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Sajal Chakraborti
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Regulation of Membrane Na^+-K^+ ATPase

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Regulation of Membrane Na^+-K^+ ATPase

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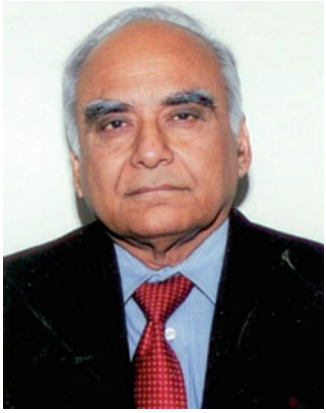

Prof. Nirmal K. Ganguly, M.D., D.Sc. (Hon)

This book is dedicated to Dr. Nirmal K. Ganguly for his outstanding leadership in promoting medical research and education throughout India. He is Honorary Professor, Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi; and Emeritus Professor, Postgraduate Institute of Medical Research and Education, Chandigarh. He established the immunological basis of chronic artery disease and made major research contributions in the field of rheumatic heart disease. He served as Director General, Indian Council of Medical Research, New Delhi, and showed extraordinary commitment for promoting biotechnology and public health.

Preface

Pluck this little flower and take it, delay not! ….. If though its colour be not deep and its smell be faint use this flower in thy service and pluck it while it is time.

Rabindranath Tagore (Gitanjali: Song of offerings)

Na^+-K^+ ATPase or Na-pump ATPase, a member of "P"-type ATPase superfamily, is characterized by association of multiple isoforms mainly of its α- and β-subunits. At present four different α- (α-1, α-2, α-3, and α-4) and three β- (β-1, β-2, and β-3) isoforms have been identified in mammalian cells and their differential expressions are tissue specific. Regulation of Na^+-K^+ ATPase activity is a complex process, which involves short-term and long-term mechanisms. Short-term regulation of Na^+-K^+ ATPase is either mediated by changes in intracellular Na^+ concentrations that directly affect the Na^+-pump activity or by phosphorylation/dephosphorylation mediated by some stimulants leading to changes in its expression and transport properties. On the other hand, long-term regulation of Na^+-K^+ ATPase is mediated by hormones, such as mineralocorticoids and thyroid hormones, which cause changes in the transcription of genes of α- and β-subunits leading to an increased expression in the level of Na^+-pump. Several studies have revealed a relatively new type of regulation that involves the association of small, single-span membrane proteins with this enzyme. These proteins belong to the FXYD family, the members of which share a common signature sequence encompassing the transmembrane domain adjacent to the isoform(s) of α-β subunits of Na^+-K^+ ATPase. Many investigators have independently demonstrated that, in addition to the classical ion transporting role, Na^+-K^+ ATPase can also relay extracellular ouabain (a cardiac glycoside that inhibits the enzyme activity) binding associated signaling into the cell through the regulation of protein phosphorylation, which includes activation of mitogen-activated protein kinase (MAPK) signal cascades, mitochondrial reactive oxygen species production, as well as activation of phospholipase C and inositol triphosphate receptor in different types of cells.

This book is an outcome of enthusiasm of renowned experts in the relevant research areas. Each chapter in this book raises many questions that eventually need

to be addressed for finding appropriate solutions in the area being dealt with. This book consists of 24 chapters, where the authors have summarized various aspects of the Na^+-K^+ ATPase regulation. The contents of this book have been organized into two major sections for the convenience of our readers, namely (1) Functional and Signaling Aspects and (2) Modulatory and Regulatory Aspects. It provides a comprehensive resource for stimulating Na^+-K^+ ATPase research and improving the modern therapeutic approaches of different life-threatening diseases that are associated with the regulation of the enzyme. It is hoped that the readers will find each chapter truly interesting and thought impelling.

Considering the extraordinary importance of Na^+-K^+ ATPase in cellular function, several internationally established investigators have contributed their articles in the monograph entitled *Regulation of Membrane Na^+-K^+ ATPase* for inspiring young scientists and graduate students to enrich their knowledge on the enzyme. We are sure that this book will soon be considered an important piece of comprehensive scientific literature in the area of Na^+-K^+ ATPase regulation in health and disease.

As editors, we are grateful to the authors for the time and effort they spent in making the book an advancement of knowledge in the field of regulation of Na^+-K^+ ATPase. We would like to thank Prof. Rattan Lal Hangloo, Vice Chancellor, University of Kalyani for his encouragement. We are grateful to Dr. Vijayan Elimban and Ms. Eva Little (St. Boniface Hospital Research Centre, University of Manitoba, Winnipeg, Canada) for all their hard work in helping to get this monograph into print. Finally, we like to express our sincere thanks to Dr. Meran Owen (Senior Publishing Editor, Springer-London) and Mrs. Leslie Poliner, Project Coordinator, Springer-New York, for their understanding and interest during the editorial process.

Kalyani, West Bengal, India — Sajal Chakraborti
Winnipeg, MB, Canada — Naranjan S. Dhalla

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Part I
Functional and Signaling Aspects

Chapter 1
Na^+/K^+-ATPase: A Perspective

Sajal Chakraborti, Sayed Modinur Rahaman, Md Nur Alam, Amritlal Mandal, Biswarup Ghosh, Kuntal Dey, and Tapati Chakraborti

"Looking for the answer:
You hunt it,
You catch it,
You fool yourself,
The answer,
is always,
a step ahead".

Jens C Skou
Nobel Prize winner in Chemistry (1997)

Abstract Na^+/K^+-ATPase (NKA), a transmembrane protein, facilitates active transport of three Na^+ out of the cell and two K^+ into the cell with the expense of an ATP. It plays an important role in regulating the ionic homeostasis and maintaining membrane potential. Additionally, NKA plays a crucial role in driving a variety of secondary transport processes such as Na^+-dependent glucose and amino acid transport.

NKA is composed of α and β subunits, which have several tissue-specific isoforms. The α subunit of NKA possesses catalytic activity of the enzyme and that contains binding sites for cardiac glycosides, ions, and ATP and also phosphorylation sites for protein kinase A and protein kinase C. The β-subunit is required for the insertion for the catalytic subunit into the membrane and also facilitates cell adhesion and associated signal transduction.

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Cardiotonic steroids, for example, ouabain, elicit their effects by inhibiting the NKA activity, thereby raising $[Na^+]_i$ leading to an increase in $[Ca^{2+}]_i$ mainly via NCX, thereby modulating ion concentrations and contractility. There are several synthetic and endogenous protein inhibitors of NKA having similar effects that mediate an increase in $[Ca^{2+}]_i$.

Activation of PKA and PKC by different stimulants, for example, thrombin, regulates NKA activity in pulmonary smooth muscle cell membrane. Regulation of NKA activity by PKA and PKC has been shown to occur upon phosphorylation of FXYD proteins, which are regulated in a tissue-specific manner. Additionally, some hormones, for instance, catecholamines, increase lung fluid clearance via β-adrenergic mediated mechanisms of active Na^+ transport across lung epithelial cells. NKA is associated with several cellular functions such as apoptosis and cellular proliferation. Dysregualtion of NKA is implicated for several metabolic and neuronal disorders.

Keywords Na^+/K^+-ATPase • FXYD • Ouabain • Phosphorylation • Na^+/K^+-ATPase inhibitors

1 Introduction

In 1997 Nobel Prize in chemistry was shared by the Danish biochemist Janes C. Skou for his discovery of Na^+/K^+-ATPase (NKA), although his involvement with this enzyme had started way back in 1950. While studying the effects of local anesthetics in nerve cells, Skou found that the anesthetics molecule affected the opening of Na^+ channels and make the nerve cells inexcitable, thereby producing anesthesia. Subsequently, Skou looked at enzymes on the plasma membrane and examined whether their properties are affected by the anesthetics. This led him to discover an enzyme, ATPase. Skou observed that its activity was optimum when right combinations of Na^+, K^+, and Mg^{2+} were added to the enzyme preparation. Although he speculated that this enzyme could play a role in the active movements of Na^+ and K^+ across the cell membrane, his studies were devoid of direct experimental proof. So, while publishing his findings in 1957, Skou was skeptical about the enzyme's involvement in active ion movements, so he did not mention the term "Na^+/K^+ pump" in the title of the paper [1].

In 1958, Skou met Robert Post at a conference in Vienna. By that time Post had already reported that in red blood cells, three Na^+ ions were pumped out of the cell for every two K^+ ions pumped in, and that ouabain, a cardiotonic steroid, inhibited the pump. After sharing his findings with Post, Skou was curious to see whether his enzyme was inhibited by ouabain. Subsequently, he observed that ouabain did inhibit the activity of his enzyme preparation. This finally led him to ascertain a link between the enzyme and the "Na^+/K^+ pump" [2].

NKA, a P-type ATPase, maintains Na^+ and K^+ gradients across the plasma membrane of animal cells [3]. The Na^+ pump is the major determinant of cytoplasmic Na^+ ($[Na^+]_i$) and plays a pivotal role in regulating cell volume, cytosolic pH, and cytosolic Ca^{2+}

($[Ca^{2+}]_i$). It also plays a crucial role in driving a variety of secondary transport processes such as Na^+-dependent glucose and amino acid transport [3].

Since NKA modulates contraction, its regulation is important in the myocardium. NKA controls the steady state $[Na^+]_i$, which subsequently determines $[Ca^{2+}]_i$ mainly via involvement of Na^+/H^+ and Na^+/Ca^{2+} exchangers. An increase in $[Ca^{2+}]_i$, in turn, is pumped into the sarco(endo)plasmic reticulum S(E)R by the SERCA. Thus, regulation of the Na^+ pump is important for proper understanding of the cardiac muscle contraction and vascular tone [4].

2 Structure of Na^+/K^+-ATPase

Na^+/K^+-ATPase is composed of α- and β-subunits. The isoforms of α-subunit are ~110 kDa, whereas the isoforms of the β subunit are ~55 kDa. Each subunit has distinct mRNA and is synthesized independently [5]. There are tissue specificities which complements gene expression for each isoform and formation of different combinations of α-β complexes [6]. Along with α and β, there is another subunit, the γ-subunit, a member of FXYD protein family, that plays an important role in regulating the enzyme activity in some systems (Fig. 1.1).

2.1 The Alpha Subunit

The α-subunit possesses catalytic activity of the enzyme and that contains the binding sites for cardiac glycosides, ions, and ATP and also possesses the phosphorylation sites for protein kinase A and protein kinase C [7]. Four α isoforms (α_1, α_2, α_3, and α_4) have so far been identified in mammalian cells, which are expressed in tissue- and cell-specific manner. There are ten transmembrane α-helical segments of the α subunit of NKA, where both the N- and C-termini of the chain are located in the cytosol [8, 9]. The smaller loop resides between transmembrane domains M2 and M3, where the larger loop resides between M4 and M5, which is ATP-binding and phosphorylation site (Fig. 1.1) [10].

The α_1 has been shown to express in all tissues examined so far [11], whereas the α_2 isoform is expressed in skeletal muscle, adipocytes, brain, and heart [12]. The α_3 isoform is present in nerves, brain, and also heart tissues [12], while the α_4 isoform has been found in the testis [13].

2.2 The Beta Subunit

Among the P-type ATPases, the β-subunit is confined to the Na^+/K^+ and H^+/K^+-ATPase. The N-terminal end of the β subunit is located in the cytosol [14], while a major portion of the –COOH end of the subunit is located outside the cell [9, 15]

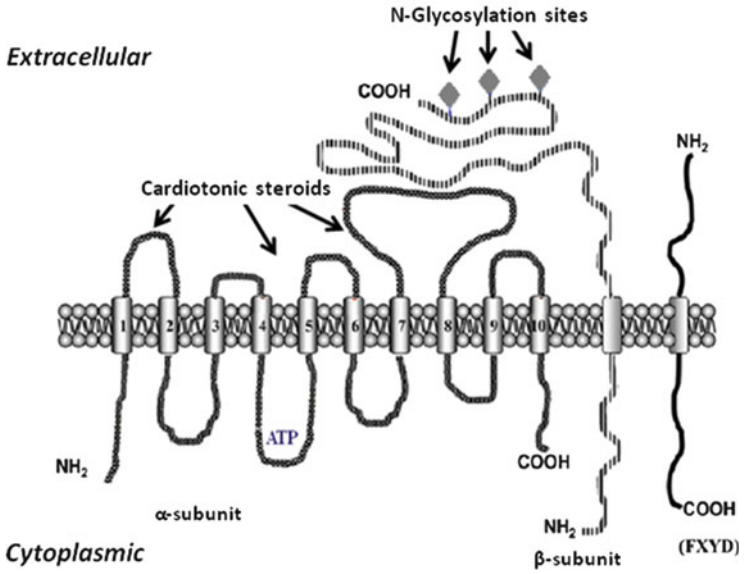

Fig. 1.1 Structure of Na^+/K^+-ATPase, which consists of α and β polypeptides in equimolar ratios. The α catalytic subunit has ten transmembrane segments. The extracellular segments of α-subunit has a binding site for cardiotonic steroids (CTS) which include TM1–TM2, TM5–TM6, and TM7–TM8 loops and several amino acids from the transmembrane regions M4, M6, and M10. The binding site for ATP is located on the intracellular loop TM4–TM5, which forms the "pocket" for this nucleotide. The phosphorylation domain located on the proximal and distal parts of intracellular loop TM4–TM5. The αβ-subunit complex of Na^+/K^+-ATPase associates with third subunit, which contains the conserved motif FXYD identical for all seven proteins from this family. FXYD2 protein is known as the γ subunit of Na^+/K^+-ATPase

(Fig. 1.1). In extracellular part of the β-subunit, three N-glycosylation sites are located [16]. The β subunit is required for the insertion of the catalytic subunit into the membrane, but does not participate in the catalytic process directly [17]. In nervous tissue, β-subunit has been shown responsible for cell adhesion [18]. In some systems, β-subunit may interact with lectins of animal origin (galectins) and thereby regulates cell adhesion and associated signal transmission [19].

The Na^+ pump consists of α and β subunits in a 1:1 ratio. The interaction between the α and β subunit is important for NKA activity and this has been exemplified by the observation that reduction of a disulfide bond between Cys158 and Cys175 of the β subunit causes loss of the enzyme activity [11, 20].

3 Mechanism of Action of Na^+/K^+-ATPase

The NKA cycles between two conformational states, E_1 and E_2. These states are characterized mainly depending on the interactions with Na^+, K^+, ATP and with cardiac glycosides, such as ouabain.

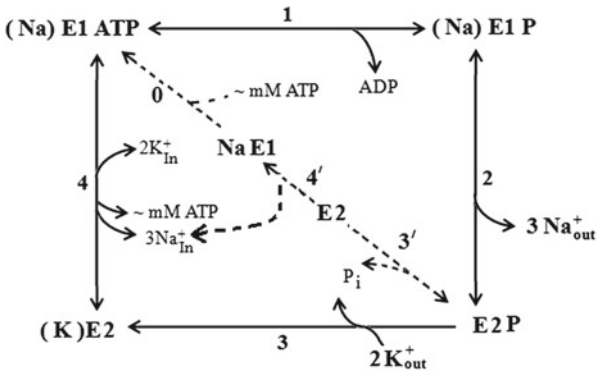

Fig. 1.2 Post-Albers mechanism for the enzymatic manifestation of Na^+/K^+-ATPase. Taken from Taniguchi K, Kaya S, Mardh S (2001). The oligomeric nature of Na^+/K^+-ATPase. J Biochem 129: 335–342 with permission

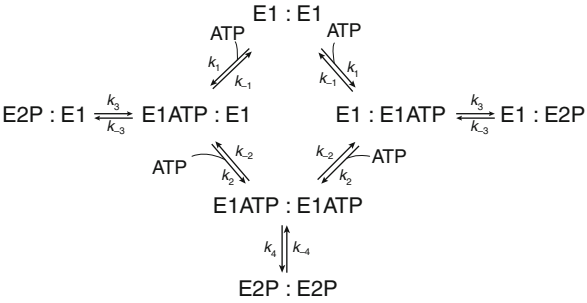

Fig. 1.3 Schematic representation for the diprotomeric model of Na^+/K^+-ATPase action. Taken from Clarke RJ, Kane DJ (2007) Two gears of pumping by the Na^+ pump. Biophysical J 93: 4187–4196 with permission

Since the discovery of NKA, accumulated evidences suggest that the active transport of Na^+ and K^+ occur according to the Post-Albers scheme. However, controversies arose concerning whether the functional unit of the enzyme is an αβ-protomer or an oligomer. Both the αβ protomeric and (αβ) oligomeric models gained support from various experiments [21]. A simple Post-Albers scheme is represented in Fig. 1.2. The scheme, however, does not take into consideration that the sodium pump might exist as a diprotomer of cooperating $(\alpha\beta)_2$ subunits and thus contain two binding sites for ATP.

The concentration–effect curve for ATP hydrolysis is biphasic, which can be explained by extrapolation of the single-site model shown in Fig. 1.2. Each (αβ) protomer has a single ATP binding site that alters from high affinity to low affinity with changes in conformation. This model is strongly supported by experiments showing that the stoichiometry of binding for either ATP, phosphate or ouabain is one per subunit, and that solubilized enzyme retains its catalytic activity [22].

A second model (Fig. 1.3) postulates that the biphasic nature of the ATP concentration curve is due to the presence of two catalytic α subunits that work cooperatively. Each catalytic subunit proceeds through the same conformational alterations that are described in the single-site model in such a way that they are shifted 180° from each other. Thus, in this model the high affinity and low affinity ATP binding sites occur

simultaneously, and there is also simultaneous transport of Na^+ out of the cell and K^+ into the cell. Several experimental results support this model [23].

Another model postulated by Plesner [24], which suggests that the cooperativity of the α subunits described by Repke occurs only in the presence of Na^+ and K^+. The partial reactions of the NKA are catalyzed by the (αβ) protomeric enzyme, as is the case with Na^+-ATPase or K^+-stimulated phosphatase.

4 Na^+/K^+-ATPase-Mediated Signal Transduction

Cellular ionic homeostasis and related signal transduction go hand-in-hand with NKA activity. Cardiotonic steroids (CTS) are known to exert their effects by inhibiting the NKA activity, thereby raising $[Na^+]_i$ leading to an increase in $[Ca^{2+}]_i$ mainly via Na^+/Ca^{2+} exchanger [25]. However, the therapeutic concentrations of CTS that produce positive inotropic effects in patients, for example, with congestive heart failure are much below than that was shown to inhibit the NKA activity [26].

4.1 Na^+/K^+-ATPase-Src Complex and $[Ca^{2+}]_i$ Regulation

Src family kinases are membrane-associated non-receptor tyrosine kinases. In response to various extracellular ligands, the Src family kinases participate in several tyrosine phosphorylation and associated signaling pathways, for example, EGFR is coupled with NKA-Src complex [27, 28]. Low doses of ouabain cause Src-EGFR activation and that in turn trigger different signal transduction pathways including Ras/Raf/MEK/ERK1–2 cascade. This subsequently leads to an increase in the production of ROS, which upon activation of PKC [26, 29], results in a rise in $[Ca^{2+}]_i$ (Fig. 1.4).

In some cell types, for example, cardiac myocytes, low doses of ouabain interacts with some membrane proteins, other than NKA, and transduce signals to intracellular signaling complexes. Ouabain has also been shown to evoke Ca^{2+} oscillations in renal epithelial cells as well as endothelial cells independent of changes in $[Na^+]_i$ [30]. Interestingly, in some types of cells, for example, cardiac myocytes activation of some signal transduction pathways by ouabain are not associated with intracellular ionic concentrations and contractility [26, 29]. Src family kinases (SFKs) cause differential regulation of NKA activity in lens epithelium. SFK family members such as Src and Lyn were reported to enhance NKA activity [31] and Fyn, another member of the SFK family, has been found to inhibit the NKA activity in lens epithelium [32].

4.2 Regulation by PKA and PKC

Cyclic AMP dependent protein kinase regulates NKA activity, which could be mediated by direct phosphorylation of the α-subunit of NKA [33]. PKA has been suggested to stimulate NKA activity by increasing its number in the plasma

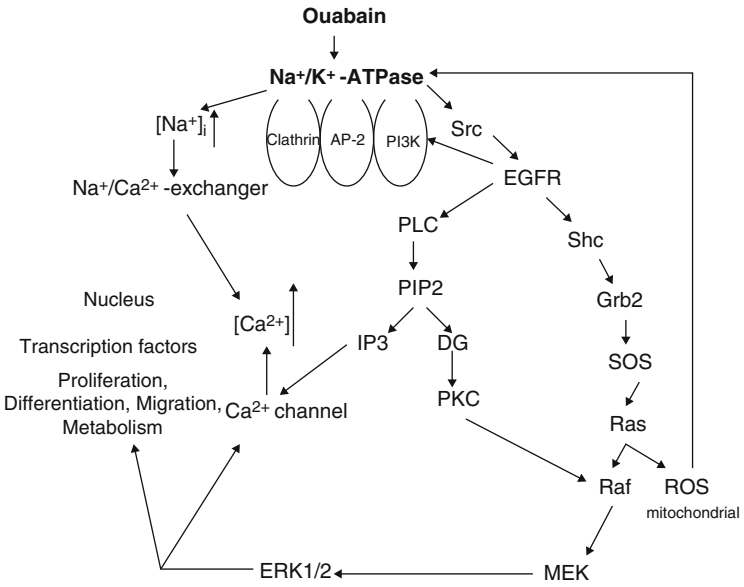

Fig. 1.4 Na^+/K^+-ATPase/Src/epidermal growth factor receptor (EGFR) complex-induced signal transduction. Ouabain binding to Na^+/K^+-ATPase in caveolae results in Src activation and subsequent transactivation of EGFR, forming Na^+/K^+-ATPase-Src-EGFR complex, followed by the assemblies and/or activations of different pathways, which include Ras/Raf/MEK/ERK-2 cascade, ROS production, PLC/PIP2/inositol triphosphate, PLC/PIP2/DG/protein kinase C, PI3K/AP2/clathrin-coated pits, and their subsequent effects (taken from Zhang L, Zhang Z, Wang Y (2008) Na^+/K^+-ATPase-mediated signal transduction and Na^+/K^+-ATPase regulation. Fundamental & Clinical Pharmacology 22: 615–621 with permission)

membrane [33]. PKA has been shown to induce the recruitment of active NKA units to the plasma membrane, for example, in rat proximal convoluted tubule cells of kidney [33].

Ouabain at micromolar concentrations has been reported to selectively inhibit NKA α_2 subunit function causing an increase in cellular cAMP level and an increase in the activities of protein kinase A (PKA) and Na^+/H^+-exchanger (NHE-1) with associated capacitative Ca^{2+} entry in rat optic nerve head astrocytes [34]. Additionally, some hormones, for example, catecholamines increase lung fluid clearance via β-adrenergic-mediated mechanism of active Na^+ transport across lung epithelial cells [35].

