabain and agrin induced membrane depolarization. The example of these publications stresses the importance to clarify the role of agrin in the regulation of Na,K-ATPase activity to avoid data misinterpretation.

Beside the Na,K-ATPase, the molecules such as α-dystroglycan (Qu and Smith, 2004), integrins (Burgess et al., 2002), and MuSK (Daniels, 2012; Ksiazek et al., 2007) have been considered as agrin receptors or related to an agrin receptor in the brain. However none of them can fully explain all the experimental results. Agrin is a large protein that exists in either secreted or transmembrane form and possesses several splicing sites. It is therefore likely that agrin induced effects in the brain are mediated by different mechanisms.

The neurotrypsin mediated cleavage of 22 kD agrin on the neuronal synapse is dependent on the NMDA receptor (NMDAR) activation (Matsumoto-Miyai et al., 2009). Recently agrin has been shown to co-precipitate with and induce phosphorylation of the NR1 subunit of NMDAR in spinal cord (Cui and Bazan, 2010). In our study NMDAR receptor inhibitor D-AP5 inhibited solubilized agrin C20-induced Ca^{2+} signaling in hippocampal neurons (Fig 4.5). Whether these oscillations were induced by agrin or ouabain however remains to be answered.

In the last few years there has been raising concern about the reproducibility of scientific studies. About 65% of the projects in the industrial labs investigating candidate cancer drug targets that are based on published studies must be terminated due to data inconsistencies. The reproducibility of published data did not significantly correlate with journal impact factors (Prinz et al., 2011). This decrease in the reproducibility of data can only from a very small part be attributed to fraud. In

many cases the lack of reproducibility can be explained by the heterogeneous experimental conditions. More importantly the lack of proper controls, use of incorrect statistical methods, avoiding the presentation of negative results, or results that are not in the line with the hypothesis, leads to misinterpretation of data. Many publishers have recently taken an action to improve their peer review process. However since there is no way to ensure that the results of the published studies are correct, it is important to critically evaluate every study even if it is published in a high profile journal.

5 CONCLUSIONS

Ca^{2+} is the most universal yet the most versatile signaling mechanism in the cell. The complexity of the Ca^{2+} signal is regulated through mechanisms controlling Ca^{2+} transport between the cytoplasm, extracellular space and cellular compartments. This thesis focuses on the regulation of Ca^{2+} release from endoplasmic reticulum *via* IP_3R. Two mechanisms inducing IP_3R mediated Ca^{2+} signal were studied; the canonical pathway triggered through activation of PLC *via* G-protein coupled AT1R signaling, and the noncanonical IP_3 independent pathway triggered through interaction with ouabain bound Na,K-ATPase. In both models the importance of the protein-protein interaction on the Ca^{2+} response was demonstrated.

Angiotensin II activates AT1R and induces Ca^{2+} release through the IP_3R. We have demonstrated that AT1R forms heterodimer with D1R in renal proximal tubule. We have shown that activation of D1R leads to rapid and reversible uncoupling of AT1R from its G protein signaling and *vice versa* activation of AT1R disabled signaling function of D1R. This represents a new mechanism of short term regulation of AT1R and D1R G protein coupled signaling.

We have shown that the cytoskeleton associated protein, ankyrin B, stabilizes the interaction between ouabain bound Na,K-ATPase and IP_3R. This scaffolding function of ankyrin B has been demonstrated to be of importance for the transmission of the ouabain-induced signal from the Na,K-ATPase to the IP_3R. In the absence of the ankyrin B, the ouabain-triggered IP_3R activation was disturbed, resulting either in complete fail of Ca^{2+} release or in the dysregulation of the Ca^{2+} signal. The importance of the precise regulation of the Ca^{2+} response was demonstrated since the ouabain-induced Ca^{2+} oscillations in ankyrin B down-regulated cells could not be properly decoded and failed to activate down-stream signaling pathway.

In the last study we revealed that the effects of 20 kD agrin fragment on Na,K-ATPase pumping and signaling function is due to ouabain contamination. Nevertheless we have described a new kind of Ca^{2+} signal in primary hippocampal neurons. Solubilized agrin C20 triggered slow Ca^{2+} oscillations that were superimposed on the spontaneous fast frequency Ca^{2+} signal. Although the origin of these Ca^{2+} oscillations, both the trigger of the signal and the Ca^{2+} entry pathway, remains unclear, we have demonstrated that two independent Ca^{2+} signals can simultaneously exists in the same cell. This adds another level to the complexity and variability of the Ca^{2+} signaling.

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