NKA-α_2 subunit is phosphorylated by PKC to a much lower extent than that of the α_1 in rat. The effects of PKC on NKA have been suggested to be species specific [36, 37]. Dopamine acts via its receptor and promotes exocytosis of NKA molecules from the late endosomes into the basolateral membrane (BLM) of alveolar epithelial cells via activation of PKC leading to an increase in NKA activity [38]. Additionally, dopamines induced NKA endocytosis via activation of G-protein-coupled receptors [39] and hypoxia-generated ROS cause PKC-ζ dependent

phosphorylation of the α-subunit of NKA [40]. Phosphorylation of the α-subunit plays an important role for internalization of enzyme in the cell membrane [41]. Protein kinase A and protein kinase C dependent modulation of NKA activity have also been shown to occur upon phosphorylation of FXYD proteins, which are regulated in a tissue specific manner [42].

4.3 Regulation by cGMP/PKG Pathway

In addition to cAMP and PKC, cGMP also regulates NKA activity in tissue and isoform specific manner. Studies showed that cGMP decreases NKA activity upon activation of protein kinase G (PKG) in the kidney [43, 44], where NKA-α_1 isoform is predominantly expressed. On the other hand, in Purkinje neurons, where the α_3 isoform is mainly expressed, cGMP increases the pump activity [45]. Hormones viz, acetyl choline that increase nitric oxide (NO) production and cGMP levels can induce a marked decrease in the activity of the NKA-α_1 isoform in kidney. In contrast, some stimulants such as carbon monoxide and glutamate markedly increase the expression of NKA-α_3 isoform in rat cerebellum leading to augmentation of NKA activity through cGMP and PKG, which are and independent of Na^+ and NO system [46].

4.4 Regulation by ROS

ROS interacts with NKA leading to its conformational change [47, 48], which is significant because in several diseases endogenous ROS level is elevated. An increase in ROS level induced by extracellular signals, for example, hypoxia increases the endocytosis of NKA that results in a marked decrease in its activity in the cell membrane [49].

5 The Na^+ Lag Hypothesis and the PlasmERosome

This hypothesis tells that a discernible inhibition of Na^+ pump leads to a transient increase in $[Na^+]_i$, which occurs in local compartments [50]. Immunochemical studies in rat arteries revealed that NCX1 and the ouabain sensitive NKA-α_2 and -α_3 isoforms of NKA, but not NKA-α_1 isoform, are present in the cell membrane close to the sarcoplasmic reticulum ('PlasmERosome', a sub-plasmalemmal space) [12, 51], which may evoke inhibitory effects of endogenous ouabain on NKA-α_2 and -α_3 isozymes. An increase in Ca^{2+} transients triggered by vasoconstrictors, for example, angiotensin II were observed during sodium pump inhibition. This

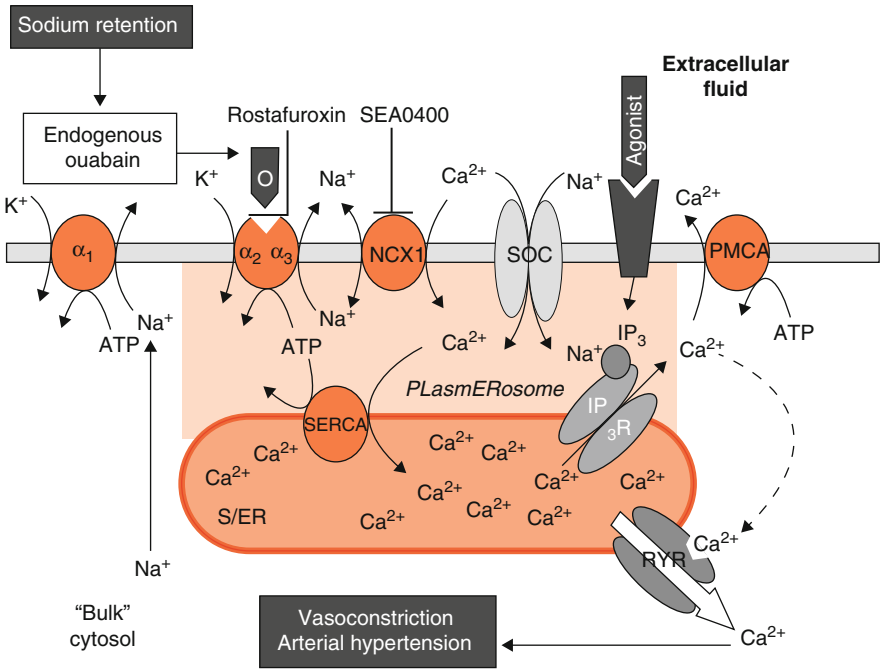

Fig. 1.5 A model of PlasmERosome and salt-dependent and ouabain-induced arterial hypertension (taken from Schoner W, Bobis G (2007) Endogenous and exogenous cardiac glycosides and their mechanisms of action. Am J Cardiovasc. Drugs 7: 173–189 with permission)

subsequently evoked opening of store-operated Ca^{2+} channels leading to the entry of both Na^+ and Ca^{2+} [51]. The increase in $[Ca^{2+}]_i$ in this microenvironment as well as in the bulk cytoplasm may activate SERCA leading to an increase in Ca^{2+} level in the SR. A considerable amount of Ca^{2+} can be released when myocytes are stimulated by ouabain, which increases Ca^{2+} in the PlasmERosome and that has been suggested to occur via activation of inositol (1,4,5) trisphosphate receptor (IP_3R) [52] (Fig. 1.5).

Mice with ouabain insensitive mutant of the NKA-α_2 subunit neither show cardiac inotropy upon ouabain treatment nor it exhibit ouabain induced hypertension [53]. A decrease in the expression of the wild type NKA-α_2 isoform causes an increase in blood pressure (BP) [54]. This indicates the possibility that α_2 and α_3 isoforms of the Na^+ pump interacts with the Na^+/Ca^{2+} exchanger and that is crucial for ouabain-induced inotropy. Functional NCX is required for an acute inotropic effect of ouabain inhibition of Ca^{2+} entry through NCX1 [55, 56]. Although the sodium lag hypothesis has been suggested as a mechanism for vascular hypertension, other mechanisms also contribute to this scenario since no strict correlation exists between hypertensive action of ouabain and subsequent inhibition of the sodium pump [57].

6 The Na^+/K^+-ATPase Signalosome

The inotropic effect produced by cardiac glycosides in the myocardium does not necessarily occur via inhibition of the Na^+ pump activity [57]. The work of Xie and Askari [58] and Xie and Cai [59] indicated that nanomolar concentrations of endogenous and exogenous ouabain-like cardiac glycosides induce cardiac inotropy, hypertension, and proliferation and also alter the life expectancy of cells [60]. In contrast to the sodium pump lag hypothesis, α-subunit isoforms are associated with the NKA in caveolae (signalosome) [61–63]. Caveolae are associated with molecules crucial for Ca^{2+} handling components such as NHE, NCX1, PMCA, and also L-type Ca^{2+} channels [64] (Fig. 1.6). Phospholipase C is coupled to the IP_3 receptor of the S(E)R and the α subunit of NKA [52, 62]. Caveolin, ankyrin, and phosphoinositide 3-kinase (PI3-kinase) binding sites are present on the α-subunit of NKA and thus form the signalosome complex [59, 65]. In caveolae, NKA interacts with several signal transducers such as Src and epidermal growth factor receptor (EGFR) [52, 62]. The signalosome complex play a role in several signal

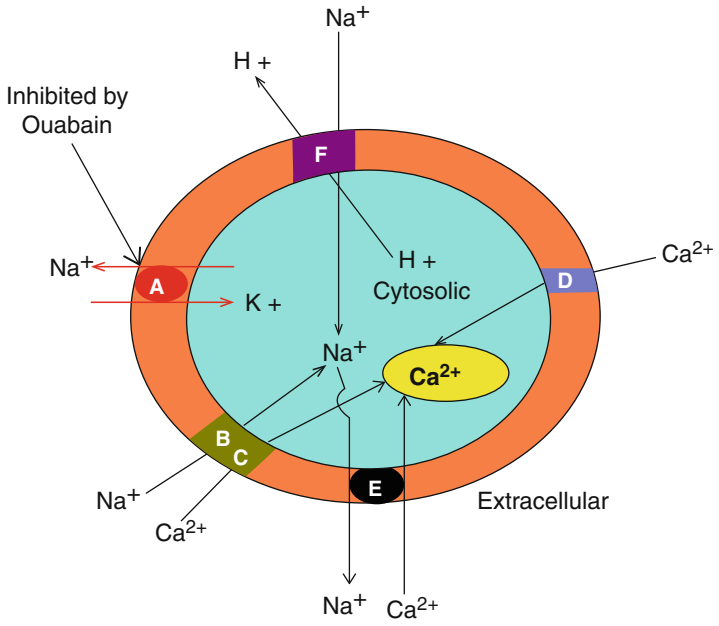

Fig. 1.6 Schematic representation of the role of Ca^{2+} influx regulatory components on an increase in Ca^{2+} level in the caveolae vesicles under Na^+/K^+-ATPase inhibition by ouabain. *A*, Na^+/K^+-ATPase; *B*, Na^+ channel; *C*, "slip-mode conductance" of Na^+ channels; *D*, voltage gated L-type Ca^{2+} channels; *E*, Na^+/Ca^{2+} exchanger; *F*, Na^+/H^+ exchanger. Taken from Ghosh B, Kar P, Mandal A, Dey K, Chakraborti T, Chakraborti S (2009) Ca^{2+} influx mechanisms in caveolae vesicles of pulmonary smooth muscle plasma membrane under inhibition of α2β1 isozymes of Na^+/K^+-ATPase by ouabain. Life Science 84: 139–148 with permission

transduction pathways, for example, regulation of NKA activity, which also involve in recycling of the plasma membrane, a process affected by CTS, $[Na^+]_i$, and adrenergic hormones [66–68].

Upon binding of ouabain with α-subunit of NKA, a change in $[Ca^{2+}]_i$ may result from stimulation of T- and L-type voltage-dependent Ca^{2+} influx, activation of promiscuous Na^+ channels, and also activation of Ca^{2+} release channels of the sarcoplasmic reticulum [69]. Ouabain treatment to cardiac myocytes leads to interaction of the NKA with Na^+ channel and make the later promiscuous in its ion selectivity and subsequently causes Ca^{2+} influx from the extracellular fluid [70]. This phenomenon was shown to be associated with stimulation of the α_2 and α_3 isoforms of the NKA [60]. Notably, the Na^+ lag hypothesis unable to explain the above phenomenon. Thus, additional pathways seem to exist and that could explain this scenario. An alteration of the α-subunit of NKA upon ouabain treatment may associate with IP_3R of the S(E)R [63] and also forms complex with PLC, whose Src-dependent phosphorylation could increase IP_3 generation and subsequently increase release of Ca^{2+} from intracellular stores [71]. Additionally, activation of PLC stimulates diacylglycerol formation leading to protein kinase C (PKC) activation [72], which upon phosphorylation may also affect voltage-dependent L-type Ca^{2+} channels [73], the Na^+/Ca^{2+} exchanger and NKA activities [71] (Fig. 1.7).

7 Interaction of Na^+/K^+ ATPase with FXYD Protein Family

In recent time, the FXYD proteins, having the signature FXYD motif in the N-terminal, are known as novel regulators of NKA. Mammalian FXYD proteins possess a single transmembrane domain with cytoplasmic –COOH and extracellular $–NH_2$ end and have been shown to interact with NKA and thereby regulate the enzyme activity [74]. FXYD proteins express in cell- and tissue-specific manner (Table 1.1). At present, the FXYD family has atleast 12 members. The γ-subunit of NKA is the only member of the FXYD family that has two alternative splice variants (FXYD2a and FXYD2b) [74, 75]. As a family, FXYD proteins are found predominantly in tissues like kidney, colon, pancreas, etc. (that are involved in solute and fluid transport) and also in electrical excitable tissues (heart, skeletal muscle, etc.) [75]. Different protein kinases can phosphorylate the FXYD proteins due to the presence of potential phosphorylation sites on to their cytoplasmic tail [76–78]. Phosphorylation of phospholemman (FXYD1) by protein kinase A (PKA) and protein kinase C (PKC) appear to influence its targeting, oligomerization, and also turnover [79].

FXYD2 has been suggested to play an important role in preserving the NKA activity in renal segments, for example, the outer medulla, which are highly prone to anoxia [80].

FXYD3 associates not only with NKA, but also with gastric and colonic H^+/K^+-ATPase [81]. Additionally, upon expression in *Xenopus* oocytes, FXYD3 modulates the processing of glycoproteins.

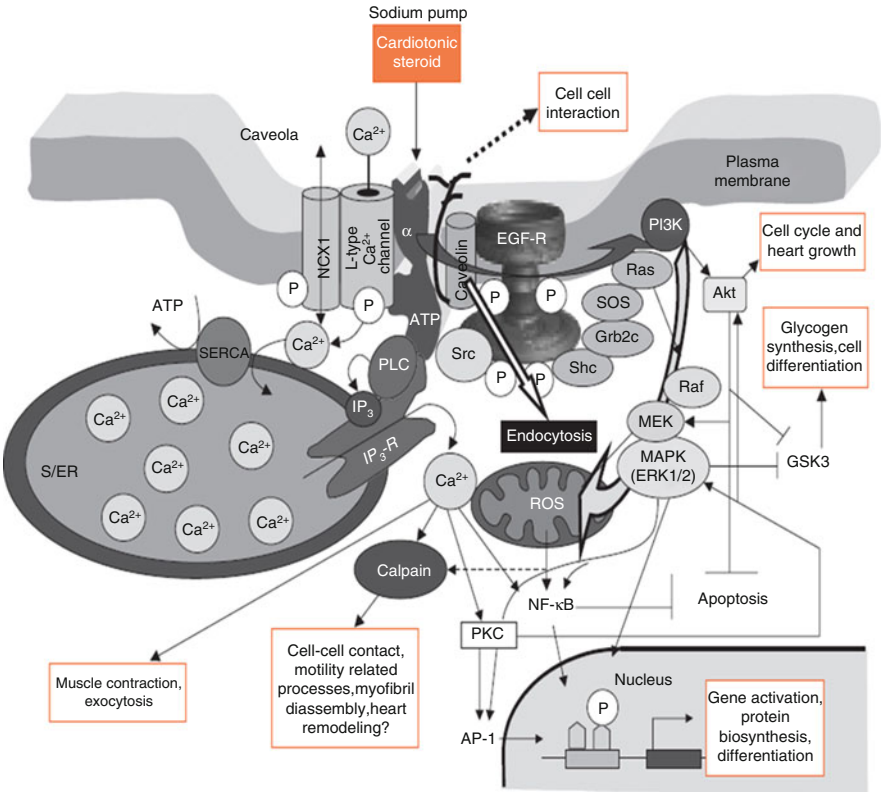

Fig. 1.7 A schematic presentation of reactions in various cells triggered by the interaction of ouabain with the Na^+ pump as a signalosome. Endogenous ouabain affects various processes using different signal transduction pathways. *Akt*, protein kinase B; *AP-1*, activator protein 1; *EGFR*, epidermal growth factor receptor; *ERKZ1/2*, extracellular signal-regulated kinase 1 and 2; *Grb2C*, growth factor receptor-bound protein 2; *GSK3*, glycogen synthase kinase 3; *IP3*, inositol (1,4,5) trisphosphate; *IP_3R*, inositol trisphosphate receptor; *MAPK*, mitogen-activated protein kinase; *MEK*, mitogen-activated protein/extracellular signal-regulated kinase kinase; *PKC*, protein kinase C; *PLC*, phospholipase C; *NF-κB*, nuclear factor κB; *PI3K*, phosphoinositide 3 kinase; *Ras*, a MAPK kinase kinase; *Raf*, a MAPK kinase kinase kinase; *ROS*, reactive oxygen species; *S(E)R*, sarco(endo)plasmic reticulum; *SERCA*, sarco(endo)plasmic reticulum Ca^{2+}-transporting ATPase; *Shc*, SH2 domain containing protein; *SOS*, mammalian homolog of Son of sevenless, a guanine nucleotide exchange factor; *Src*, sarcoma kinase. Taken from Schoner W, Bobis G (2007) Endogenous and exogenous cardiac glycosides and their mechanisms of action. Am J Cardiovasc. Drugs 7: 173–189 with permission

FXYD4 (a.k.a. corticosteroid hormone-inducing factor: CHIF) is expressed in kidney medullary collecting duct and papilla, in the distal colon, and modulates their transport properties [82]. Although biological activity of FXYD4 is mediated through its interactions with NKA, it remains elusive whether this is the only function of FXYD4 or whether it regulates other partner proteins [83].

Table 1.1 Tissue distribution of some FXYD protein family members

FXYD protein family	
FXYD	Tissue distribution
PLM (FXYD1)	Heart, liver, skeletal muscle
γ (FXYD2)	Kidney, heart, stomach
Mat-8 (FXYD3)	Colon, stomach, uterus
CHIF (FXYD4)	Kidney collecting duct, distal colon
RIC (FXYD5)	Heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis
Phosphohippolin (FXYD6)	Brain and kidney
FXYD7	Brain (cerebellum, cerebrum hippocampus, and stem)

FXYD5 (a.k.a. dysadherin) expression is induced in cells transformed by the oncogene E2A-PBX1 [84]. A role of FXYD5 in tumor progression and metastasis has been suggested, based on the observation that transfection of FXYD5 into cancer cells results in a decrease in E-cadherin-mediated cell-cell adhesion [85].

FXYD6 (a.k.a. phosphohippolin) is expressed in several tissues [86, 87]; for example, its expression in brain suggests a role in neuronal excitability during postnatal development.

In the brain, FXYD7 associates preferentially with the widely expressed $\alpha_1\beta_1$ isozyme of NKA and modulates its transport activity in a tissue-specific manner [88]. Thus, tissue- and isozyme-specific interaction of NKA with FXYD proteins contributes to regulated Na^+ and K^+ movements by the NKA.

8 Na/K-ATPase Inhibitors

A wide range of endogenous substances including ouabain-like compounds are known to inhibit NKA activity [89]. An endogenous NKA inhibitor is defined as a substance, which should be specific for and has a high affinity for NKA and functionally inhibits NKA activity [90]. There are several reports demonstrating the presence of NKA inhibitors in different tissues [91]. They are also found in plasma [92] and urine [93]. Marinobufagenin (MBG), a bufadienolide, acts as mammalian endogenous cardiotonic steroid and also inhibits NKAα_1 isoform [94]. The main source of MBG is the parotid and skin gland secretion of the toad Bufo marinus. Another compound gamabufotalin, a cardiotonic steroid from toad venom, has shown to curb COX-2 expression through IKK β/NF-κB signaling pathway in lung cancer cells [95]. Some of the NKA inhibitors known to date are proteins having NKA inhibitory activity [21, 96, 97]. The inhibitors exhibit different binding specificities toward the NKA isoforms. Some of them, for example, a 15.6 kDa inhibitor, can bind at the ouabain-binding site or in its close proximity, i.e., at the E_2 state of the enzyme [96], whereas, some other, for instance, a 70 kDa inhibitor, can bind at

the E_1 state of the enzyme [21]. Additionally, there are reports of some synthetic compounds, which showed different binding modes and affinities toward NKA [98]. One such representing example is the chromium complex $Cr(H_2O)_4$Ado*PP*[CH_2]P, which did not change the E_2 conformation, but alter the E_1 state of the enzyme. It did not affect the "backdoor" phosphorylation, but inhibited the "forward" phosphorylation. From this mode of binding study, it could be possible to ascertain the structural aspect of the enzyme, i.e., whether they exhibit monomeric ($\alpha\beta$) or oligomeric $(\alpha\beta)_n$ form [21, 96].

The 70 kDa inhibitor [21] and the 15.6 kDa inhibitor [96] elicit different affinities toward the α_1 and α_2 isoforms of NKA in pulmonary artery smooth muscle cells. The $\alpha_2\beta_1$ isoform of the enzyme was found to be more sensitive towards the inhibitor than the $\alpha_1\beta_1$ isoform for both the 15.6 and 70 kDa inhibitors. Importantly, both the 15.6 and the 70 kDa inhibitors do not show any apparent inhibitory activity toward Ca^{2+}-ATPase activity from smooth muscle microsomes or on the ouabain-insensitive Mg^{2+}-ATPase from SR membrane of pulmonary arterial smooth muscle cells [21, 96].

Fuller et al. [99] demonstrated that a cardiac- and brain-specific inhibitor of NKA, whose production is linked to oxidant stress, accumulates intracellularly during ischemia. In pulmonary artery smooth muscle cells, an increase in the inhibitory activity of the 70 kDa and the 15.6 kDa inhibitors of NKA have been demonstrated during NADPH oxidase derived $O_2^{\bullet-}$ generation under U46619 (thromboxane A_2 mimetic) treatment without any change in the expression level of the inhibitor protein [74].

The physiological significance of the 15.6 and the 70 kDa inhibitors is currently not clear. In pulmonary artery smooth muscle cells, an increase in the inhibitory activity of the inhibitors manifest during oxidant generation by the vasoactive, TxA_2 and subsequently inhibit NKA activity leading to pulmonary hypertension [100–102].

9 Hormonal Regulation

9.1 Corticosteroids

Steroid hormones especially corticosteroids have both long- and short-term regulatory effects on the NKA activity. Long-term effects are exemplified by alteration of mRNA/protein synthesis stimulated by direct interactions of receptor/corticosteroid complexes with nuclear DNA. Among the corticosteroids, aldosterone (a mineralocorticoid) and dexamethasone (a glucocorticoid) are well studied and have been shown to mediate regulation of the NKA activity [103].

Aldosterone plays an important role in Na^+ and K^+ transport in epithelial tissues, for example, in the kidney, and its physiological role is thought to be in long-term adaptations to decrease in Na^+ or increases in K^+ intake [104, 105]. The main role of aldosterone and dexamethasone on the NKA is to sustain a long-term increase in the

expression of Na^+ pump, which has been observed in toad bladder [106] and in many mammalian tissues like kidney [107], brain [108], heart [109], and also vascular smooth muscle cells [110] and cardiomyocytes [111].

The long-term upregulation of NKA by corticosteroids is isoform specific. Oguchi et al. [110] first demonstrated that the α_1 isoform, but not the α_2 and α_3 isoforms, is upregulated by aldosterone in vascular smooth muscle cells [110]. However, α_3 isoform is the main target for aldosterone-mediated regulation in brain [108, 112], whereas α_2 isoform responds to aldosterone treatment in heart [109].

Aldosterone has also been shown to elicit short-term effects on NKA activity, which are mediated by specific membrane-associated receptors [113]. Specifically, two distinct types of aldosterone-mediated short-term effects have been described. The first type is dependent on an increases in $[Na^+]_i$, while the second type is independent of $[Na^+]_i$. The underlying mechanism for the first type seems to be involved in an increase in Na^+ permeability, leading to an increase in $[Na^+]_i$ [114, 115], but the exact mechanism(s) is currently unclear. The second type, Na^+-independent aldosterone-induced isoform-specific increase in NKA activity, is observed in the rat cortical collecting tubule of kidney [116, 117] and A6 cells [118]. Interestingly, this type of modulation is sensitive to nucleic acid and protein synthesis inhibitors and is partly stimulated by the thyroid hormone, T_3 [117, 119]. The increase in activity may be secondary to changes in the number of plasma membrane Na^+ pumps and/or to an increase in the intrinsic affinity of the enzyme for Na^+ [120].

9.2 Catecholamines

Catecholamines have been shown to affect NKA activity. Interestingly, norepinephrine and dopamine of this group act differently to elicit their roles in regulating salt reabsorption in the kidney [121, 122].

Dopamine, a natriuretic factor synthesized in the kidney proximal tubule, acts in both paracrine and autocrine fashion [123, 124]. Dopamine inhibits Na^+/K^+-ATPase in the kidney proximal convoluted tubule [125], the medullary thick ascending limb [126], and cortical collecting duct [127]. It also inhibits NKA activity in vascular smooth muscle cells [128] and lung [129]. Dopamine-dependent Na^+ pump modulations are often weakened in aged [130, 131] and hypertensive rats [132]. Apart from a considerable number of evidence that dopamine (DA) is a specific inhibitor of the NKA, it has been shown that activation of DA1 receptors in striatal neurons results in Na^+ pump inhibition [133], whereas in another study DA2 agonists coupled to a pertussis toxin-sensitive G protein stimulates NKA activity through a decrease in cellular cAMP levels [134].

In contrast to dopamine, epinephrine and norepinephrine stimulate Na^+ pump activity [135]. Their effects, like dopamine, on the enzyme activity are also tissue specific. For example, epinephrine stimulates K^+ uptake in skeletal muscle after exercise [136]; however, norepinephrine, acting as a dopamine antagonist, plays a role in Na^+ reabsorption in the nephron [121]. Most of the hormones that regulate the NKA activity do

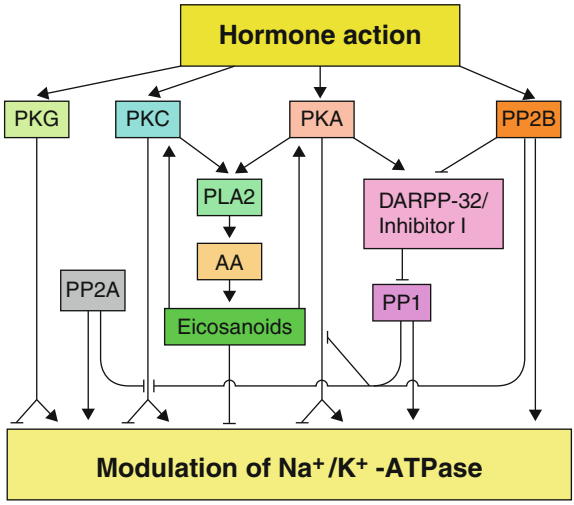

Fig. 1.8 Summary of the major mechanisms of hormonal regulation of the Na^+/K^+-ATPase. The main effectors of hormonal regulation of the sodium pump and their interactions are shown. Activation (*arrow*) and inhibition (*crossbar*) are indicated. *PKA, PKC, and PKG*, protein kinases A, C, and G; *PLA_2*, phospholipase A_2; *AA*, arachidonic acid; *PP1 and PP2B*, protein phosphatases 1 and 2B; *DARPP-32*, dopamine and cAMP-regulated phosphoprotein [taken from Therien AG, Blostein R (2000) Mechanisms of sodium pump regulation. Am J Physiol 279: C541–C546 with permission]

so through signaling mechanisms that modulate the activities of a group of protein kinases, phospholipases, and phosphatases. The interplay between the main effectors in regulating the Na^+ pump and their effects on the NKA are shown in Fig. 1.8.

10 Na^+/K^+-ATPase in Pathological Conditions

10.1 Neural Diseases

Downregulation of NKA has been demonstrated during ischemic conditions. During ischemia, reduced NKA activity was observed in both cortex and basal ganglia [137]. This could be due to the fact that ischemia or hypoxia lowers ATP level [138], increases production of ROS, and enhances inhibitory activity of the endogenous inhibitors of NKA [139]. The functional subunits α_2 and α_3 were reported to be mostly affected during ischemia [140]. An inhibition of Na^+/K^+-ATPase activity seems to delay membrane depolarization of cortical neurons after ischemic brain injury [141]. A decrease in NKA activity appears to be insufficient to maintain ionic balance during and immediately after episodes of ischemia. A reduction and/or inhibition of NKA contribute to the central neuropathy [142]. Reduced NKA activity has been demonstrated in chronic neurodegenerative diseases, for example, the

mRNA expression of the NKA-α_3 subunit has been determined to be ~45 % lower in Alzheimer's brain relative to controls. A marked decrease in the NKA activity is considered a common pathogenesis in patients with central nervous system disorders like CNS glioma, multiple sclerosis, and systemic lupus erythematosis [143]. Overall, a noticeable decrease in NKA activity could lead to several neurodegenerative and metabolic diseases.

10.2 Pulmonary Hypertension and Edema

During NKA inhibition, in pulmonary artery smooth muscle cells (PASMCs), as in other muscle types, an increase in $[Ca^{2+}]_i$ occurs, which is important for the initiation of pulmonary hypertension (PAH) [144]. The underlying mechanism(s) for PAH is currently unclear. However, available mechanisms suggest that in the contractile response in the cells, a decrease in NKA activity in response to hypoxia or hyperoxia generally results in an influx of extracellular Ca^{2+} [145]. Extracellular Ca^{2+} enters to the cytosol of the smooth muscle cells following activation of voltage-operated Ca^{2+} channels (VOCC) (L- and T-type Ca^{2+} channels) along with nonvoltage-operated Ca^{2+} channels (NVOCC). Additionally, receptor operated calcium channels (ROCC) [146] and capacitative or store-operated calcium channels (SOCC) that are activated upon depletion of Ca^{2+} from S(E)R [147] have been implicated in pulmonary hypertension.

Pathological manifestation of lung edema may occur due to both an increase in permeability of the alveolo-capillary barrier and a substantial decrease in the ability of alveolar epithelium to clear fluid from the lung [148]. An important stimulus for pulmonary edema in humans and in other animals is hypoxia. Hypoxia not only impairs active Na^+ transport across the alveolar epithelium [149], but also inhibits endothelial Na^+ channel (ENaC) and NKA activity [150]. Acute hypoxia decreases NKA activity upon phosphorylation of the Na^+ pump, thereby triggering endocytosis of NKA via activation of PKC by increased production of ROS [151]. Chronic hypoxia triggers proteolytic degradation of the NKA with the involvement of the ubiquitin/proteosome pathway.

10.3 Na^+/K^+-ATPase, Cellular K^+ Depletion, and Apoptosis

Prolonged decrease in NKA activity may eventually lead to apoptosis [152]. During apoptosis, dysregulation K^+ homeostasis occurs [153]. In apoptotic cells, a substantial decrease in cytoplasmic K^+ ($[K^+]_i$) has been observed. The cellular K^+ concentration can be reduced from ~100 to 30 mM in apoptotic cells [154, 155]. Conceivably, this reduced $[K^+]_i$ plays an important role for executing a number of apoptotic processes, including a decrease in cell volume, caspase-3 cleavage and cytochrome C release [156]. Mitochondrial membrane potential has

also been affected due to a decrease in $[K^+]_i$, which is a part of apoptotic process [155]. Depleting $[K^+]_i$ by the K^+ ionophore, valinomycin or overexpression of K^+ channels can cause apoptosis in central neurons and peripheral cells [157, 158]. It has been reported that even a slight deficiency of NKA activity may markedly increase the susceptibility of central neurons to some apoptosis-related pathogenesis. Such a situation may be contributed by endogenous inhibitors of NKA when they are released under some pathological conditions, for example, ischemia [158, 159].

10.4 Na^+/K^+-ATPase and Cytoskelatal Proteins in Ischemia-Reperfusion Injury

NKA, a transmembrane heterodimer protein, is composed of α- and β-subunits. The cytoplasmic domain of α-subunit interacts with ankyrin [160], a protein that connects the NKA to the fodrin-based membrane skeleton [161]. A decrease in protein levels of NKA-α_1 and -α_2 subunits of NKA has been demonstrated in cardiomyocytes of ankyrin-B mutated mice [162]. Additionally, dissociation of fodrin-ankyrin complex contributes to the loss of NKA transport to the plasma membrane [163].

Calpains, a group of nonlysosomal Ca^{2+}-dependent cysteine proteases, have been demonstrated to proteolytically degrade α-fodrin and ankyrin [164]. Activation of calpain and degradation of both the proteins were found during early reperfusion after prolonged ischemia [164]. In isolated rat hearts subjected to global ischemia, inhibition of calpain prevents degradation of fodrin and ankyrin. The underlying mechanism(s) of the activation has been suggested to be due to a decrease in sarcolemmal fragility and cell death during reperfusion [165].

Ca^{2+} influx through reverse-mode NCX has been shown to contribute to cardiomyocyte hypercontracture and death at the initial stage of reperfusion [165, 166], where an increase in $[Na^+]_i$ due to inhibition of Na^+/K^+-ATPase activity is the key determinant of reverse mode of NCX [167]. Thus, calpain activation, which causes an increase in $[Ca^{2+}]_i$ by reverse-mode NCX due to an impairment of NKA activity, eventually leads to cardiomyocyte death [138] (Fig. 1.9). Impairment of NKA activity during early reperfusion after prolonged ischemia has been shown to decrease the amount of the enzyme associated with the membrane cytoskeleton complex and that can be prevented by calpain inhibitors.

11 Conclusions

Caveolae signalosomes have been identified as the site for ouabain-mediated regulation of $[Ca^{2+}]_i$, where NKA acts as the transducer. The binding of ouabain activates Src family kinases in different types of cells [29, 168]. However, how ouabain activates Src and regulates these interactions are currently unclear.

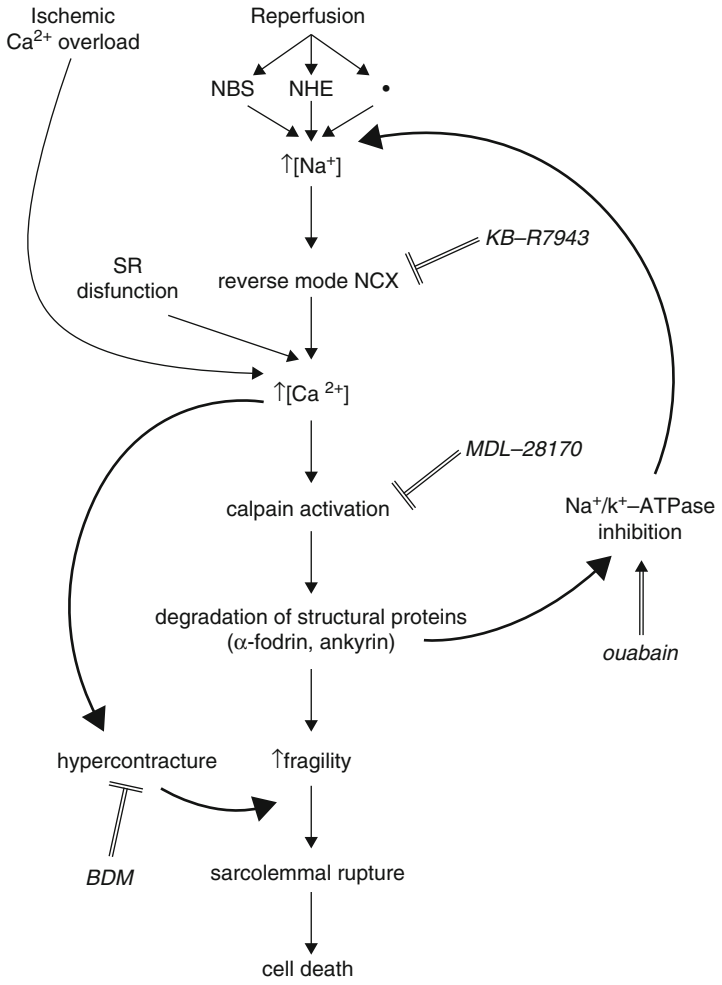

Fig. 1.9 Proposed mechanisms by which calpain activation may contribute to immediate cell death during reperfusion. The *broken arrow* indicates a probably less important mechanism. $[Ca^{2+}]_i$ indicates intracellular Ca^{2+} concentration; $[Na^+]_i$, intracellular Na^+ concentration; NHE, Na^+/H^+ exchanger; NBS, Na^+/HCO_3^- cotransporter; F, other mechanisms contributing to Na^+ overload. MDL-28170, calpain inhibitor; KBR-7943, inhibitor of NCX (reverse mode); ouabain, Na^+/K^+-ATPase inhibitor; BDM, contractile inhibitor [taken from Inserte J, Garcia-Dorado D, Hernando V, Soler-Soler J (2005) Calpain-mediated impairment of Na^+/K^+-ATPase activity during early reperfusion contributes to cell death after myocardial ischemia. Cir Res 97, 465–473 with permission]

In rat heart cells, tetrodotoxin (TTx)-sensitive Na^+ channels are opened by membrane depolarization and favored the passage of Na^+ into the cell [169]. Nanomolar concentrations of ouabain switched the ion selectivity of Na^+ channels to a state of promiscuous permeability called "slip-mode conductance." In rat heart cells, the "slip-mode conductance" of the Na^+ channels is stimulated by some

agonists [70]. TTx markedly inhibited ouabain induced increase in Na^+ and Ca^{2+} levels in caveolae of pulmonary artery smooth muscle cells [64]. Thus, in caveolae of heart and pulmonary vascular smooth muscle cells, involvement of "slip-mode conductance" has been suggested as a mechanism for an increase in $[Ca^{2+}]_i$ by ouabain.

The role that Ca^{2+} regulatory components in the caveolae microdomains play on an increase in $[Ca^{2+}]_i$ in smooth muscle cells during NKA inhibition by its endogenous protein inhibitors, for example, in hypertensive patients, has, however, not been clearly determined. Future studies on the correlation among the expression of the Ca^{2+} influx regulatory components, $\alpha_2\beta_1$ isozymes of NKA, and inhibitors of NKA in the caveolae microdomains in normal and hypertensive animals may provide better insights to establish several therapeutics, implicating these proteins to ameliorate vascular diseases, for example, pulmonary hypertension.

The α_2 isoform plays an important role in fine-tuning of contractility. Downregulation of the α_2 isoform, for example, during heart failure, could impair tight control of the excitation-contraction coupling. Such downregulation would increase $[Na^+]_i$ in the sub-sarcolemmal pool (plasma ERosome). This would impair the possible contribution of rapid reversal of the NCX to trigger Ca^{2+} release and causes dyssynchronous Ca^{2+} release as observed in models of heart failure yyy. Also, reduced Ca^{2+} extrusion through the NCX would increase SR Ca^{2+} load. However, in heart failure, SR Ca^{2+} load decreases [69, 170, 171]. This suggests that the downregulation of the α_2 isoform is a compensatory mechanism to limit the decrease in SR Ca^{2+} load. Low doses of ouabain improve contractile properties by enhancing the compensatory mechanism. Further studies are needed to determine the causes and consequences of α_2 isoform down regulation in heart failure.

At least one functional role of FXYD protein is the regulation of NKA and that occurs in a tissue- and isoform-specific manner. Although the functional effects of FXYD proteins on the transport and kinetic properties of NKA are known to some extent, yet physiological relevance of these effects and the potential implications of a loss of FXYD regulation of NKA in pathophysiological states are not clearly known and require further investigation.

ROS induced changes in $[Ca^{2+}]_i$ may be correlated to the regulation of NKA. Ouabain and ROS were shown to exhibit additive effects on $[Ca^{2+}]_i$, indicating a common target for both the stimuli. This was supported by the findings that ouabain potentiated the effects of ROS on protein synthesis and gene expression. Thus, ROS may not only regulate protein kinase cascades directly, but also affect NKA, which in turn leads to hypertropic signaling pathways. In this connection, it is worth noting that ouabain binding to the NKA stimulates ROS production in a Src- and Ras-dependent manner in cardiac myocytes [171]. Thus, it is likely that ouabain-induced ROS could further affect the receptor, thereby amplifying the associated signal transduction mechanisms, which needs to be experimentally verified.

NKA activity reduces during apoptosis or vice versa; that is, considerable reduced activity of NKA leads cells to undergo apoptosis, while its activity increases at the time of cell proliferation. For example, TNF-α exerts a time-dependent opposite effect on the pump. The early 1 h response is inhibitory and suggestive of an

apoptotic state, while the cell proliferation response was determined at 4 h. Importantly, in both the responses JNK has been shown to be activated and is the key player [172]; however, the exact mechanisms by which JNK exerts opposite effect on its targets such as NF-κB and caspases, which mediate cell survival and apoptosis, respectively, are currently unclear and need further experimental proof.

In cardiomyocytes inhibition of NKA during early reperfusion after prolonged ischemia was determined, at least partly, to be due to calpain-mediated detachment of the NKAα-subunit from the membrane cytoskeletal complex. This contributes to Ca^{2+} influx through reverse-mode of NCX and plays a critical role in reperfusion induced cell death. Although this provided a novel mechanism for explaining the impairment of NKA activity that occurs in hearts during early reperfusion after prolonged ischemia, calpain-independent mechanisms also contribute to NKA dysfunction under ischemia-reperfusion injury to the vasculatures.

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Chapter 2
Na/K-ATPase and Its Role in Signal Transduction

Moumita Banerjee and Zijian Xie

Abstract The Na/K-ATPase was discovered as an essential ion pump that maintains intracellular ionic balance by transporting potassium and sodium ions into and out of eukaryotic cells at the expense of ATP. The efforts of numerous investigators during the last two decades have revealed several important non-pumping functions of Na/K-ATPase. This chapter focuses on the molecular mechanism of Na/K-ATPase-mediated signal transduction and its potential regulatory role in animal physiology and diseases.

Keywords Na/K-ATPase • Ouabain signaling • Cardiotonic steroids • Src kinase • Signalosome

1 Introduction

The Na/K-ATPase (NKA) was discovered by Skou about 60 years ago as an ion pump [1]. It belongs to a large family of P-type ATPases. Unlike other P-type ATPases, NKA contains binding site for both endogenous and exogenous cardiotonic steroids (CTS) that include ouabain, digoxin, and marinobufagenin. Because digoxin and related CTS have been widely used as drugs for heart failure, both physician and basic scientists have had a long history of interest in knowing whether these drugs affect gene expression and cell growth [2, 3]. By early 1970s, several groups of scientists had demonstrated the regulatory effects of ouabain on cell growth and gene expression. At that time, these regulatory effects of ouabain were all ascribed to the pump inhibition and the resulted change in intracellular ion concentration [4–6]. About 20 years ago, a series of studies conducted first in neonatal cardiac myocytes and subsequently in renal epithelial cells showed that ouabain could activate a number of cell growth-related pathways, of which many are

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independent of changes in intracellular ion concentration. These studies have led to a great effort by many laboratories and subsequent demonstration that the NKA actually has many non-pumping functions [7, 8]. In this chapter, we briefly look into the structural properties of NKA that is responsible for direct protein interaction, and then discuss different aspects of NKA-mediated signal transduction and its role in animal pathophysiology.

2 Structure of NKA

The NKA is an oligomeric protein comprising of two main subunits—a ~100 kDa ten-transmembrane-spanning alpha (α) subunit and a 50–60 kDa highly glycosylated single-membrane-spanning beta (β) subunit. In certain types of cells, a third subunit called gamma (γ) also associates with α and β subunits. The α subunit is the catalytic subunit that contains binding sites for the pump substrates as well as CTS. The three dimensional structure of the NKA is rather elongated, with short extracellular region that serves as the binding site for CTS [9]. The ten transmembrane domains are embedded inside the plasma membrane and are usually numbered as M1 to M10. The cytoplasmic region is rather large, and is formed by three main intracellular structures—the amino terminal tail of about 90 amino acids, the second cytoplasmic peptide of ~136 amino acids that connects M2 and M3, and a third rather large peptide of ~434 amino acids that connects M4 and M5. These cytoplasmic regions are named based on their functionality, according to the X-ray crystal structures of SERCA (sarcoplasmic reticulum Ca^{2+} ATPase) and then NKA [10–12]. The amino terminal tail and the second cytoplasmic peptide together form a domain called actuator domain or A domain. On the other hand, the large cytoplasmic loop connecting M4 and M5 can be divided into two domains: the P domain or phosphorylation domain containing the aspartate (D371) that accepts the terminal phosphate during ATP catalysis, and N or nucleotide-binding domain that binds ATP. During ion pumping cycle, the NKA undergoes a large-scale domain rearrangements while transitioning between two unphosphorylated states of E1 and E2, and two phosphorylated states of E1P and E2P [9–12]. Interestingly, many membrane and cytoplasmic proteins have been identified to interact with either the A or N or both A and N domains as reviewed in references [7, 13, 14]. Moreover, these studies have also revealed that such protein interactions are not only important for control of pump trafficking and enzymatic activity, but may also play an essential role in regulating the function of these pump-interacting proteins. The latter provides the molecular basis for the non-pumping functions of NKA.

3 Regulation of Cell Growth by Cardiotonic Steroids

As early as 1930s, Christian postulated that digoxin delays the symptoms of cardiac insufficiency in patients with heart diseases by affecting cardiac enlargement [15]. In the 1970s, several laboratories had obtained evidence of CTS-induced changes

in cell growth and gene expression including the inhibition of mitogen-induced differentiation of lymphocytes isolated from both normal and leukemic subjects, and up-regulation of NKA [4, 16, 17]. Initially, these growth-promoting/inhibiting effects of CTS were logically attributed to the alteration of intracellular ion concentrations. However, it gradually became an issue because investigators realized that CTS could affect cell growth and gene expression at very low concentrations (lower than 1/10th of the IC50 on NKA activity) that do not likely affect the ion pumping capacity of cells. In mid-1990s, we reported that ouabain could stimulate protein synthesis and hypertrophic growth in cultured cardiac myocytes by activating a number of growth-related signaling pathways, including the activation of several proto-oncogenes such as Src and EGFR, induction of transcriptional factors such as AP-1 and NF-κB, and increased production of reactive oxygen species (ROS) [18–22]. Most importantly, we showed some of these pathways could be activated by ouabain in the absence of detectable changes in intracellular Na^+, and in the absence of extracellular Ca^{2+}, raising the possibility of NKA/ouabain interaction having ion pumping-independent signaling function [20]. During this period of time, several laboratories also demonstrated growth stimulatory effects of ouabain on vascular smooth muscle cells and endothelial cells at sub-nM concentrations that were insufficient for changing cellular pump capacity [23–25]. These early studies have now been well documented by different laboratories around the world in a variety of cell types including cardiac myocytes, endothelial cells, renal epithelial cells, vascular smooth muscle cells, cancer cells, and sperm and neuronal cells [26–36]. Taken together, they have led credentials to the hypothesis that the NKA can regulate cellular phenotype by acting as a signal transducer. Moreover, they have illustrated that the growth effects of CTS are cell specific. To this end, it is of interest to mention several studies. First, a potential role for CTS in pathogenesis of autosomal dominant polycystic kidney disease (ADPKD) has been suggested by Blanco's laboratory. They have demonstrated that physiological concentrations of CTS is capable of stimulating proliferative growth of ADPKD, but not normal human kidney epithelial cells, by activating Src/EGF receptor/ERK pathways [37, 38]. Second, we and others have shown that CTS are potent stimuli of dermal and other types of fibroblasts. They could increase collagen synthesis at concentrations well below 1/10th of their IC50 on the NKA activity by activating Src and PKC as well as by increasing the production of ROS [39–41]. Third, it is important to point out that the fetal bovine serum we all use in our cell culture may contain sufficient amount of CTS to promote cell growth according to the studies from Lichtstein's laboratory [42]. Finally, it is equally important to recognize that CTS could also inhibit cell growth in a wide variety of cancer cell lines such as prostate, lung, colon cancer cells and neuroblastoma cells by stimulating several different pathways, including apoptosis and autophage-related processes [43–51]. Taken together, it is clear that the NKA possesses some other functions distinct from its function as an ion pump, through which it can regulate gene expression and cell growth in a cell-specific manner.

4 Src Kinase and NKA-Mediated Signal Transduction

4.1 The Identification of NKA/Src Receptor Complex

Although there is still some degree of uncertainty in regarding to the chemical identity and biosynthetic pathways of endogenous CTS, their existence and physiological role have now been firmly established, especially after the publication of transgenic animal work of Lingrel laboratory [52, 53]. It is also most agreed among investigators that endogenous CTS are circulating at the concentrations far below the IC50 of these compounds (e.g., below 1/10th of IC50). Naturally, it has been questioned how CTS work as hormones in vivo.

Blaustein and colleagues have postulated based on their initial work on smooth muscle cells that localized increase in intracellular Na^+ due to CTS-induced pump inhibition would increase Ca^{2+} influx through Na^+/Ca^{2+} exchanger (NCX) [54]. This is certainly true when high concentration of ouabain is applied to both cardiac and smooth muscle cells. It might be also in operation when smooth muscle cells are exposed to certain low concentration of CTS [55]. However, most cells express a large number of NKA and cells contain a large pool of reserved NKA. Localized increase in intracellular Na^+ will certainly increase the turnover rate of CTS-free NKA, resulting in a smaller change in intracellular Na^+, and thus the overall signal amplitude. Moreover, it is less likely that such mechanism is responsible for CTS-induced activation of protein kinases.

Several of our early studies showed that ouabain could stimulate tyrosine phosphorylation of multiple protein kinases in different types of cells including cardiac myocytes, smooth muscle cells, and renal epithelial cells [19, 21, 22, 56, 57]. The findings in renal epithelial cells were more interesting because these cells, unlike muscle cells, express only α1 isoform of NKA and also much less NCX. In accordance, ouabain-induced protein tyrosine phosphorylation was detected even when these cells were cultured in Ca^{2+}-free medium [20, 22]. These new findings at the time led us to look for protein kinases that could be activated by the binding of CTS to NKA. This effort resulted in the discovery of the receptor NKA/Src complex [56–58].

Several lines of evidence support this contention. First, time course studies showed Src to be one of the first activated kinases when cells were stimulated by ouabain. Concomitantly, inhibition of Src by Src-specific inhibitors attenuated all of ouabain-induced signaling pathways including ouabain-induced increases in intracellular Ca^{2+}. This was independently confirmed in SYF mouse fibroblasts where three major Src family kinases (Src, Yes, and Fyn) are knocked out. Most importantly, expression of Src restored ouabain-induced protein tyrosine phosphorylation and other signaling events in SYF cells [56]. Second, Src was detected in the highly purified NKA preparations from dog and pig kidneys. In accordance, co-immunoprecipitation experiments indicated the formation of NKA/Src complex. Ouabain-induced Src activation further recruited more Src to this complex. This is consistent with the detected co-localization of these two proteins in the plasma

membrane microdomains and further supported by the FRET analyses. It is important to note that most of above findings has now been confirmed by different laboratories working with other types of cell cultures and in vivo [28, 37, 44, 51, 56–61]. Third, using GST- or his-tagged Src or NKA fragments we were able to show direct binding between these two proteins. At least two independent binding sites from the α1 subunit of NKA have been identified. Interestingly, these two binding sites are separately located in the A and N domains. While NKA N domain binds to Src kinase domain, the A domain interacts with Src SH2 domain. Because the binding of ouabain or pump substrates to NKA results in a large movement of both A and N domains, it is conceivable that such conformational changes could alter the interaction between the NKA and Src. To this end, we were able to show that ouabain was capable of disrupting the interaction between the N domain of NKA and Src kinase domain, which was further supported by live-cell FRET analyses [56, 57].

4.2 Src Kinase

An insight into the regulation of Src kinase is necessary for understanding its interaction with NKA; therefore this paragraph provides a short summary of Src kinase structure and regulation. Src family kinases, consisting of nine family members, are proto-oncogenic non-receptor tyrosine kinases that play intermediary roles in signal transduction in a variety of growth factor-stimulated pathways. The first identified member of this family was Src kinase, the viral form (v-Src) of which was the first identified oncogene [62]. Src kinase and other family members have been found to be responsible for regulating several fundamental cellular functions such as cell division, attachment, migration, and survival [63, 64]. Src family kinases share a common structure containing six functionally distinct domains: (a) a membrane anchoring N terminal SH4 domain that contains sites for myristoylation or palmitoylation, (b) followed by a unique region, (c) SH3 domain that preferentially binds polyproline motifs, (d) SH2 domain that binds to a specific sequence containing phosphorylated tyrosine, (e) kinase domain that is the catalytic domain responsible for phosphorylating Src substrates, and (f) a short regulatory C terminal tail. Detailed studies into crystal structure of Src kinases have revealed that Src SH2 and SH3 domains play an important role in the regulation of Src kinase activity. Two regulatory tyrosine residues—Y418 in the kinase domain and Y529 in the C terminal tail—are also very important. In general, when Src SH2 binds the phosphorylated Y529, it aids the binding of SH3 domain to a short motif in the linker region connecting SH2 and kinase domain. These double-intramolecular interactions keep the overall structure in a closely compacted inactive conformation and hinder the phosphorylation of Y418 that is required for the full activation of Src. Thus, dephosphorylation of Y529 could lead to the activation of Src. However, under most physiological conditions, Src appears to be activated by its SH2 binding to membrane receptors. For example, when receptor tyrosine kinases such as EGF receptor are tyrosine phosphorylated, they are capable of recruiting and then activating Src

through the SH2-mediated binding without affecting Y529 phosphorylation [65, 66]. Thus, the binding of SH2 to the phosphorylated membrane proteins (receptors) could actually serve two important regulations of Src: namely targeting and activation. As discussed in the next paragraph, NKA represents another important regulatory mechanism of Src, but works differently from receptor tyrosine kinases.

4.3 NKA and Src Regulation

In comparison to the widely accepted mechanism of Src regulation, the newly described NKA/Src interaction has not been greatly appreciated. Therefore, it is important to compare and contrast the NKA vs. other cellular protein-mediated Src regulation, especially in the following three aspects. First, NKA regulates Src differently from that of receptor tyrosine kinases. For example, EGF receptor is one of the most prominent receptor tyrosine kinases that Src engages in. It is capable of recruiting Src kinase to the signaling complex, and resulting in Src activation, which consequently increases tyrosine phosphorylation of EGF receptor and other signaling proteins [67]. Like EGF receptor, NKA is also a plasma membrane protein, capable of recruiting Src via its interaction with the SH2 and kinase domains. However, unlike EGF receptor, these interactions actually keep Src in an inactive state. This has now been well documented in test tube, live cells, or in vivo [56, 57]. For example, knocking down of NKA expression significantly increases cellular Src activity under the basal culture conditions. This increase is fully reversible and can be blocked by the expression of either pumping-competent or -null NKA [68]. Thus, NKA serves not only as a Src scaffold for targeting Src to the plasma membrane domains where NKA is highly expressed, but also as a Src inhibitor. Furthermore, the formation of NKA/Src complex produces a functional receptor, allowing NKA ligands such as CTS to activate the NKA-associated Src. Therefore, the NKA/Src interaction could allow a dynamic and NKA-specific on- and off-mechanisms of Src regulation. Second, the number of expressed NKA is much higher than that of any other membrane receptor. Most mammalian cells express more than one million NKA molecules in the plasma membrane, and the number goes to more than ten million in kidney epithelial cells [69]. Our FRET studies indicate that approximately 25 % of these pump molecules in renal epithelial cells have the capability of interacting with Src kinase [56]. In contrast, one of the most highly expressed receptor tyrosine kinases, EGF receptor, is expressed in a range of about 10,000–100,000 molecules per normal cell [70]. Src can also be regulated by G protein-coupled receptors and the expression of these receptors is in the range of few hundred and few thousand molecules per cell depending on cell type. Thus, NKA by virtue of its capacity of stable interaction with Src kinase could represent one of the most important regulatory mechanisms of Src kinase in mammalian cells. This is in accordance with both in vitro and in vivo data showing that knocking

down of NKA increases the basal Src activity [68, 71]. Third, the location of NKA is also unique in comparison to other receptors. Most of NKA is expressed in the plasma membrane of epithelial cells. Moreover, it has been demonstrated that more than 30 % of NKA is concentrated in caveolae together with Src and other signaling partners in these cells. This is clearly different from most of receptor tyrosine kinases or G protein-coupled receptors [58, 72].

Over the years, several proteins have been identified as important negative regulators of Src kinase. It is also of interest to compare and contrast them with NKA. Among the negative regulators, Csk and Chk are most prominent. They phosphorylate the Y529 and inactivate Src [73]. However, they appear to require other membrane proteins to exert their effects on membrane domain-specific pool of Src. Interestingly, while CTS stimulate the phosphorylation of Y418, they have no effect on Y529 phosphorylation [56]. Examples of other negative regulators include Wiskott-Aldrich syndrome protein (WASP). Like, NKA, it could directly inhibit Src kinase by binding to the catalytic site of Src [74]. However, WASP is a cytoskeletal protein and its effect on Src kinase activity lies far downstream of most receptor signaling pathways. In short, NKA provides a unique mechanism of Src regulation, different from those of receptor tyrosine kinases, G protein-coupled receptors, and other positive and negative regulators.

4.4 Development of pNaKtide as a Functional Antagonist of Receptor NKA/Src Complex

Realization that the N domain of NKA interacts with Src kinase domain and inhibits Y418 phosphorylation had prompted us to map and develop a peptide from the N domain of NKA that could bind/inhibit Src Y418 phosphorylation and also disrupt the formation of a functional NKA/Src receptor complex. This effort led to the identification of NaKtide, a 20-amino acid peptide from the α1 subunit of NKA [75]. To make it cell permeable, we tried several membrane-penetrating peptides. Interestingly, addition of a 13-amino acid TAT peptide to NaKtide (named as pNaKtide) not only made it permeable to cell membrane, but also localized a majority of pNaKtide to the plasma membrane. Several unique properties of this peptide were subsequently demonstrated. First, pNaKtide is a potent inhibitor of Src Y418 phosphorylation in vitro with IC50 of about 4 nM comparable to the commonly used small molecular Src inhibitor PP2. However, unlike PP2, pNaKtide does not affect much of basal Src activity in live cells. It becomes effective when cellular Src activity is increased due to the downregulation of NKA. Second, it seems to be more specific to Src than to other Src family kinases, and it is not an ATP analog. Third, it has no effect on PKC family of kinases and on receptor tyrosine kinases such as EGFR and IGFR. Finally, pNaKtide is effective in reducing the formation of NKA/Src receptor complex and thus acts as a specific antagonist of CTS [75, 76].

4.5 Identification of NKA Mutants that Pump but Are Defective in Signal Transduction

Structure/activity studies indicate that the helical structure of pNaKtide is important for the peptide to inhibit Src Y418 phosphorylation. This led us to test whether we could generate a pumping-competent mutant NKA with no ability to regulate Src Y418 phosphorylation. Several mutants were generated in the helical region of NaKtide, and the mutant expression vector was transfected into PY-17 cells where the endogenous NKA was knocked down. Expression of mutant NKA further reduced the expression of endogenous NKA, making the stable cell line almost exclusively express the mutant. Functional characterization of these mutant cell lines led to the identification of A420P mutant that pumps normally, but is incapable of regulating Src Y418 phosphorylation. In accordance, ouabain was no longer able to activate Src and Src effectors in the mutant-expressing cells [77]. Thus, we have made a Src regulation-defective NKA mutant that has normal pumping function.

It is known that NKA adapts several conformations (e.g., E1 and E2) during pump cycle and that CTS stabilizes the enzyme in E2-like conformation [78, 79]. In view of the fact that NKA regulates Src via direct protein interaction and that ouabain could activate the NKA-associated Src, we have postulated that the interaction between NKA and Src might be conformation dependent. Subsequent studies using chemical modifiers to stabilize the NKA in either E1 or E2 conformation are supportive of this notion. For example, fluoride analogues such as beryllium fluoride stabilize purified NKA in E2-like state and result in the activation of NKA-associated Src as ouabain whereas stabilization of NKA at E1-like state by *N*-ethylmaleimide completely inhibits Src Y418 phosphorylation. To further test this conformation-dependent regulation of Src by NKA, we also studied the effect of extracellular K^+ and intracellular Na^+ on cellular Src activity. We found that lowering of extracellular K^+ concentration or increasing intracellular Na^+ stimulated the formation of pY418 Src, and consequently activated Src effectors in renal epithelial cells by accumulating NKA in E2-like state [57]. To further affirm that NKA can regulate Src in a conformation-dependent manner and to identify other NKA mutants that are defective in signal transduction, we have generated several stable cell lines that express either E1-like or E2-like NKA mutants. Characterization of these cell lines provides further evidence of conformation-dependent regulation of Src by NKA. While E1- and E2-like mutants have similar degree of overall deficiency in their pumping functions, they regulate Src differently. Unlike E2-like mutant, expression of E1 mutant caused much stronger inhibition of basal Src activity, suggesting its capacity of forming a functional receptor complex. This is in accordance with the fact that ouabain activated signal transduction in E1-, but not E2-mutant, expressing cells [80]. Taken together, these studies provide strong support of the notion that NKA has pumping-independent signaling function due to its interaction with Src kinase and that NKA may regulate Src in a conformation-dependent manner.

In short, NKA appears to represent a unique regulatory mechanism of cellular Src-mediated signal transduction. In most epithelial cells, this mechanism allows a

spatial, dynamic, and high-capacity regulation of Src by NKA, and its ligands such as CTS. However, several important issues are worthy of further discussion. First, the Src family consists of at least nine members. They share similar overall structure but are expressed in a tissue-specific manner. Thus, it is conceivable that NKA could interact with other members of Src family in a tissue-specific manner as suggested by recent studies [81, 82]. This may also be applied to other isoforms of NKA [83–87]. Moreover, because H/K-ATPase may interact with Src [56, 88, 89], there is possibility of formation of another group of receptor complex. All together, the diversity of these interactions could produce a large population of receptor species and provide a tissue-and ATPase-specific response. Second, although evidence of direct protein interaction between NKA and Src is overwhelming, other modes of Src regulation by CTS have been proposed [90, 91]. Needless to say, these issues have to be further investigated.

4.6 Formation of Cell-Specific Signalosomes for Signal Initiation, Amplification, and Termination

Studies of last two decades have revealed that CTS could activate a variety of cellular signaling pathways in a cell-specific and dose-dependent manner. It involves both short- and long-term regulations. To simplify the discussion, we will focus on a few important short term regulations that could be stimulated by CTS at concentrations that do not affect the overall pumping capacity of cells. As illustrated in Fig. 2.1, the receptor NKA interacts with several proteins to perform cell-specific signal transductions including the Raf/MEK/ERK, PLC/PKC, PI3K/Akt, Ca^{2+} signaling, and the generation of ROS. One of the most important signaling partners trans-activated by NKA/Src receptor complex is EGF receptor, which is recruited and phosphorylated at several phosphorylation sites other than its major phosphorylation site Y1173 when cells are exposed to CTS [21]. The activated EGF receptor then recruits the adaptor protein Shc, which in turn binds the protein complex Grb2 and SOS. SOS is a guanine nucleotide exchange factor that activates Ras by exchanging GDP for GTP. Activated Ras then stimulates Raf/MEK and p42/44 ERK cascade [21, 22]. Activation of this cascade by CTS appears to occur in most of cell types [28, 37, 44, 72]. In addition to the ERK cascade, the activation of Src/EGF receptor is also required for CTS-induced activation of PLC and consequently the generation DAG, which leads to the activation of PKC [92–94]. The NKA/Src complex also plays an important role in CTS-induced Ca^{2+} signaling. Several mechanisms appear to be in operation depending on cell types. In most of the cells, NKA is important for the formation of Ca^{2+} signalosomes [94–101]. While Src is involved in the phosphorylation of IP3 receptor and the generation of IP3 in epithelial cells [94, 95, 99], it appears to play a role in assembly of TRPC6, NCX1, and NKA into a signaling complex in response to ouabain stimulation [55, 102]. Finally, CTS activate PI3K/Akt pathways in several cell types. However, the activation of Src/

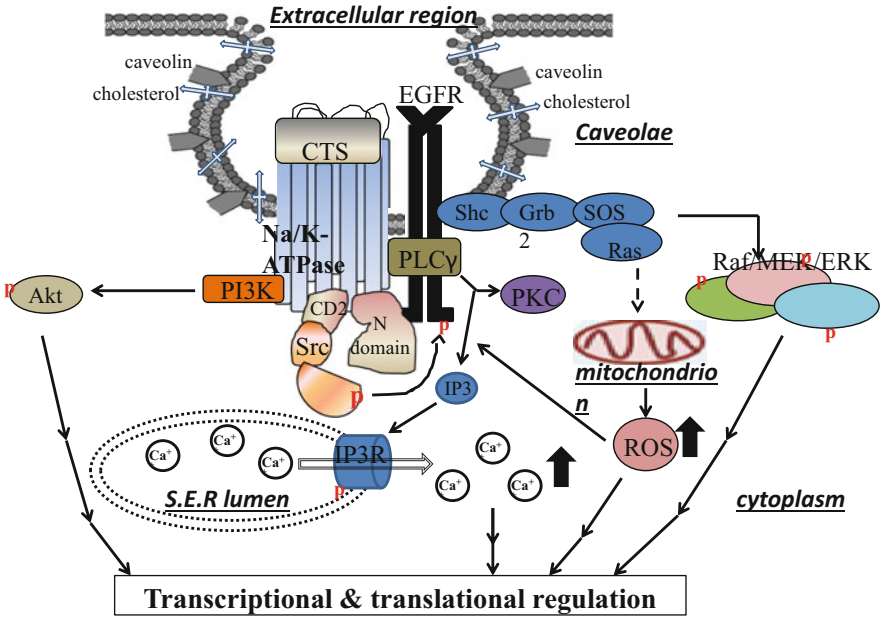

Fig. 2.1 Schematic diagram showing different molecular components of the signalosome formed by Na/K-ATPase/Src receptor complex in response to cardiotonic steroid binding. Cardiotonic steroid binding activates multiple signaling pathways, induces calcium signaling and generation of ROS, and ultimately regulates cellular transcription and translational events. The abbreviations used are as follows: *EGFR*, epidermal growth factor receptor; *PKC*, protein kinase C; *CD2*, second cytoplasmic domain of Na/K-ATPase; *N domain*, nucleotide-binding domain of Na/K-ATPase; *Src*, Src kinase; *PI3K*, phosphatidyl inositol 3 kinase; *Grb2*, growth factor receptor-bound protein 2; *Sos*, son of sevenless protein; *Shc*, src homology collagen like protein; *PLCγ*, phospholipase Cγ; *IP3*, inositol 3,4,5; *IP3R*, inositol 3,4,5 receptor; *S.E.R*, sarco/endoplasmic reticulum; *MEK*, MAPK/ERK activating protein; *ERK*, extracellular regulated kinase (MAPK); *ROS*, reactive oxygen species; *CTS*, cardiotonic steroids (like ouabain, digoxin, marinobufagenin, etc.). P (*red*) denotes phosphorylated amino acids. *Solid line* denotes proved signaling pathway, *dashed line* denotes speculated signaling pathway, *thick black line* denotes upregulation

EGF receptor appears to be important, but not necessary for the activation of PI3K/Akt [77, 80, 103, 104]. Moreover, it appears that CTS fail to activate this pathway in certain types of cancer cells, leading to the downregulation of surface expression of NKA and the inhibition of cell growth [104].

Several important features of this newly appreciated signaling mechanism are worthy of further discussion. First, unlike the suggested NKA/NCX coupling mechanism of CTS signaling, the activation of protein and lipid kinase cascades and the generation of second messengers ensure the formation of a positive feed-forward loop that could amplify CTS-provoked signal transduction, and also allow signal diversification and transcriptional and translational regulation of gene

expression [21, 32, 39]. This is best exemplified by the recruitment of additional signaling partners into the receptor complex [21, 58, 94] and by the ROS-induced signal amplification [105–107]. In accordance, this could explain as to why endogenous CTS could have profound effects on animal physiology at concentrations well below 1/10th of IC50 [108]. Second, the fact that CTS could provoke signal transduction through kinase cascades, and the generation of second messengers points the need of reexamination of CTS physiology and exploring the potential new pharmacology of exogenous CTS. Looking back, most of the pharmacological studies of CTS were focused on their ability to inhibit NKA. As such, they were used as NKA inhibitors to increase myocardial contractility. Even in this application, clinical studies have demonstrated that the use of lower, but not higher, doses of digoxin is associated with a decrease in mortality in patients with CHF [109]. Moreover, we and others have shown recently that the activation of NKA signaling, but not inhibition of cellular pump capacity, by CTS is capable of protecting the heart from ischemia/reperfusion injury [110–112]. This is consistent with the findings that CTS may be protective of organ development during malnutrition [113] and that CTS may be important for fetus development [114, 115]. Furthermore, CTS at doses lower than 1/10th of IC50 are effective stimuli of collagen synthesis, suggesting the potential use of these compounds in skin care and wound healing [39, 41]. Thus, the new data suggest that CTS as NKA/Src receptor agonists may be explored for new clinical implications at low doses that do not produce cardiac toxicity. Third, it has been reported that the endocytosis of NKA/Src receptor complex, like many other membrane receptors, is stimulated by its ligands such as CTS [116–118]. This occurs via clathrin-coated pits, early and late endosomes, and depends on the activation of Src and PI3K. Although it remains to be further investigated, it is conceivable that CTS-induced endocytosis of receptor NKA/Src could represent a pathway of signal termination. Of course, it might also generate an effective way of communication with intracellular compartments during the signal transduction process [119].

Because NKA has to interact with Src and other proteins to relay CTS signal, it is postulated that NKA and its partners may pre-assemble into signalosomes in caveolae. Caveolae are membrane microdomains that were first identified as flask-shaped invaginations of plasma membrane enriched in cholesterol and marked by caveolins [120]. Caveolins are 21–24 kDa membrane-associated scaffolding proteins that directly interact with multiple membrane proteins as well as cholesterol. Two potential caveolin-1 binding motifs are identified in the α1 subunit of NKA and they are highly conserved [7, 58]. Several studies suggest that NKA may directly interact with caveolin-1 via its N-terminal caveolin-binding motif, and is highly concentrated with Src in caveolae [25, 58, 72]. Functionally, this appears to be important for CTS-induced signal transduction and for the formation of signalosomes [7, 58, 121]. Because caveolin-1 expression varies among different cells, this could provide some specificity to receptor NKA/Src-mediated signal transduction. Moreover, recent studies reveal an important functional interaction among NKA, Src, caveolin-1, and cholesterol in cell culture as well as in vivo [71, 122].

5 NKA-Mediated Signal Transduction in Animal Physiology and Diseases

It is now clear that endogenous CTS represent a class of important hormones. The appreciation of endogenous CTS in animal physiology and diseases has grown in recent years as the mechanisms by which the Na/K-ATPase/Src complex functions as a receptor have been elucidated. There is ample evidence for a role of this receptor complex in the regulation of cell growth, fetus development, maintenance of organ structure and function, and regulation of blood pressure [2, 25–27, 42, 81, 113–115, 123–126]. Furthermore, a chronic stimulation of the receptor appears to operate as mal-adaptive response under many pathological conditions contributing to tissue fibrosis, inflammation, and pathogenesis of ADPKD, to name a few [37, 127–131]. Thus, endogenous CTS appear to operate like other important hormones and neurotransmitters such as angiotensin II and norepinephrine in animal physiology and diseases. The following two cases are discussed to illustrate this point of view.

5.1 NKA-Mediated Signal Transduction in the Regulation of Renal Salt Handling

In kidney, the renal proximal tubule mediates about 60 % of total Na^+ exchange in the body. NHE3 is largely responsible for Na^+ intake on the apical side of the proximal tubule whereas the NKA mediates Na^+ extrusion on the basolateral side. NHE3 belongs to a family of Na^+/H^+ exchangers (NHE) that are responsible for Na^+ and H^+ exchange across cell membrane. It is widely accepted that coordinated regulation of NHE3 and NKA is essential for maintaining Na^+ homeostasis and blood volume [119, 132].

The first suggestion that receptor NKA/Src complex is important for coordinated regulation of NKA and NHE3 came from in vitro studies conducted in LLC-PK1 cells in culture. LLC-PK1 cells are derived from pig proximal tubules and the surface expression of NKA and NHE3 are polarized as in proximal tubules, when cells are cultured on an insert. As expected, addition of ouabain (at concentration of less than 1/10th of IC50) to the basolateral but not apical side of insert was sufficient to cause a decrease in surface expression of NKA with concomitant NKA increase in the early and late endosomes [116]. This regulation is dependent on the activation of Src and PI3K as well as the expression of caveolin-1. Surprisingly, there was no detectible change in intracellular Na^+ when surface expression of NKA was significantly reduced by ouabain exposure. Further studies revealed that ouabain also increased endocytosis of NHE3 and thus a significant decrease in apical expression of NHE3 and naturally Na^+ influx. Flux studies further confirmed the ability of ouabain to reduce trans-cellular movement of Na^+

across the monolayer [133]. Moreover, ouabain also suppressed the transcription of NHE3. These studies indicate that the receptor Na/K-ATPase/Src complex may be important for salt handling in renal tubular cells. Several ex vivo and in vivo studies support this notion. First, infusion of CTS or fed animal with high salt was equally effective in activating renal tubular Src and inducing the endocytosis of both NKA and NHE3, which resulted in an increase in renal excretion of Na^+ [134, 135]. Second, ex vivo studies confirmed that CTS activated Src and induced natriuresis in isolated proximal tubules [136]. Moreover, by using transgenic mice, Lingrel's group was able to demonstrate greater natriuretic response to Na^+ loading in mice expressing a mutant form of NKA α1 isoform that is much more sensitive to CTS than that in wild-type mice [137]. Finally, Liu and his colleagues showed that high salt intake or CTS could activate NKA signaling and consequently induce the coordinated down-regulation of surface expression of NHE3 and NKA only in Dahl salt-resistant but not in Dahl salt-sensitive rats [134]. Taken together, the studies of last 10 years have provided strong evidence that endogenous CTS may regulate renal salt handling through the activation of receptor NKA/Src complex.

5.2 NKA Signaling and Tissue Fibrosis

It has been well documented that endogenous CTS are elevated in patients suffering from chronic renal failure, uremic cardiomyopathy, congestive heart failure, hyperaldosteronism, and preeclampsia [127–131]. We suggest that while this increase might enhance the functionality of target organs such as the heart and kidney, it would become mal-adaptive if the receptor is chronically stimulated. This is best illustrated by a series of studies conducted by Shapiro laboratory using partially nephrectomized animal models [128, 138]. As expected, partial nephrectomy increased plasma level of marinobufagenin, one of the endogenous CTS, in rat. This increase was associated with cardiac hypertrophy and tissue fibrosis, quite analogous to patients suffering from end stage renal disease [40, 128, 138]. Subsequent investigations demonstrated that infusion of CTS was sufficient to increase tissue fibrosis and that neutralization of the increase in endogenous CTS by either passive or active immunization against CTS was effective in reversing tissue fibrosis in partial nephrectomized rats or mice [139, 140]. At molecular level, CTS stimulated collagen synthesis by activating the signaling function of NKA, triggered epithelial to mesenchymal transition, and increased the transcription as well as translocation of the protein Snail in renal tubular cells [141, 142]. In short, chronic stimulation of receptor NKA/Src complex appears to be pro-fibrotic. Thus, it would be desirable to develop effective receptor antagonists and test whether they are effective in reducing or reversing CTS-induced tissue fibrosis and organ remodeling. To this end, it is of interest to mention that spironolactone may antagonize CTS-induced signal transduction and prevent partial nephrectomy-induced cardiac fibrosis in rats [143].

6 Conclusion

Studies over the last two decades have revealed that NKA has important signaling functions in addition to pumping ions. We have also begun to appreciate the signaling function of NKA in animal physiology and diseases. However, there is a clear need to develop new animal models and tool chemicals that will enable the field to better probe the intricacies and complexities of NKA-mediated signal transduction in live animals. With the better understanding of this newly appreciated signaling mechanism, it is most likely that the NKA signaling-specific new therapeutics may be developed for various human diseases.

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Chapter 3
Na, K-ATPase Cell Signaling Pathways and Cancer

Marco Túlio C. Pessôa, Vanessa F. Cortes, and Leandro A. Barbosa

Abstract Recently, cardiotonic steroids (CTS), used in heart failure treatment, have shown interesting antitumor effect acting by several intracellular signaling pathways triggered by Na, K-ATPase. Several signaling pathways activated by CTS are involved in tumor progression, metastasis, and proliferation, leading an interesting perspective to study the relationship of the Na, K-ATPase and cancer. Here we highlight the major signaling pathways modulated by Na, K-ATPase and the chemical modification of traditional CTS that are under study to develop new anticancer drugs.

Keywords Na • K-ATPase • Signaling • Cancer • Cardiotonic steroids

1 Introduction

For many years, Na, K-ATPase has been known by its ionic transport effect of maintaining an electrochemical gradient (high Na^+ concentration and low K^+ concentration in extracellular side) which enables essential functions in organisms, such as glucose uptake by enterocytes and synaptic activities performed by neurons. In 1785, the English botanist and physician William Withering described the properties and medicinal uses of the flowering plant *Digitalis purpurea*, which is widespread throughout Europe, mainly for the treatment of hydropsy. Within 14 years, it was discovered that extracts from this plant had a direct action on the heart. For many years after, and even to some extent today, the dried leaves from this plant have been used as a heart failure treatment. Finally, cardiotonic steroids (also called cardiac glycosides or digitalis-like compounds), the main isolated molecules of *Digitalis* extract, were demonstrated to be ligands of the Na, K-ATPase.

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The digitalis mechanism of action is well established. Briefly, cardiotonic steroids bind to the extracellular domains of the Na, K-ATPase leading to blockage of this enzyme in E2 form. The partial inhibition of the Na, K-ATPase causes a delay in Na^+/K^+ ionic restoration before the next cell depolarization. In this way, there is a temporary increase in intracellular Na^+ leading to a decrease in Na^+ uptake by the Na^+/Ca^{2+} exchanger, and consequently, a decrease in extrusion of intracellular Ca^{2+}. The high levels of intracellular Ca^{2+} bind to the troponin C complex and activate the myosin II/actin fibers, resulting in the inotropic effect (increasing of contractility) of cardiac glycosides [1]. However, high levels of cardiotonic steroids, increasing the Na, K-ATPase inhibition, promote toxic effects leading to cardiac arrhythmias.

Starting in the 1970s, new evidences for gene expression and cell proliferation involving the Na, K-ATPase were demonstrated. Researchers have observed, in cultured cells, alterations in the expression of the Na, K-ATPase subunits after chronic inhibition of this enzyme either by lowering extracellular K^+ or by exposition to inhibitors of the Na, K-ATPase, such as ouabain (a cardenolide cardiac glycoside). This change in expression of the Na, K-ATPase subunits has been observed in multiple cell types, including HeLa cells [2], rat astrocytes [3], rat cardiocytes [4], and suspension of rat outer medullary tubule segments [5]. Subsequently, many studies showed chronic inhibition of Na, K-ATPase (via low extracellular K^+ levels or presence of the Na, K-ATPase inhibitors) also altered the levels of many gene transcripts other than Na, K-ATPase subunits [6–8]. Finally, several works revealed growth-related effects of ouabain on some cell lines, effects that were dissociated from ouabain-induced changes in osmotic balance [9, 10]. These effects have encouraged the scientific community to look for additional information to explain how the Na, K-ATPase communicates with the intracellular side of different cell types.

2 Signal Transduction Through Na, K-ATPase

The first evidences of signal transduction involving the Na, K-ATPase have arisen in rat cardiomyocyte models. Several studies have showed that nontoxic ouabain concentrations trigger multiple-chain protein phosphorylation [11, 12]. Moreover, the discovery that Src tyrosine kinase family forms a complex with the Na, K-ATPase revealed a method by which the Na, K-ATPase could transduce signal, as the Na, K-ATPase does not present intrinsic tyrosine kinase activity [12–14].

The Xie group postulated that the Na, K-ATPase could exist in two distinct pools: a classical pool, in which the main function would be ion pumping, and a signal transduction pool, in which protein-protein interactions would lead to activation of a set of signaling intermediaries [15]. Interestingly, this signal transduction pool of the Na, K-ATPase was found to colocalize with caveolin-1, the main constitutive protein of caveolae, demonstrating that the Na, K-ATPase can bind to this protein through two conserved caveolin-binding motifs present in α1 isoform [13]. In fact, caveolae are the main signal transduction compartment of many cell types, corroborating these interesting discoveries. Despite several studies having

demonstrated the cardiac steroids effects through Na, K-ATPase-Src complex inside caveolae, a couple of works demonstrated interesting findings. Askari group recently did a comparative between properties of caveolar and noncaveolar preparations of kidney Na, K-ATPase [16]. In this paper they demonstrated that the Na, K-ATPase found in noncaveolar fraction of kidney can be modulated by ouabain and be able to modulate signaling pathway. Also, Souza and coworkers observed that ouabain treatment on Caco-2 (a cell line that does not contain caveolae) resulted in modulations on expression and redistribution of junctional proteins as well as Na, K-ATPase subunits [17]. Deeper investigations demonstrated that previous effects were dependent on the MAPK signaling activation. Altogether, these findings suggest that caveolae seems to be very important to the Na, K-ATPase signaling pathways; however these effects are completely dependent on cell type and that the presence of caveolae is not essential for Na, K-ATPase signaling.

2.1 The Src Complex

Src family proteins comprise a set of nonreceptor kinases associated with cell membrane that compartmentalizes many signaling pathways through caveolae. The ability of Src to bind directly to the Na, K-ATPase to form a stable signaling receptor complex in response to cardiotonic steroids has been demonstrated by several laboratories through many experiments like immunofluorescence analysis, FRET analyses, co-immunoprecipitation assays, and in silico simulations [18, 19]. This binding appears to occur at the level of the CD2 and CD3 domains of the α1 Na, K-ATPase, interacting with SH2 and kinase domains of Src, respectively, keeping Src protein in an inactive state [13]. It is interesting to note that the binding of ouabain to this complex releases the Na, K-ATPase-attached Src protein, allowing the signaling function of Src. In fact, the effects of cardiotonic steroids are blocked by addition of Src inhibitors in many cell types [20–22]. Thereby, most signaling events triggered by cardiotonic steroids seem to be connected with the release of Src from this intricate complex found on caveolar fractions of cell membrane. Interactions between the Na, K-ATPase and caveolar fractions are so coordinated that cardiac fibroblasts extracted from mice without caveolin-1 demonstrate alterations in several Na, K-ATPase signaling functions [23].

2.2 EGFR Transactivation

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor capable of relaying the growth factor external message to intracellular side of the cell and exerts a central role in signal transduction [24]. Briefly, the epidermal growth factor (EGF) binds to its receptor (EGFR) inducing dimer formation leading to self-phosphorylation on tyrosine residues. Then, the phosphotyrosine residues can act as docking sites for adaptor proteins and cytosolic kinases.

Recently, several studies have provided evidence that EGFR performs cross-communication with other signaling systems to integrate the extracellular signal variety in some pathways such as cytokines, hydrogen peroxide, and G protein-coupled receptors [13, 25–27]. This kind of activation is called *transactivation*, to distinguish it from the EGFR activation by its natural ligand EGF [28]. EGFR transactivation has been shown to be present upon ouabain signaling, and probably other cardiotonic steroids, to relay the signals to cytosolic kinases through the Na, K-ATPase-Src complex [22]. It is noteworthy that ouabain stimulates phosphorylation of EGFR at different amino acids than the natural EGFR ligand. EGF binding to EGFR leads to the phosphorylation of several tyrosine residues such as Y845, Y992, Y1068, Y1086, Y1148, Y1173, whereas EGFR phosphorylation in response to ouabain does not include Y992 and Y1173 residues in HeLa cell lines [29].

2.3 Ras/MAPK Signaling Pathway

The main downstream signaling pathway associated with EGFR activation is Ras/MAPK. This signaling pathway is activated in a particular way to guide essential cell functions like proliferation, cell growth, differentiation, apoptosis, migration, and neuronal activity [30]. Briefly, three adaptor proteins (Shc, Grb2, and Sos), containing SH2 and SH3 domains, act as linkers between activated EGFR-exposed phosphotyrosines and Ras. Afterward, activated Ras leads to the subsequent activation of Raf/MEK/MAPK pathway. This pathway regulates essential cell functions in many ways. For example, a transient activation followed by light signaling, but sustained, of Ras/Raf/MEK/MAPK is a common cell proliferation aspect in many systems [31].

It is interesting to note that some cardiotonic steroids acting through the Na, K-ATPase have been demonstrated to trigger the Ras/Raf/MEK/MAPK signaling cascade. Specially, ouabain was found to trigger this pathway in SH-SY5Y and MDA-MB-435S cell lines, leading to cell death [32, 33]. Many of cardiotonic steroids-related signaling pathways were demonstrated to be linked to Src activation. Ouabain-induced activation of the MAPK cascade in A7r5 cells was abolished by cell treatment with Src inhibitors. In SYF cells, which harbor functionally null mutations in both alleles of Src family kinase members Src, Yes, and Fyn, ouabain was not able to activate the MAPK signaling pathway. The restoration of c-Src activity in SYF cells, by stable transfection, restored the ability of ouabain to activate the MAPK cascade, confirming Src-Ras-MAPK pathway [34]. Ex vivo assays with rat isolated kidney showed that bufalin (a bufadienolide and cardiotonic steroid) activates MAPK signaling pathway only with pre-activation of Src [20]. On the other hand, a specific study with ouabain and cinobufagin demonstrated that both compounds induce cell cycle arrest to block the growth of human hepatoma cell lines by decreasing MAPK phosphorylation [35]. This divergent point was revisited by Yin and coworkers, who demonstrated that cytotoxic and antiproliferative effects of bufalin can be a result of either MAPK stimulation or inhibition depending on the cell type [36].

2.4 PI3K/Akt Signaling Pathway

Another signaling pathway able to guide essential functions in cells is the PI3K/Akt cascade. Briefly, this pathway involves the presence of phosphoinositides acting as scaffolds to cytosolic signaling proteins to promote cell growth and survival [37]. PI3K is able to bind to phosphotyrosine residues of tyrosine kinase receptors and phosphorylates the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 acts as a scaffold for many intracellular signaling proteins, such as Akt and PDK1, leading to the cell effects aforementioned.

Some studies have shown the activation of the PI3K pathway, in response to the Na, K-ATPase inhibition, leading to varied effects. The Na, K-ATPase endocytosis was demonstrated by a clathrin-dependent mechanism, in LLC-PK1 cells, after the treatment with ouabain [38]. Another work with the same cell line showed that the Na, K-ATPase inhibition was able to activate PI3K/Akt pathway and inhibit apoptosis [39]. Ouabain was also found to stimulate cardiac myocytes hypertrophy through activation of PI3K/Akt signaling pathway, and it was demonstrated that this effect is abolished after cell treatment with PI3K and Src inhibitors [40]. In fact, all these findings are in accordance with other studies that demonstrate that PI3K binds to a proline-rich motif in the Na, K-ATPase α subunit and has the capacity to regulate its trafficking [41]. Other studies demonstrated that Na, K-ATPase β1 subunit expression suppresses cell motility by reorganization of the actin cytoskeleton in MSV-MDCK cells [42].

2.5 $[Ca^{2+}]_i$ Oscillations

It is well established that the Na, K-ATPase inhibition by cardiotonic steroids increases the $[Na^+]_i$, and subsequently, increases the $[Ca^{2+}]_i$ momentarily. This is the known mechanism of action for digitalis to increase contractility in heart [43]. On the other hand, Ca^{2+} is a well-known second messenger inside most of the cell types and can activate many protein kinases, such as protein kinase C (PKC). Two second messenger pathways are activated by phospholipase C (PLC)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), which releases the second messenger molecules inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol [44]. Briefly, PKC binds and phosphorylates a variety of proteins, as cytoskeletal proteins, enzymes, and nuclear proteins that regulate gene expression, affecting neuronal and immune function and the regulation of cell division.

In 2001, Aperia group demonstrated that ouabain signals to the cells through slow calcium oscillations, leading to NF-κB activation, depending on Inositol trisphosphate receptors and capacitative calcium entry via plasma membrane channels [45]. This ouabain effect happens in concentrations known to cause only partial Na, K-ATPase inhibition, and other inhibitory stimuli for Na, K-ATPase, such as low extracellular

K^+ and depolarization of cells, do not present calcium oscillations. Later, the same group showed that the Na, K-ATPase organizes in a functional microdomain, together with the IP_3 receptor ($InsP_3R$), to create the calcium oscillations which signal after cardiotonic steroids bind to the Na, K-ATPase. Also, the induction of slow calcium oscillations is dependent on the Src activation, since this effect is practically abolished upon treatment of Src inhibitors [46, 47].

3 Na, K-ATPase and Cancer

After many grounded studies regarding cardiotonic steroids and Na, K-ATPase interaction in the cardiomyocytes model, scientists have observed several interesting effects for cardiotonic steroids in cancer cells. First evidence of these effects dates back to 1967, when Shiratori described the growth inhibitory effect of cardiac glycosides and aglycones on neoplastic cells by in vitro and in vivo approaches [48].

Since then, several laboratories confirmed the cytotoxic and antiproliferative effects of these compounds. Ouabain inhibits breast cancer cell proliferation by the Na, K-ATPase inhibition and subsequent signal transduction in a dose- and time-dependent manner [33]. Digoxin was demonstrated to induce apoptosis in human acute T-cell lymphoblastic leukemia and also to inhibit neuroblastoma tumor growth in mice [49, 50]. Lopéz-Lázaro and coworkers have demonstrated an antitumor effect for digoxin and digitoxin, in nanomolar concentrations, on many human cell lines, such as renal adenocarcinoma cells (TK-10), breast cancer cells (MCF-7), melanoma cells (UACC-62), and chronic myelogenous leukemia cell lines (K-562) [51]. Importantly, assays performed with human malignant hematological cultures (Jurkat T cells and Daudi B cells) showed remarkable sensitivity to digoxin and digitoxin, demonstrating an apoptotic phenotype when treated at nontoxic concentrations for normal tissues [52].

Five different cardiac glycosides (ouabain, peruvoside, digoxin, digitoxin, and strophanthidin) were able to inhibit the Na, K-ATPase and sensitize several cancer cells lines (PCC-1 and PC-3 prostate, HeLa cervical, OVCAR3 ovarian, and T47D breast cancer cells) to anoikis (a form of programmed cell death), preventing metastasis. These effects may be due to ouabain being involved with the mitochondrial pathway of caspase activation [53]. Bufalin was shown to induce apoptosis in several cancer cell lines, such as lung, breast, prostate, leukemia, gastric cancer cells, and hepatocellular carcinoma [36]. Moreover, interaction between cardiac glycosides and the Na, K-ATPase could circumvent several chemoresistance pathways [54].

Regarding the direct interaction of cardiotonic steroids and the Na, K-ATPase, and also downstream signaling events, studies have suggested a dual role to these compounds. Micromolar to millimolar concentrations of cardiac glycosides inhibit the Na, K-ATPase activity. However, nanomolar concentrations were demonstrated to upregulate the Na, K-ATPase activity [55].

All these previous effects were considered important and scientists have allied the cell biochemistry of cardiotonic steroids to organic chemistry, specifically the hemi-synthesis of new cardiotonic steroids from the naturally isolated prototypes.

This particular area, in association with in silico studies, has helped researchers to describe the interactions between cardiac glycosides and the Na, K-ATPase and also to postulate groups to add to the cardiac glycosides to enhance desired effects. Principally, the addition of steroidal and sugar moieties in particular to cardiac glycosides has resulted in improvements in the Na, K-ATPase-cardiotonic steroids interactions, selecting the desired effects regarding cell cytotoxicity, Na, K-ATPase activity modulation, cell signaling pathways, and so on.

Juncker and coworkers demonstrated the apoptotic effects for a new hemi-synthetic cardenolide, UNBS1450, on leukemic cell lines, with incredibly low nanomolar concentrations [56]. The molecular mechanism for UNBS1450 apoptotic effect was demonstrated to be the inhibition of NF-κB signaling pathway with concomitant cleavage of pro-caspases 8, 9 and 3/7, decreasing the expression of the antiapoptotic factor Mcl-1 and recruiting proapoptotic proteins as Bak and Bax [56]. Jensen and coworkers have synthesized bivalent steroids, using sulfur linked ethylene glycol moieties of varying length, and have assessed their potencies to inhibit the Na, K-ATPase and for their cytotoxic effect on MCF-7 cancer cell lines, demonstrating that the steroid bioactivity is dependent on the ethylene glycol chain length [57]. Recently, Elbaz and coworkers demonstrated the apoptotic effect, through caspase-9 cleavage, of a synthetic digitoxin analog, D6-MA, on NCI-H460 cancer cell lines [58]. The cells arrested in G2/M phase after the treatments, demonstrating downregulation of cyclin B1/cdc2 complex and survivin, as well p53 downregulation, suggesting a p53-independent cell cycle arrest mechanism [58]. A new steroid, the 21-benzylidene digoxin (21-BD), was synthesized using the natural isolated cardenolide digoxin [59]. A vinylogous aldol reaction was performed to add an aromatic ring on digoxin lactone portion. Together with computational models, it was demonstrated that the 21-BD has the ability to bind to the Na, K-ATPase α1 subunit, similarly with other cardiotonic steroids, leading to interesting downstream effects. This new molecule was able to induce cytotoxicity on HeLa and RKO cancer cell lines, whereas no cytotoxicity was observed on MDCK normal cells. An apoptotic mechanism was found to 21-BD previous effects. Interestingly, 21-BD has no effect on isolated Na, K-ATPase, but this new steroid has been able to upregulate Na, K-ATPase α1 and β1 subunits at micromolar range in intact cells. Regarding the cell tight junctions, 21-BD was found to increase TER (transepithelial electrical resistance) as well as modulate cell tight junction proteins such as claudin-2, claudin-4, and ZO-1, increasing the tight junction sealing. Finally, 21-BD, instead of digoxin, was able to inhibit Pdr5p activity, a member of ABC transporters family from yeast that shares many substrates and inhibitors with the mammalian P-glycoprotein [60].

4 Conclusions

Na, K-ATPase has been investigated extensively in the past and continues to be studied today. Many important functions have been attributed to the Na, K-ATPase, such as (1) a multifunctional protein with key roles in the formation and

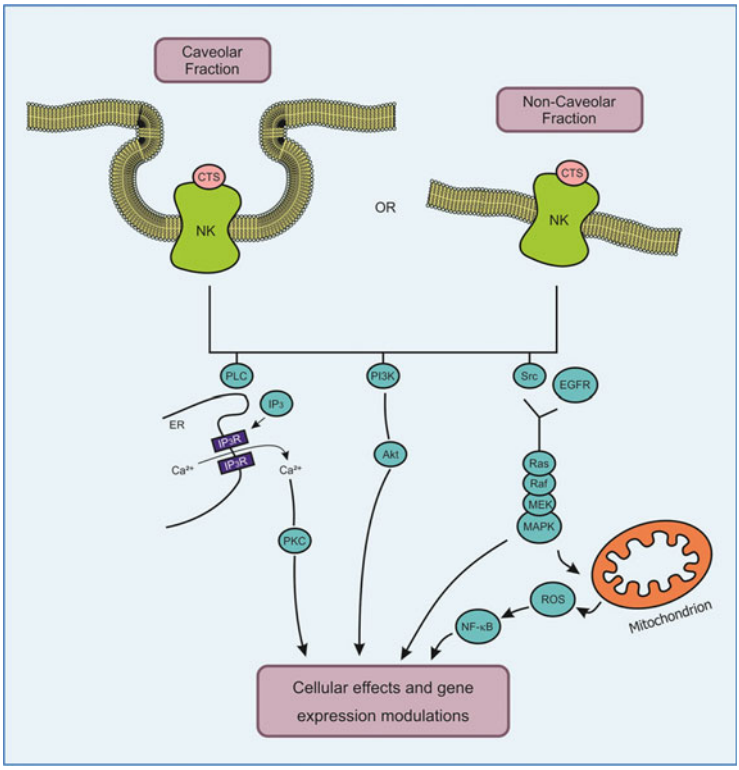

Fig. 3.1 Na, K-ATPase signaling pathways. Binding of CTS on the Na, K-ATPase (in caveolar or noncaveolar pool) activates several intracellular signaling proteins that will be responsive to modulate several cellular effects

maintenance of adhesion complexes, as well as induction of epithelial cell tight junctions and polarity; the Na, K-ATPase therefore has roles in cell adhesion, motility, and actin dynamics, (2) a signaling protein, and (3) a valuable novel target in anticancer therapy and progression of a growing number of cancers, and Fig. 3.1 shows the pathways of Na, K-ATPase. With an endogenous ouabain stimulus, the Na, K-ATPase can also trigger a cascade of signaling events, including binding and subsequent activation of c-Src, EGFR, MAPK, and PI3K. Activation of this pathway ultimately results in the assembly of clathrin-coated pits and the subsequent endocytosis of Na, K-ATPase. Src kinase is able to form a complex with the Na, K-ATPase and might serve as a critical mechanistic link between inflammation and cancer, mediating and propagating a cycle between immune and tissue cells that can ultimately lead to the development and progression of cancer. Activated Src phosphorylates a diverse spectrum of substrates that results in upregulation of several cancer-associated pathways, including EGFR signaling, which acts by synergism with Src to promote cancer. As we can see, the Na, K-ATPase can be involved in cancer regulation in many different ways.

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Chapter 4
Calcium Controls the P2-ATPase Mediated Homeostasis: Essential Role of NaAF

Tushar Ray

Abstract This chapter reveals a new unique story of Ca-signaling in upholding cellular homeostasis. The cytosolic activator (regulatory) protein, NaAF (of 170 kDa mass), for the ubiquitous P2-ATPase (and 80 kDa HAF solely for the gastric H/K-ATPase) is essential for P2-ATPase function. The NaAF and HAF function as the allosteric operator-cum gate-keeper of the dual channel P2-ATPase system (where mirror-image orientation of the two α-subunits serves as the membrane-embedded in-and-out gates) for simultaneous transport of two ions. The entire cyclic operation is in turn fine-tuned by local Ca (μM) as top (allosteric) controller of the P2-ATPase to maintain homeostasis. Thus at lower range Ca (<2) stimulates, but at higher range (>2) Ca abruptly inhibits the HAF-stimulated H/K-ATPase abolishing it at 4 μM Ca. At this point the (K ± HAF)-independent basal (Mg-dependent) activity of the H/K-ATPase acts as a provisional Ca-ATPase pump in an altered state to remove excess Ca, thus resuming the initial Ca-activated HAF-regulated state of a new cycle. Identical Ca-signaling operations also control the universal NaAF-regulated Na/K-ATPase system.

Keywords P2-ATPase (s) • Cytosolic activator protein • Allosteric regulation • Ca-signaling • Homeostasis

1 Introduction

Michael Berridge [1] has recently reviewed the details of many universal Ca-signaling processes, which he initially mapped over a decade ago [2]. The present work describes the delicate control mechanism of active ion transport by μM Ca published in the 1980s based on a new dual-topology model for the P-2 ATPase

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system, since the existing Post-Albers single topology scheme (for sequential ion transport) reining the P2-ATPase field was inadequate to explain this. The new dual-topology P2-ATPase model first introduced in 1986 [3] accommodates all the limitations of PA scheme and also demonstrates the universality of active ion transport by cytosolic regulatory protein and μM Ca mentioned above.

Our bodily cells have built-in ion gradient; the intracellular concentrations of different metal ions (such as, Na, K, and Ca) are very different from those present in the blood transporting them to tissues all through the body; the circulating blood of an individual maintains a fixed concentration (~4 mM) of Na (~140 mM), K (~4 mM), and Ca (~5 mM) compared to the cell interior having Na (~10 mM), K (~140 mM), and Ca (in varying μM range) respectively. So, it is essential for a living cell to maintain the ion gradient (homeostasis) which is at a constant flux with the extracellular environment for carrying out numerous essential functions. As a result, the cells have built-in 24/7 ion-pumps, such as H/K-ATPase, Na/K-ATPase (Na-pump), and Ca-ATPase (Ca-pump), called the P2-ATPase system, on the plasma membrane to pump out the excess metal ions maintaining homeostasis. The cell Ca is used for many important tasks like membrane fusion, turning on and off the key intercellular processes as a final regulatory and other cellular signaling [4, 5].

The plasma membrane Ca-ATPase (PMCA) pumping out excess Ca is indeed a provisional operation of the basic P-2 ATPase systems [5] like the gastric H, K-ATPase [6] and the Na, K-ATPase, working in altered state as a Ca-ATPase based on local need of the host cell (see below). After the job is done the Ca-pump switch back to the former state pumping H or Na as the case may be. The entire operation of the provisional Ca-Pump depends on the nature of the cytosolic regulator (discussed below) running the original pump (H- or Na-Pump). This chapter deals with the HAF-dependent Proton-Pump and Na-Pump belonging to the apical (secretary) membrane (APM) and basolateral membrane (BLM) of parietal cell, and then extending further to the analogous nonparietal Na-Pump as needed for clarification. The details on Ca-signaling will follow.

Let me introduce at first the new dual-topology model of gastric proton-pump giving a unified view of the HAF-regulated pumping of H/K, Ca/H and Na/K, Ca/K across the APM and BLM where the signaling role of Ca is revealed under different conditions of local pH and Ca.

2 The Dual-Topology H, K-ATPase System Is a General Model for the Simultaneous Bidirectional Transport of H/K, Na/K, and Ca/H Across the Apical Plasma Membrane

The construction of the dual-topology model is based on hard data on the orientation of critical ligand sites within a functional H, K-ATPase complex associated with tightly sealed gastric microsomal vesicles of uniform orientation that are capable of ATP-dependent accumulation of H in exchange for K [3]. The dual-topology H,

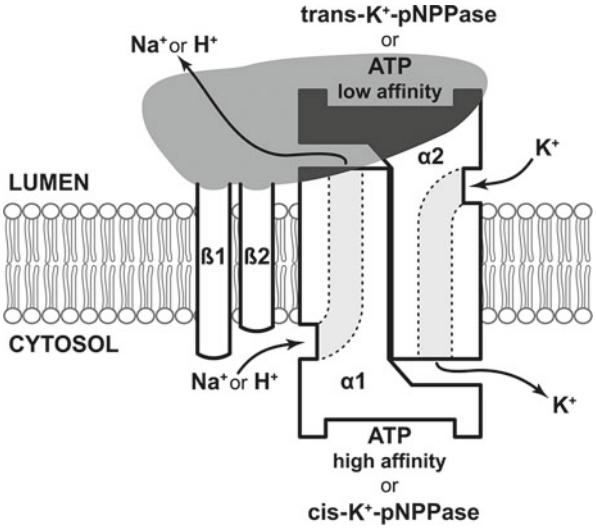

Fig. 4.1 The dual-topology Na (H), K-ATPase showing bilayer orientation of α and β subunits with related ion channels. Two identical subunits, α1 and α2 (not isomers), are shown in mirror images across the membrane with embedded ion channels in contact, and are held with two closely associated β-subunits facing the lumen. The ATP hydrolytic site (separate from the *cis*-pNPPase site) on the α1, and *trans*-cytosolic non-hydrolysable ATP-binding site and the corresponding *trans*-pNPPase site on the α2 are shown. Besides ATP-binding the intimate association among α2, β1 and β2 on the cell exterior is expected to modulate the ATPase function in various ways including the reception of extracellular signals. The high-affinity K^+ site for ATPase stimulation is located across the bilayer on α2 and the corresponding high-affinity H^+ or Na^+ site is on the cytosolic side of the α1 enabling access gating. The low-affinity K binding sites responsible for releasing the transported ions are present at or near the exit end of the related ion channel on each side of the bilayer. Under appropriate conditions of local ionic milieu, the versatile P-2 ATPase pump transports Na/K, H/K, and Ca/H in altered states [3, 4]

K-ATPase described recently [3, 4] is a paradigm shift from the 50-year-old single topology model dominating the P2-ATPase field. We observed earlier that the K-stimulated para nitro phenyl phosphatase (K-pNPPase), co-purified with the gastric H, K-ATPase, was not a partial reaction of the gastric ATPase [3, 4] as postulated in the single topology Post-Albers scheme. As the name "dual topology" implies, new model of H, K-ATPase has two 100 kDa α-subunits in mirror-image orientation across the membrane, in contrast to existing "single topology" one having only one α-subunit facing the cytosol (Fig. 4.1). The new model has two low-affinity K-para nitro phenyl phosphatase (K-pNPPase) sites (one on each α-subunit) regulating simultaneous transport of H/K across (Fig. 4.1). Generality of the dual-topology model was revealed by the identical orientation of K-pNPPase across the isolated surface epithelial cell outer membrane capable of dose-dependent ^{86}Rb uptake inhibitable by ouabain [7].

The HAF is intimately involved in pump operation (Fig. 4.1) from the beginning of the pumping process, such as binding of ATP to the ATPase catalytic site, up to

the end of pumping cycle by carrying out the bidirectional transport of H and K across, and then beginning a new phase. In fact, during its allosteric pumping execution the HAF creates the high-affinity K-effector site of the dual-topology H, K-ATPase (facing the lumen) for exchange with cytosolic H. It must be noted that without the availability of luminal high-affinity K site the pump cannot function. Such crucial role of HAF was confirmed by the use of mono-specific anti-HAF antibody that blocks the HAF-stimulated H, K-ATPase activity in vitro, as well as blocked the production of protons in vivo when inserted into digitonin-permeabilized rabbit gastric glands (Zenodo, DOI: 10.5281/zenodo.7093).

The adjoined α subunits with embedded ion channels in mirror-image orientation (Fig. 4.1) are believed to oscillate laterally within the plane of the membrane during the pump operation [4]. Oscillation of the ion channels is initiated by cytosolic activator (HAF)-dependent activation of the enzyme (E) forming E*.ATP at the catalytic α_1 site with simultaneous binding of high-affinity H (Na) to a nearby site in the cytosol (see below). Binding of H (Na) induces domain-domain interaction between the membrane-embedded helixes of α_1 and α_2 with the resultant binding of high-affinity K at the *trans*-cytosolic α_2 site. This H (cytosolic) and K (luminal) bound transitional complex (E*ATP.H.K) spontaneously hydrolyze the ATP helping the H, K-ATPase molecule return to its original configuration (E) following a harmonious shift of the adjacent ion channels back to its initial state. The entire process creates a peristaltic movement of both ion channels for the bidirectional transport of H and K across. Please note that in the case of nongastric tissue, the P2-ATPase pump transports Na and K mediated by an analogous regulatory protein, NaAF (of 170 kDa mass), universally present in all bodily cells except gastric parietal cells. The detail on HAF regulation of the proton-pump is discussed in the following section.

3 Allosteric Regulation of the Gastric ATPase System by Its Cytosolic 80 kDa HAF and Ca (μM)

In the active state, the 80 kDa HAF is a dimer of two identical 40 kDa subunits; the monomers are totally inactive [3]. The dramatic nature of HAF activation of the gastric H, K-ATPase system is shown in Fig. 4.2. During the rapid allosteric activation of the pump (Hill coefficient = 4.5), eight to ten molecules of HAF interact cooperatively with each H, K-ATPase pump unit for optimal stimulation. The negatively charged HAF (80 kDa mass) consisting of 39 % nonpolar, 33 % polar uncharged, 5 % positively charged, and 20 % negatively charged amino acids [8] is suited for domain-domain interaction amongst themselves as well as interfacing the neighboring ATPase catalytic domain. Previous reconstitution studies on gastric microsomal H, K-ATPase following inactivation by mild perturbation of annular lipids [9, 10] suggest the HAF molecules to be loosely anchored to some phosphatidyl choline (PC) having distinct fatty acid (FA) compositions (80 % saturated, 20 % mono- and di-unsaturated lacking in polyunsaturated FA). It appears that the

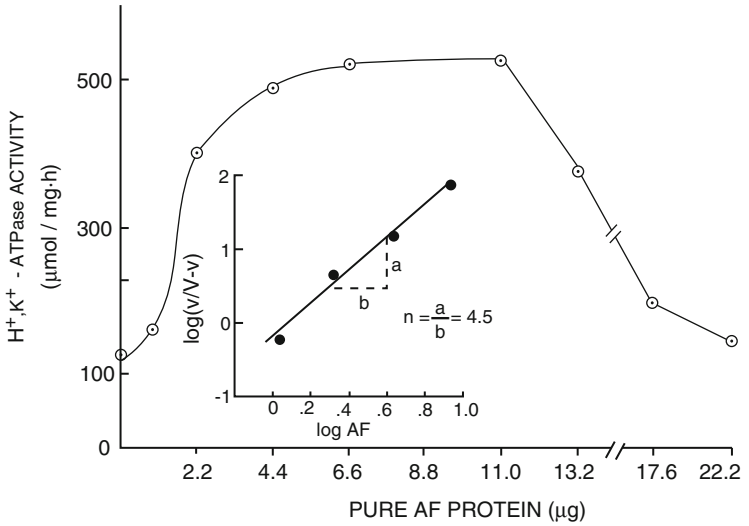

Fig. 4.2 Effects of increasing concentrations of pure HAF (described as AF) on the APM-associated pig gastric H, K-ATPase activity. 4 µg of the pig H, K-ATPase and indicated amount of the pure HAF were preincubated for 10 min at 37 °C in Pipes buffer (pH 6.8) and assayed [6]. Data are the average of triplicates. The *inset* shows the Hill plot. This figure shows highest cooperative activation with 11 µg HAF and 4 µg nearly homogenous ATPase. Assuming the MW of gastric H, K-ATPase to be 320,000 we estimated that each nmol of H, K-ATPase binds 10 nmol of HAF. Note the dramatic downregulation with further increase in HAF level. It is noteworthy in this connection that the activity of K-pNPPase is also significantly increased without any alteration of the low-affinity K site [Taken from Ref. 8]

negatively charged HAF molecules are anchored to the cytosol-facing head group of PC at the annular zone by entropy-driven process discussed below.

Inspection of the various thermodynamic parameters of the ATPase activation process reveals that the HAF activation of the H, K-ATPase is entropy driven [8]. About eight to ten molecules of HAF as a single unit boost the ability of each H, K-ATPase to generate optimal transition state (E*.ATP) complex by cooperative interaction in their cytosolic ambience (37 °C) by increasing the entropy of activation ($\Delta S‡$) of the system, thereby causing the simultaneous allosteric binding of high-affinity Na (or H) and high-affinity K in the lumen across. This is how the HAF appears to initiate the lateral movement of the transmembrane helixes of mirror-image (α1α2) orientation for simultaneous binding and transport of both ions during ATP hydrolysis [8], then shifting back immediately to the original state (E) to begin the a new cycle with fresh formation of E*.ATP.

However, following the optimal activation of H, K-ATPase at 1:10 (mentioned above) the K-stimulated activity is drastically reduced being eliminated at 1:14 (ATPase to HAF) reaching the basal (Mg) state. This might be the way proton-pumps enjoy momentary rest prior to returning to the intracellular tubulovesicular (TV) storage pool staying fused to it.

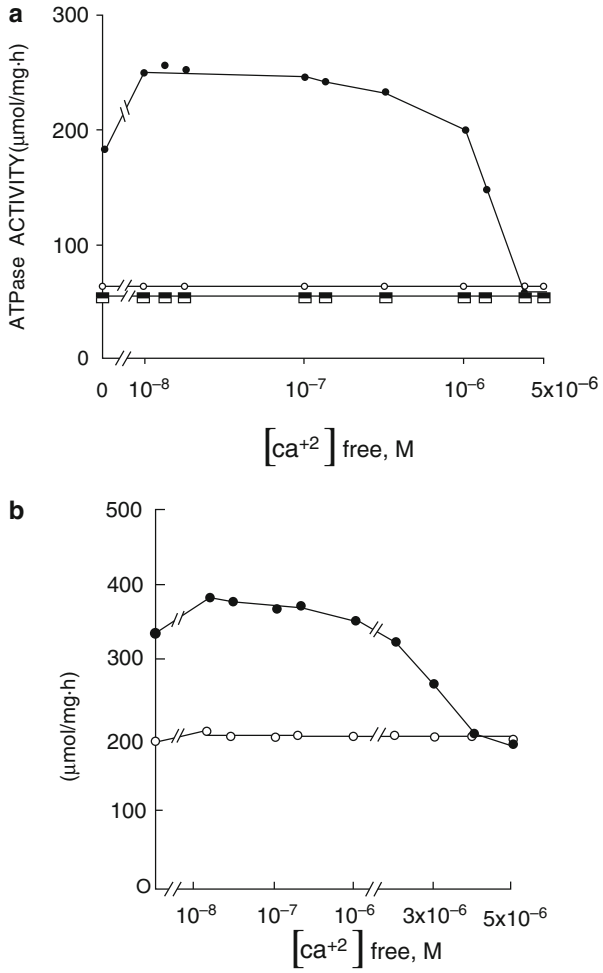

Fig. 4.3 (**a**) Effects of µM Ca on HAF-dependent H, K-ATPase activity associated with low-density APM. Without HAF without K (*open square*) and with K (*closed square*); with HAF without K (*open circle*) and with K (*closed circle*). (**b**) Effects of µM Ca on HAF-dependent H, K-ATPase activity associated with high-density BLM in the absence (*open circle*) and presence (*closed circle*) of the HAF. Note the similar stimulation followed by inhibition in both **a** and **b** with increasing µM Ca. Note that similar to APM above only the HAF-dependent activity associated with BLM is abolished. Data taken from Ray et al. [11]

The critical role of µM Ca in the regulation of the HAF-stimulated gastric H, K-ATPase system associated with the APM and BLM is shown in Fig. 4.3. The dramatic sensitivity of the HAF-regulated gastric H, K-ATPase system to physiological Ca level is obvious from Fig. 4.3 [11].

The HAF-stimulated H, K-ATPase activity under steady-state condition shows additional allosteric stimulation at very low level of Ca (<2 µM); beyond this the

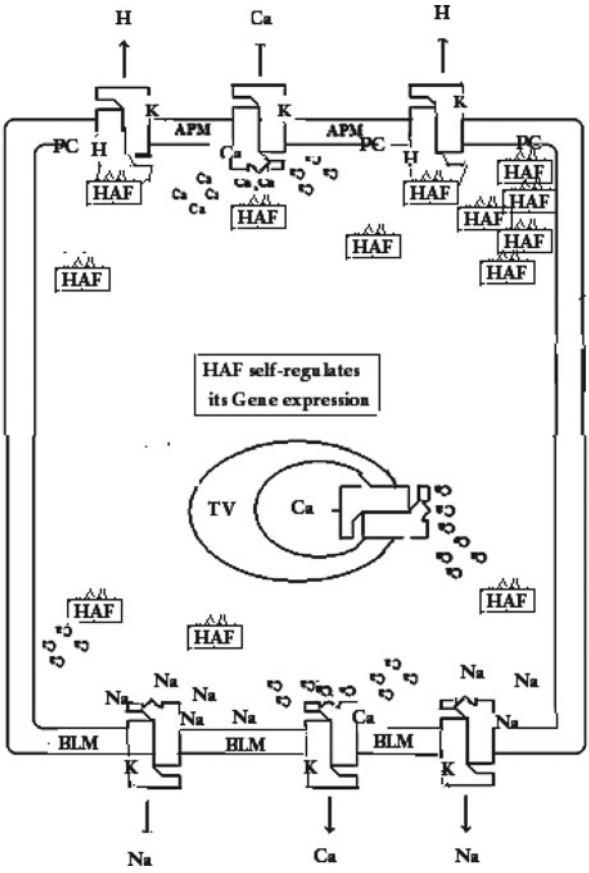

Fig. 4.4 Critical interplay of Calcium in the HAF regulation of the gastric H, K-ATPase Pump (pumping H against a millionfold gradient compared to intracellular pH) at the APM showing oscillation of the Pump between H- and Ca-transporting modes depending on the local Ca level. The APM pump works well between 1 and 2 μM Ca but abruptly stops between 2 and 4 μM Ca. In a similar fashion the H-pump at the basolateral membrane (BLM) works as a Na-Pump and a Ca-pump based on local levels of high Na, high Ca, and higher pH (bicarbonate tide) due to proximity to blood supply. This figure also shows clustering of the HAF molecules bound to the APM-associated polar group of phosphatidyl choline that facilitates allosteric activation (E*.ATP) of the proton-pump by forming domain-domain interaction neighboring HAF mentioned earlier. Note that the annular PC molecules providing the proper fluid environment for the H, K-ATPase function have been identified to consist of saturated (16:0 and 18:0) and unsaturated (18:1 and 18:2) fatty acids lacking in 20:4 [9, 10]

HAF-stimulated H, K-ATPase is drastically downregulated, coming to a halt at 4 μM. At this point the H-pump acts as a provisional Ca-pump to pump out excess Ca, thus resuming the H-pump activity (Fig. 4.4). Hence, higher Ca (2–4 μM) acts as a feedback control switch for turning off/on the HAF-operated H-pump as the top regulator.

4 Critical Interplay of Calcium in the HAF-Regulated H, K-ATPase and Na, K-ATPase Pumps in Gastric Parietal Cell

For each mole of HCl secreted by the parietal cell, one mole of ATP is consumed. Hence, the mitochondria-enriched parietal cells have a constant supply of metabolic substrates, which are in turn co-transported with Na across the BLM. As a result, for each mole of H transported across the APM, an equivalent amount of Na enters into the cell across the BLM border, which is promptly pumped out again to maintain ionic homeostasis. So, for each mole of HCl secreted the parietal cells spend two moles of ATP and produce two moles of HCO_3 (byproduct) which is promptly transported out (by Cl/HCO_3 exchanger) in exchange for Cl across the BLM to maintain pH homeostasis. These activities together with other major membrane processes undergoing gastric acid secretion, such as consistent trafficking of the H, K-ATPase molecule back and forth between the APM and intracellular reserve as tubulovesicles (TV), make the parietal cell membranes most active next to brain cells of the human body. The TV acting as the intracellular reservoir of H-pumps saves substantial energy for the cells. As shown in Fig. 4.4, the cytosolic HAF appropriately controls both the proton-pump on APM and the BLM (basolateral) Na-pump of the gastric P2-ATPase system acting alternatively as a provisional Ca-pump to maintain homeostasis. Please note that similar to parietal cell (Fig. 4.4, below) the NaAF-regulated ubiquitous Na, K-ATPase system belonging to all other tissues shows similar altered function as a provisional Ca-pump [1–3].

It is clear from the preceding information (Figs. 4.1, 4.2, 4.3, and 4.4) that the allosteric operation of the gastric H, K-ATPase system, pumping H, K, Na and Ca, is totally dependent on the cytosolic HAF acting as the operator of the bidirectional H/K-ATPase pump. During the pump operation (Figs. 4.1 and 4.4), the HAF helps to bind concomitantly the cytosolic H and luminal K (both with high affinity) for the simultaneous transport across the APM in opposite direction. Same thing happens with the H, K-ATPase at the BLM where in the alkaline environment the H/K--ATPase pump acts as the Na/K-pump; and in both situations the HAF acts as a faithful gate-keeper of ions for the parietal cell P2-ATPase system [5]. Thus, to carry out the dynamic functions of the H, K-ATPase the HAF appears to function as an operator-cum gate-keeper for managing the heavy ion-traffic across the plasma membrane gates, where the dual-topology ($\alpha^2\beta^2$-isoform) setting (Fig. 4.1) only serves as double gates for the passage of ions. In an analogous manner, the NaAF acts as the operator-cum gate-keeper of the ubiquitous Na, K-ATPase system.

5 Extracellular and Intracellular Ca-Environments of the Parietal Cell Under the Resting and Hormone-Stimulated Conditions

Under resting condition, the luminal or secretary environment of the parietal cell is generally smooth with neutral or slightly alkaline pH. In the cytosol, there are numerous mitochondria and tubulovesicular (TV) membranes (highly enriched in gastric H, K-ATPase activity) acting as a proton-pump reserve [12]. Following stimulation of acid secretion by the secretagogues, a spectacular transformation takes place within few minutes. Numerous TV membranes migrate towards the apical (secretary) plasma membrane (APM) causing fusion of proton-pumps with the resultant appearance of numerous secretary cannelicular projections into the lumen. During peak secretion the parietal cells secrete acid against a concentration gradient of over a millionfold (luminal pH nearing 0.1).

5.1 The State of [Ca] Under Resting Condition

Waves of Ca arising from the BLM locale have been reported in parietal cells [13, 14]. Most likely Ca enters the parietal cells through BLM via its InsP3R (receptor operated Ca-channel) creating Ca-waves along the intracellular tubulovesicles, TV (storage for proton-pumps), reaching the APM site in mild waveforms. In resting cell Ca remains pretty active in the mid-cell region TV pool in the following way. The Proton-Pumps (H, K-ATPase molecules) associated with this inside-out TV (vesicles) have the ATP hydrolytic (catalytic) site facing cytosol surrounded by the HAF pool. The presence of high Ca-waves (2–4 μM) in that mid-cell region will prevent the HAF from interacting with the ATPase site, forcing them to pump Ca (Fig. 4.4) into the vesicle interior for storing (as Ca-sink), thus keeping the proton-pumps truly at rest without wasting further energy. Upon receiving the signal for acid secretion, the Ca-loaded TV migrates towards the APM and the BLM for transferring the pumps to these sites. The mitochondria-loaded parietal cells would also store Ca in the mitochondrial matrix to activate a key Krebs cycle enzyme, thus meeting the ATP demands during acid secretion.

5.2 The State of [Ca] Under Stimulated Conditions

Following stimulation of the parietal cells Ca would be needed for the organized cytoskeletal movement of the TV towards the secretary APM site (of lighter buoyant density compared to TV) for the incorporation of new Proton-Pumps. As for the BLM site, however, there seems to be a different kind of mechanism at work,

since no such organized movement of TV towards BLM has ever been reported. In this case the Ca-loaded TV vesicles surrounded by the HAF pool seem to move towards the BLM environment by virtue of having identical ($d = 1.115$) buoyant densities [11]. In this environment TV easily mingles with the BLM by the whirlpool movement created by the highly active Na/K-pump, thus bringing them close together for the transfer of new pump molecules by Ca-mediated fusion. Such fusion between the Ca-loaded TV and high Ca BLM locality will be spontaneous due to identical lipid make up [9] of the ion-pumps.

6 Signaling Roles of Ca Under the Resting and Stimulated States of Parietal Cell

The specialized plasma membrane microdomains, "Caveolae or lipid rafts," made up of Sph and cholesterol have been implicated in Ca mobilization [15]. The lipid rafts would control the polar region of the parietal cell and regulate various Ca-signaling events. The critical constituent of lipid rafts, Sph, is very high in APM (66 %) and TV/BLM (59 %) consisting entirely of saturated fatty acid (SFA), such as the unique 14:0 (35.7 %), along with 16:0 and 18:0 with traces of unsaturated FA [11, 16]. Following stimulation, massive movement of TV towards the secretary cannelicular region of APM takes place, causing extensive membrane fusion, where the caveolae (by virtue of its cytoskeleton dynamics) would be acting as scaffolds for Ca ion channels, thus connecting the intracellular stimuli to extracellular milieu of the cell. Also, during the fusion of approaching TV with the APM (following hormonal stimuli), the PI [11] content of APM (23.6 μmol/mg protein) and TV (13.1 μmol/mg protein) would be involved in InsP3-mediated targeting of Ca to the specific fusion sites.

Recent studies by Fujimoto Toyoshi [17] reveal that a transmembrane protein structurally similar to the type-I IPR and the plasma membrane PM Ca-pump (Ca-ATPase) are concentrated in the caveolae. The HAF-stimulated H, K-ATPase activity under steady-state condition shows additional allosteric stimulation at very low level of Ca (<2 μM); beyond this the HAF-stimulated H, K-ATPase is drastically downregulated, coming to a halt at 4 μM. At this point the H-pump acts as a provisional Ca-pump to pump out excess Ca, thus resuming the H-pump activity (Fig. 4.4). Hence, higher Ca (2–4 μM) acts as a feedback control switch for turning off/on the HAF-operated H-pump as the top regulator [17, 18]. Type-I IPR in the ER is a Ca^{2+} channel that opens upon IP binding implicating that the caveolae associated IPR-like proteins are most likely plasma membrane Ca-channel regulating the intracellular Ca. In capillary endothelium the caveolae are closely related to the endoplasmic reticulum (ER) and it was suggested that the non-muscle cells storing Ca in ER should have similar relation [18].

In the case of parietal cells, the intracellular TV/BLM pool stores Ca, hence should have similar caveolae-connection mentioned above. So, the alteration in local Ca concentration should influence the nearby caveolae protein to be involved

in Ca-transport. In the case of parietal cell, Perez et al. [13] demonstrated (using the fluorescent signal of Fura-2) a Ca-transient at the secretary APM prior to the onset of acid secretion; the acid secretion occurred 3 s after carbachol stimulation. Simultaneously, a different group, Caroppo et al. [14] demonstrated using immunohistochemical staining the actual colocalization of H, K-ATPase, Ca-ATPase, and CaR in both the APM and TV membranes of parietal cell. It must be noted, however, that CaR was not detected in the BLM by these authors. Similar coexistence of PMCA and CaR was also revealed in the peptic cells, but not in any other cells like the mucus secreting and surface epithelial cells of gastric mucosa [14]. The parietal cells extrude Ca from cytosol through the APM into lumen, and take up Ca from the nutrient side coming through the BLM, hence is consistent with the absence of CaR in BLM just mentioned. Pancreatic acinar cells and salivary glands display similar initial increase in Ca at the lumen followed by Ca-wave spreading towards the BLM, consistent with immunochemical localization of PMCA on APM [19]. Thus, even though the BLM (analogous to APM) has provisional Ca-ATPase pump functioning as an altered form of the HAF-regulated Na-pump, there is no evidence of CaR involvement.

Thus, it is clear that the observed "Ca-transients" and CaR are intimately related. During the transfer of new proton-pump (from TV) on to APM, the newly incorporated pump will have Ca leftover (at fusion site) near the catalytic center that is pumped out at first by the provisional Ca stimulated Mg-ATPase (discussed earlier) for its subsequent operation as the HAF-regulated proton-pump. At this point, the CaR (facing the lumen) by way of its Ca-sensing devices [20–22] is likely to act as a sensor of cytosolic Ca and program itself as a regulator of the forthcoming Ca-transport events from the provisional Ca-pump across the bilayer. As soon as the CaR senses the unwanted level of Ca, the proton-pump turns into a provisional Ca-pump until the safe Ca level is reached. Similar role of CaR has been suggested in the voltage-gated channeling of Ca in the nerve terminal [5]. The detailed function of CaR as a Ca-sensor, a self-programmed timer, as well as a fine regulator of the Ca-channel function remains to be elucidated.

7 Emerging Picture of Ca-Signaling in Maintaining the Ionic Homeostasis

In view of the preceding information, and our data on the up- and down-regulation of the activator-regulated allosteric P2-ATPase pumps by μM Ca, the following unified picture is emerging:

- Calcium signaling is the absolute controller of homeostasis of our bodily cells equipped with allosteric P2-ATPase pump.
- The entire bodily network is operated by two different cytosolic regulatory proteins, namely the NaAF (170 kDa) and HAF (80 kDa) for the ubiquitous Na, K-ATPase and the distinctive gastric H, K-ATPase (proton-pump) respectively.

- Ca effects vary with local pH; the apical pump (operating at pH, 6.8) is shut off at 4 μM Ca while the basolateral (operating at pH > 8.0) needs five- to sixfold higher Ca for shutting off.

It may be noted that there are a lot of information in the literature dealing with the natures of the PMCA isoforms in Ca-signaling [23]. Based on current report, the tissue-specific isoforms of the plasma membrane Na-pump act as provisional Ca-pump (or PMCA) to pump out excess local Ca for maintaining homeostasis. So, the nature of the isoforms of the PMCA and Na-pump in any particular tissue should be identical. The current report will thus help in such tissue-specific identification of the related pumps as well as in identifying the area-specific isoforms of the P2-ATPase system in brain function [24].

8 Conclusions

The endogenous HAF is an allosteric regulator of the gastric H, K-ATPase system, which also seems to regulate its own intracellular level by regulating gene expression [25]. This chapter reveals that the HAF-regulated H, K-ATPase system is, in turn, allosterically regulated by the cytosolic free Ca in a pH-dependent manner. It will be important to know if Ca has any feedback influence on the genetic self-regulation scheme of the HAF that is so vital in allosteric ion transport by the gastric H, K-ATPase system.

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Chapter 5
Na, K-ATPase α4: An Isoform Dedicated to Sperm Function

Gladis Sánchez and Gustavo Blanco

Abstract Several proteins that play essential roles in the cell exist in multiple different molecular forms. This variability in structure often results in the production of isoforms with properties that are distinct from those of the original protein. The discovery and study of isoforms represents one of the most fascinating areas in biology, since it has uncovered the elaborate mechanisms that cells have developed to fulfill specific tasks. One protein system characterized by a high molecular heterogeneity is the Na-K-ATPase, the ion transport mechanism that maintains the transmembrane Na^+ and K^+ concentrations across the plasma membrane of cells. Na, K-ATPase results from the association of different molecular isoforms of an α- and a β-subunit. One of the Na, K-ATPase α polypeptides, α4, is solely produced in male germ cells of the testis, where it serves an important role in sperm function. This review discusses the particular expression, functional properties, regulation, mechanism of action, and role of Na-K-ATPase α4 in the context of the physiology of the male gamete. The current experimental evidence shows that the appearance of α4 during evolution is not a redundant event but rather a sophisticated mechanism to adapt Na^+ and K^+ active transport to the requirements of sperm, which carry the amazing mission of swimming relatively long distances to find and fertilize the egg.

Keywords Ouabain • Sperm motility • Sperm capacitation • Male fertility

1 Introduction

Compared to their surroundings, animal cells maintain low Na^+ and high K^+ concentrations by the activity of the membrane-bound Na, K-ATPase or Na pump. This protein system is a plasma membrane-embedded enzyme that utilizes the energy from the hydrolysis of ATP to catalyze the movement of intracellular Na^+ in

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exchange for K^+ in a $3Na^+$:$2K^+$ fashion [1, 2]. Ion movement through Na, K-ATPase is achieved by a series of Na^+ and K^+ induced conformational changes and alternating phosphorylated and dephosphorylated states of the transporter [3]. The asymmetric transmembrane ion distribution established by Na, K-ATPase participates in the maintenance of cell plasma membrane potential at rest and fuels the Na^+-coupled transport of many solutes and water across the plasma membrane [4–6].

Na, K-ATPase is a heterodimer complex, constituted by α and β subunits [7, 8]. The α polypeptide, considered the catalytic subunit of the enzyme, contains the binding sites for ATP, Na^+, K^+ and several regulators of the Na, K-ATPase, including the cardiotonic steroid inhibitor ouabain. The α polypeptide is a 110–112 kDa ten transmembrane spanning protein, with cytoplasmic N- and C-termini, a large intracellular region, and five small extracellular loops [9]. The β polypeptide is a 40–60 kDa single membrane spanning protein, heavily glycosylated with most of its mass facing the extracellular medium [7]. The β polypeptide does not directly participate in ion transport and enzymatic activity of the enzyme; however, it plays an important role in the folding, stability, and targeting of the α subunit to the plasma membrane [10, 11]. A third subunit, which includes a series of tissue-specific hydrophobic polypeptides, accompanies and regulates the activity of the Na, K-ATPase [12–15].

Studies on the effects of ouabain on the catalytic and transport properties of the Na, K-ATPase from different tissues provided the first indication that functionally distinct forms of the enzyme existed. Later, evidence for the molecular heterogeneity of the Na, K-ATPase was obtained from the finding of differential migration of the Na, K-ATPase α subunit from different tissues in SDS-polyacrylamide gels (reviewed in [16–20]). Then, with the advent of molecular biological tools, a family of genes encoding for not only different α (α1, α2, α3) but also various β (β1, β2, and β3) polypeptides was discovered in mammals [16, 21–24]. More recently, an additional α polypeptide, α4 was revealed [25, 26]. The identification of Na, K-ATPase isoforms also in zebra fish and hydra suggested that the divergence of the genes occurred early in evolution [27, 28]. The α and β subunits are characterized by a high degree of primary structural homology. The β subunits share a lower degree of amino acid identity and, in addition, exhibit differences in the number and composition of their carbohydrates [16, 21]. The α and β subunits are expressed in different combinations and in a cell type specific and developmentally regulated manner. The α1 and β1 isoforms are widely expressed in most cells, α2 predominates in adipocytes, muscle, heart, and brain, α3 is expressed in nervous tissues, and α4 is confined to the testis male germ cells. The β2 polypeptide is found in skeletal muscle, pineal gland, and nervous tissues and β3 is expressed in testis, retina, liver, and lung [17, 18]. Distinct association of α and β isoforms results in multiple αβ dimers or isozymes of the Na, K-ATPase with different functional properties. The α isoform is responsible for most of the functional dissimilarities between the Na, K-ATPase isozymes, with the β subunit having only modest effect on the enzyme affinity for ligands [29–33]. The recent use of genetic approaches and transgenic technology has allowed a better understanding of the role of Na, K-ATPase isoforms in the context of the whole animal [34–39].

2 The Male Specific Na, K-ATPase Isoform

Early reports have shown that, similar to somatic cells, spermatozoa maintain transmembrane gradients for Na^+ and K^+ [40]. Later, a Na^+, K^+, and Mg^{2+}-dependent ATPase activity was detected in flagellar fractions of boar epididymal spermatozoa [41] and then in sperm from other species [42–45]. Additional studies showed ^{3}H-ouabain binding to bull sperm [46]. Moreover, ouabain affected the transmembrane Na^+ and K^+ gradients, depolarized the plasma membrane, and reduced flagellar motility of bull sperm [47]. In some species, ouabain also inhibited the acrosomal reaction, a process consisting on the release of hydrolytic enzymes that is necessary for fertilization of the egg [48, 49]. Altogether, these results supported the presence of Na, K-ATPase in the male gamete.

Following these original studies, it was discovered that the Na, K-ATPase of sperm consisted of more than one molecular form. This started with experiments using restriction mapping, Southern blot hybridization, and sequencing of a leukocyte human genomic library, which allowed the identification of DNA fragments corresponding to partial sequences of a previously unknown P-type ATPase α isoform [26]. This form, originally named αD, resembled an α subunit of Na, K-ATPase but shared a nucleotide and amino acid identity that was lower (between 66 and 76 %) than that existing between the other Na, K-ATPase α isoforms. It was unclear if this novel partial DNA encoded a functional catalytic form of the Na, K-ATPase, or if it corresponded to another closely related cation ATPase. Moreover, the possibility existed that the DNA sequences uncovered corresponded to a pseudogene. Once the full DNA of the new α isoform was isolated and the amino acid primary structure was deduced, it was apparent that the αD clone was structurally related with the Na, K-ATPase. The isoform was then named Na, K-ATPase α4, following the nomenclature used for the other α isoforms [25]. The full sequence of α4 was firstly reported for the rat and showed α4 to be a 1028 amino acid polypeptide that shared the lowest degree of identity with the other isoforms, with only 78 %, 78 %, and 76 % amino acid identity with the rat α1, α2, and α3 isoforms respectively. As occurs with the other isoforms, the N-terminal portion of the polypeptide, rich in positively charged amino acids, is the region with the highest structural variability. In contrast, the homology of α4 across species is higher [25]. Comparison of the primary structure of α4 with that of the other Na, K-ATPase α isoforms is reviewed in [50]. Northern blot analysis in a collection of human and rat tissues identified the ~3.9 kb RNA for the α4 isoform, abundantly expressed in the testis and absent from other major tissues, except for the skeletal muscle, in which α4 RNA was identified at very low levels. The appearance of α4 RNA in skeletal muscle may have probably been due to some cross-reactivity of the used probes with the α2 mRNA, since subsequent studies were not able to find α4 in this tissue [25]. Chromosomal mapping of the α4 gene, *Atp1a4*, showed it located on mouse chromosome 1, proximal to the *Atp1a2* gene that encodes for Na, K-ATPase α2 [51], which suggested that α4 originated from the α2 gene [23, 51]. Later, the human *ATP1A4* gene was characterized, mapped to chromosome 1q23, and its exon/intron structure determined [52]. Eventually, as will be discussed below, the α4 polypeptide was identified to be expressed in the male germ cells of the testis and to be abundant in sperm.

3 Identification and Enzymatic Properties of Na, K-ATPase α4

The new Na, K-ATPase gene discovered in testis still required functional confirmation before it could be ascribed as a catalytically competent subunit of the Na, K-ATPase. This information came from studies performed on the recombinant α4 protein from rat exogenously expressed in Sf9 insect cells [53]. Thus, co-expression of rat Na, K-ATPase α4 and β1 resulted in a ouabain sensitive, Na^+, K^+, and Mg^{2+}-dependent hydrolysis of ATP and a ouabain-sensitive uptake of ^{86}Rb in the host cells. Also, α4β1 presented a ouabain-sensitive phosphorylation from ATP, another typical characteristic of Na, K-ATPase. Furthermore, activity of α4 was inhibited by the generic P-type ATPase inhibitor vanadate but was unaffected by thapsigargin or Sch-28080, compounds that inhibit the sarcoplasmic reticulum Ca-ATPase or the gastric H, K-ATPase respectively. In addition, α4 showed an optimal pH for activity of 7.4 and was inactivated by divalent cations, such as Ca^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}, demonstrating that H^+ or divalent cations are not natural substrates of the enzyme and that α4 displays the properties of a Na, K-ATPase [53]. A Na, K-ATPase activity with similar characteristics of those of α4 expressed in the insect cells was found in rat testis [17, 53].

The ability to produce α4 separated from other Na, K-ATPase isoforms in Sf9 cells allowed the characterization of its enzymatic properties [53]. This showed that, compared to the Na, K-ATPase α1, α2, and α3 isoforms, α4 had a relatively higher apparent affinity for Na^+, a lower apparent affinity for K^+, and an intermediate affinity for ATP. Interestingly, α4 exhibited a high sensitivity to ouabain, with a calculated $K_{0.5}$ in the low nanomolar range [17, 53]. A comparison of the kinetic properties of Na, K-ATPase α4β1 and the ubiquitous α1β1 is shown in Fig. 5.1. The biochemical characteristics of rat α4 were also studied after transfection and stable selection with

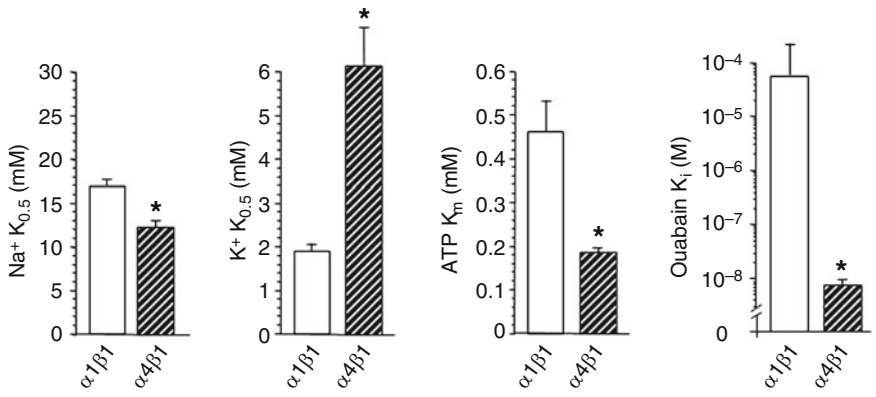

Fig. 5.1 Comparison of the kinetic characteristics of rat Na, K-ATPase α4β1 and α1β1 expressed in Sf-9 insect cells using the baculovirus expression system. Apparent affinities ($K_{0.5}$), K_m, and inhibition constant (K_i) for ouabain were calculated from dose-response curves of Na, K-ATPase activity to the indicated ligands. Values are the mean ± SEM and *asterisks* indicate statistically different values compared to α1β1. Data have been re-plotted from values taken from Ref. 16

neomycin in murine NIH 3T3 cells [54]. Using this system, the interaction of α4 with Na^+ and K^+ was determined by exploring the effects of the cations on [^{3}H] ouabain binding/displacement, which further proved that α4 exhibited the characteristics of a Na, K-ATPase and not those of another P-type ATPase. [^{3}H] ouabain binding self-competition assays in membrane preparations from the α4 expressing NIH 3T3 cells confirmed the high affinity of α4 for the cardiac glycoside, although the calculated K_D for ouabain was higher than those of previous reports, which could depend on differences in the membrane preparations used. The high ouabain affinity of α4 was also reported for the human isoform stably expressed in HeLa cells, by assessing the survival of the cells under different amounts of ouabain in the culture medium [55]. Later, the enzymatic properties of human α4 were directly measured in the native environment of human sperm and showed that the reactivity of α4 to Na^+, K^+, and ouabain is overall conserved with respect to those of the rat ortholog [56].

4 Tissue Cell and Subcellular Specific Expression of Na, K-ATPase α4

The α4 polypeptide is the Na, K-ATPase isoform with the most restricted pattern of expression being uniquely present in the testis. However, expression of α4 in the testis is not exclusive, since Na, K-ATPase α1 is also present. In contrast, testis does not express the Na, K-ATPase α2 and α3 isoforms [17, 25]. In vitro hybridization and immunochemical techniques have shown that, in contrast to the ubiquitous α1, the α4 isoform is present only in the testis seminiferous tubules, where it is abundant in the adluminal side of the tubules [57, 58]. Na, K-ATPase α4 is found in most mature male germ cells and in spermatozoa but not in Sertoli, Leydig, or undifferentiated male germ cells. These results suggested that α4 is confined to the testis male germ cells and showed that these cells express α4 along with the Na, K-ATPase α1 subunit. Figure 5.2 shows the pattern of expression of Na, K-ATPase α4 in mouse

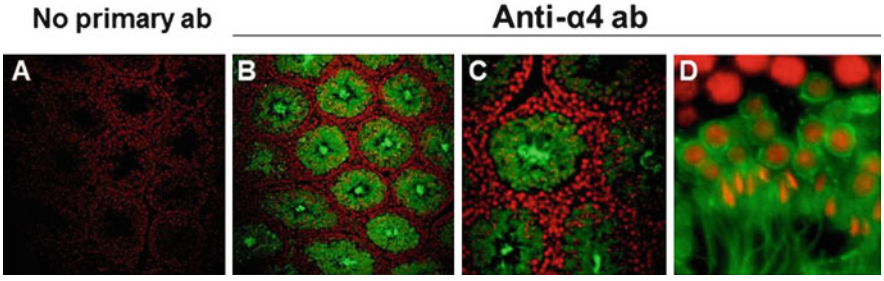

Fig. 5.2 Immunocytochemical localization of Na, K-ATPase α4 in mouse testis. Labeling was performed using a chicken generated antiserum against α4, followed by an anti-chicken antibody conjugated to FITC. Propidium iodide was used to stain the cell nuclei. Panel **a** shows the negative control in the absence of primary antibody. Panels **b** and **c** show two different magnifications of testis seminiferous tubules. Panel **d** is a high magnification of the seminiferous epithelium within a tubule

testis seminiferous tubules. Analysis of the ouabain inhibition profile of Na, K-ATPase activity revealed that, in rat sperm, approximately two thirds of the total Na, K-ATPase activity of the cells correspond to α4, the remaining being α1 [59]. The primary expression of α4 in sperm was further supported by the drastic reduction of the protein in mice that are oligospermic due to ablation of the transcription factor Egr4 [57].

Immunocytochemical studies have shown that in rat and mouse sperm α4 is expressed in the flagellum, being more abundant in the midpiece of the sperm tail. Little or no α4 is found in the sperm head [58, 59]. This particular flagellar localization has also been described for human sperm [56]. However, while human α4 was reported to be mainly localized in the sperm flagellar midpiece in one study, it was shown in the principal piece in another, a fact that may depend on the different antibodies used [55, 56]. In any case, it is clear that Na, K-ATPase α4 in human sperm has a flagellar distribution. Different from α4, the α1 polypeptide appeared to be more evenly distributed along the sperm flagellum, and, as α4, was barely detected in the sperm head [59]. This suggests the existence of isoform-specific mechanisms for the targeting and particular retention of α1 and α4 at restricted domains of the plasma membrane of the male germ cells. Further evidence for the flagellar distribution of α4 was obtained in transgenic mice overexpressing the rat α4 isoform tagged at its C-terminal portion with GFP [60]. However, staining for GFP extended beyond the midpiece of the flagellum, which is not surprising, since the addition of GFP to α4 may have altered targeting of the whole protein. Alternatively, overexpression of α4 may have overwhelmed the protein delivery mechanisms in the cells. In conclusion, the subcellular localization studies indicated a primary flagellar localization of the protein and suggested a role for Na, K-ATPase α4 at the sperm tail.

5 Changes in Na, K-ATPase α4 Expression During Development

Several findings suggested that α4 expression was regulated during spermatogenesis. Thus, α4 was found to be highest in spermatozoa and lower in the external side of the seminiferous tubules, where immature male germ cells are located [58]. Also α4 is scarce in a male germ cell line, GC-1, which does not fully differentiate in vitro [57]. First evidence for a developmental regulation of α4 was obtained by Northern blot analysis of rat whole testis RNA, which showed that α4 is not expressed until 4 weeks of age and that it reaches maximal levels at week 6. In contrast, RNA for the α1 isoform in testis was found to remain at constant levels throughout the life of the animal [58]. Immunocytochemical studies identified α4 expression starting at 6 weeks of age and becoming maximal at 8 and 12 weeks of age. Therefore, expression of α4 protein closely followed that of RNA [58]. Overall, these results indicated that α4 expression is regulated in parallel with the onset of sexual maturity in the rat.

We have found that absolute values of Na, K-ATPase activity on testis homogenates increased approximately twofold between week 1 of age to adulthood [59].

Ouabain inhibition profiles of Na, K-ATPase activity on testis homogenates showed the biphasic ouabain dose-response curves corresponding to expression of α4 and α1 isoforms. However, the relative contribution of each α isoform to the total Na, K-ATPase activity of the gonad varied with age. ATP hydrolysis sensitive to relatively low ouabain concentrations (3×10^{-6} M), corresponding to α4, increased from 10 % at week 1 to approximately 20 % at day 18 after birth, and became almost half of the total Na, K-ATPase in the adult gonad. Instead, the highly ouabain-resistant activity of the α1 isoform remained approximately constant throughout those timepoints. In agreement with the functional assays, immunoblot analysis showed that the α4, but not the α1, polypeptide is augmented during maturation of the male gonad [59]. The timepoints in testis development that we had chosen reflected critical stages in male germ cell development, with 1 week after birth containing spermatogonia, 18 days of life, having in addition preleptotene, leptotene, and pachytene spermatocytes; and adult animals presenting cells at all stages of spermatogenesis, including spermatids and spermatozoa [61]. To more directly assess the developmental regulation of Na, K-ATPase, we studied the expression and function of α4 and α1 during spermatogenesis in highly enriched fractions of different male germ cell types, obtained after testis cell dissociation and unit gravity sedimentation, or counterflow elutriation [59, 62]. Ouabain inhibition profiles of Na, K-ATPase activity showed that spermatogenesis was accompanied by an approximately twofold increase in absolute values of total Na, K-ATPase activity and a more than sevenfold increase in Na, K-ATPase α4, compared to α1. Activity of α4 was very low in undifferentiated spermatogonia and pachytene spermatocytes, and augmented with the development of the round spermatids into spermatozoa. Ion transport function of the Na, K-ATPase of male germ cells, measured as the ouabain-sensitive uptake of ^{86}Rb by the cells, showed a similar pattern. Maximal α4 activity was found in spermatozoa; however, no significant further differences in α4 function were detected between sperm obtained from the caput and cauda of the epididymis. This suggests that α4 is not subjected to additional changes during transit of the sperm along the epididymal ducts. RT-PCR and immunoblot analysis of each male germ cell type mirrored the functional assays and showed that transcriptional upregulation of the *ATP1a4* gene in pachytene spermatocytes is followed by a burst in protein synthesis later during spermatid development and in spermatozoa [59]. These studies in isolated male germ cells allowed a detailed characterization of α4 expression in undifferentiated male germ cells, which may have not been detected in previous studies of whole testis, due to masking by the high expression in differentiated testis spermatozoa. From all these results, it is clear that α4 is subjected to important developmental changes that correlate with sexual maturation of the testis and the onset of sperm formation.

To further determine the temporal and spatial pattern of expression of the α4 subunit, we have also used a genetic, knock-in strategy in mice, using expression of the green fluorescent protein (GFP) as a reporter for the expression driven by the endogenous *Atp1a4* promoter. This approach confirmed that the *Atp1a4* promoter guides testis-specific expression, as shown by the typical GFP green fluorescence and the presence of GFP protein in the gonad, but its absence in a series of other

tissues. In addition, GFP expression was developmentally regulated, appearing in adult but not in mouse embryos or the sexually immature 7- and 18-day-old mice. Immunocytochemistry of whole testis sections identified GFP only in differentiated male germ cells but not in spermatogonia, Leydig, or Sertoli cells. Further studies, combining immunoblot analysis of fluorescently sorted testis cells with cell type-specific markers, detected GFP only in spermatocytes, spermatids, and spermatozoa [63]. Altogether, these studies provided evidence, beyond previous studies of the α4 RNA or protein that the *Atp1a4* promoter drives expression of Na, K-ATPase α4 exclusively in male germ cells of the testis, at late stages of spermatogenesis. This postmeiotic expression pattern of α4 is shared with that of other genes that are essential for sperm function.

To better understand the mechanisms regulating α4 expression, we studied the transcriptional control of the human Na, K-ATPase *ATP1A4* gene [64]. We focused on a region of approximately 1 kb upstream the first methionine codon of *ATP1A4*, which had been predicted as the proximal promoter region of the isoform by in silico studies [52]. In this region, we identified the transcription initiation site of the *ATP1A4* promoter to an adenosine located 472 bp upstream of the ATG translation start codon of *ATP1A4* and experimentally confirmed that this 5′ untranslated region of the *ATP1A4* gene exhibits promoter activity in luciferase reporter assays. Computer analysis of this promoter region revealed the presence of potential binding sites for several transcription factors, including two partial consensus sites (GTCA) for the cyclic AMP (cAMP) response element modulator (CREM). This transcription factor is of particular interest in the testis, since a testis-specific splice variant of CREM (CREMt) is involved in the expression of a series of genes that are essential for sperm function [65]. Using luciferase assays and exogenous expression of CREMt, we demonstrated that this transcription factor along with cAMP is an activator of the *ATP1A4* promoter in a dose-dependent manner. Further characterization of CREMτ on deleted constructs of the *ATP1A4* promoter and on ATP1A4 promoter regions carrying mutations in the CRE sites showed that a CRE like motif, located 263 bp upstream the transcription initiation site, was essential for CREMτ effect. The usage of the CREMt site was further supported by electrophoretic mobility shift assays (EMSA), which directly showed the physical interaction between CREMt and the CRE target sequence [64]. In the native environment of the testis, CREMt expression is temporally coincident with the upregulation of a series of postmeiotic genes [66]. Therefore, the transcriptional regulation of *ATP1A4* gene expression by CREMτ places Na, K-ATPase α4 within the cluster of genes that are upregulated after meiosis to serve a role in the physiology and fertility of spermatozoa.

6 Biological Relevance of Na, K-ATPase α4 to Sperm Function

The particular cell type and developmentally regulated expression as well as the unique kinetics of α4 suggested that it performs a specific function in the male gamete. As shown in Fig. 5.1, in the rat, ouabain affinity of α4 is approximately 10,000-fold

higher than that of α1 [16]. This property provided the opportunity to selectively inhibit α4 and determine its function, separate from that of α1. Initial studies tested the effects of relatively low ouabain doses to define the effects of α4 on sperm motility, using simple visual determinations of sperm movement [58]. Then, computer sperm analysis (CASA) was introduced, which provided a higher resolution to the analysis of α4 action on different components of flagellar beat [67]. These approaches showed that blocking the activity of α4 caused inhibition of sperm total motility and multiple parameters of sperm movement, including progressive motility, straight line, curvilinear and average path velocities, lateral head displacement, beat cross frequency, and linearity. The use of higher ouabain concentrations that also inhibited α1 did not cause additional reduction in sperm motility [58, 67]. These results revealed a specific role of α4 in sustaining multiple aspects of sperm flagellar movement. The broad effect of inhibition of α4 activity on the various components of sperm motility suggested that this isoform maintains sperm movement by affecting multiple different vital parameters of sperm physiology.

The idea developed that α1, which is present in all tissues, functions as the isoform that maintains sperm basal Na^+ and K^+ transport in sperm and that α4 plays sperm-specific roles. Activity of α4 is primarily involved in maintaining sperm intracellular Na^+ ($[Na^+]_i$). In addition, ouabain inhibition of α4 causes depolarization of the sperm plasma membrane [67]. Since the Na, K-ATPase is not the only determinant of membrane potential, to influence plasma membrane excitability of the male gamete, α4 action must be linked to sperm K^+ channels. An adequate membrane potential is essential for sperm motility and cell membrane depolarization has been shown to be associated with infertility in asthenozoospermic patients [68]. Therefore, one of the mechanisms by which α4 isoform influences sperm motility is through its key role in maintaining the uneven transmembrane distribution of Na^+ and K^+, and the electrical potential of the sperm plasma membrane. Besides its direct role in Na^+ and K^+ transport, α4 secondarily controls proton levels in spermatozoa, and ouabain inhibition of α4 caused a decline in pH of the sperm cytoplasm [58, 67]. The effects on pH appear to be secondary to the inwardly directed Na^+ gradient that provides the electrochemical energy to drive the secondary movement of protons out of the cell, via the Na^+/H^+ exchanger (NHE). This is supported by different lines of evidence. First, several NHE transporters are expressed in sperm, including the NHE1 and NHE5 of somatic cells and the sperm-specific NHE, sNHE [69]. Second, NHE1 and NHE5 have been found to be co-localized with α4 [70]. Finally, it has been observed that the ionophores nigericin and monensin, which allow leakage of H^+ out of the cells, are able to reestablish the motility that ouabain causes in sperm [70]. Variations in the proton concentration modulate sperm flagellar bending pattern. In this manner, α4 activity may be preventing the rise of protons that takes place in the sperm cytoplasm as a consequence of active movement and metabolism of the cells [58].

We have also found that α4 activity is functionally coupled to the regulation of sperm Ca^{2+} and ouabain inhibition of α4 augments the intracellular sperm calcium ($[Ca^{2+}]_i$) [67]. Since our experiments were performed in the absence of extracellular Ca^{2+}, the increase in $[Ca^{2+}]_i$ is not due to Ca^{2+} internalization from the media but rather depends on a decrease in Ca^{2+} clearance from the cell cytoplasm, possibly via

the Na^+/Ca^{2+} exchanger (NCX). Interestingly, NCX has been shown to be expressed at the sperm flagellum [71, 72]. Maintenance of sperm $[Ca^{2+}]_i$ within a relative limited range is critical to the motility of the male gamete [71]. In this manner, the ability of the α4 isoform to control $[Ca^{2+}]_i$ may represent another mechanism by which this Na, K-ATPase polypeptide sustains sperm motility. Different from rodents, ouabain affected progressive, but not total, of bull sperm motility [49]. Also, ouabain did not affect $[Ca^{2+}]_i$ in bull sperm and induced sperm capacitation through activation of kinases and the phosphorylation of proteins in tyrosine residues [73]. These differences may be reflecting dissimilarities in species, in the amounts of ouabain, which in the experiments with bull sperm included concentrations higher than those needed to bind to α4, and in the incubation times with ouabain, which were longer for bull sperm. Further studies are needed to ascertain these dissimilarities in sperm response to ouabain.

7 Regulation of Na, K-ATPase α4 Function

Spermatozoa are cells that move through environments with very diverse composition and they need to constantly adjust their motile activity. In the epididymis, sperm are in a noncapacitated state and have little motility. Once released into the female track, sperm increases its motility, acquires the hyperactive pattern of motility, and undergoes the capacitated state, gaining the ability to fertilize the egg [74]. We have investigated whether activity of the α4 isoform is regulated and if this event is relevant to the capacitated state of sperm. We have performed this by following α4 function through its selective inhibition with ouabain in rat sperm before and after inducing capacitation in vitro [75]. Sperm capacitation was accompanied by a time-dependent increase in α4 ion transport and enzymatic activity that was prevented by selective blockage with low ouabain concentrations. This indicates that the α4 isoform is subjected to regulation and that its activity is stimulated as sperm becomes capacitated. Interference of α4 activity with ouabain blocked the increase in sperm motility and prevented the plasma membrane hyperpolarization and hyperactive pattern of sperm motility that is commonly associated with sperm capacitation. In contrast, ouabain inhibition of α4 did not affect the progression of the spontaneous sperm acrosomal reaction that follows capacitation. Concomitant with the functional changes mentioned, we found a capacitation-dependent rise in levels of active α4 isoform at the sperm surface. This was reflected by an increase in sperm labeling with the fluorescent indicator bodipy-ouabain and by an increase of α4 in biotinylated and streptavidin precipitated sperm plasma membrane proteins. Thus, the upregulation of α4 during sperm capacitation appears to involve mechanisms that consist in both increases in molecular activity and changes in the level of α4 at the sperm plasma membrane. Mature spermatozoa cannot express new protein, in this manner; the augment of α4 at the plasma membrane must be the result of translocation of preformed α4 molecules from intracellular compartments to the

sperm surface. Currently, we are performing additional experiments to ascertain the location and place of the putative reservoirs of the α4 isoform in the sperm cytoplasm, as well as the molecular mechanisms involved in the subcellular translocation of α4. These experiments show that α4 activity is regulated and that the phenomenon is important to sustain the changes in motility that sperm undergoes in their journey to find the egg.

8 Role of Na, K-ATPase α4 in Male Reproduction

While the studies based on ouabain inhibition allowed important progress in understanding the function of Na, K-ATPase α4, a more direct approach was required to elucidate the overall role of this isoform in male fertility. We investigated this in genetically modified mice, in which α4 was either deleted or overexpressed [60, 76]. Knockout mice were made by removing a region spanning exons 5–8 of the *Atp1a4* gene, which encodes for the ATP binding and phosphorylation sites of the catalytic domain of Na, K-ATPase α4. Homozygous knockout male mice lacked expression of α4 mRNA and protein. In addition, compared to sperm from wild-type mice, sperm from α4-null mice exhibited a significantly lower level of total Na, K-ATPase activity, with a specific disappearance of the high ouabain affinity component corresponding to the α4 isoform, and lost their ability to bind bodipy-ouabain. The α4-null mice were overall phenotypically normal, showing testis with size and morphology indistinguishable from wild-type mice. Also, α4 knockout mouse presented normal sperm numbers. However, homozygous male, but not female, mice were completely infertile. Heterozygous male mice were reproductively competent, suggesting that partial expression of α4 is sufficient to support male fertility. Not only were the α4-null mice sterile, but sperm from these mice were incapable of fertilizing oocytes in vitro. Deletion of α4 leads to severe reduction in sperm motility and drastic reduction of all parameters of sperm flagellar beat. Furthermore, sperm hyperactivation was almost completely abolished in α4 knockout mice. Other alteration of sperm from α4-null mice consisted in a bend in the sperm flagellum, indicative of abnormal sperm ion regulation and osmotic imbalance; and cell plasma membrane depolarization. Deficient expression of α4 in α4-null mice was not compensated through upregulation of expression and activity of α1. Overall, these results demonstrate that while α4 is not needed for sperm production, it is an absolute requirement for male fertility. The inability of α1 to compensate for α4 function supports the unique role of α4 in male fertility.

Further evidence for the role of α4 in male reproduction came from exogenous expression of α4 in transgenic mice [60]. Sperm from mice expressing the α4 isoform from rat, fused at the C-terminus to green fluorescent protein (GFP), under the protamine-1 promoter had higher than normal total sperm motility. In contrast, overexpression of α4 did not significantly affect sperm acrosome reaction. Mating trials with WT females showed that despite having higher motility, transgenic α4

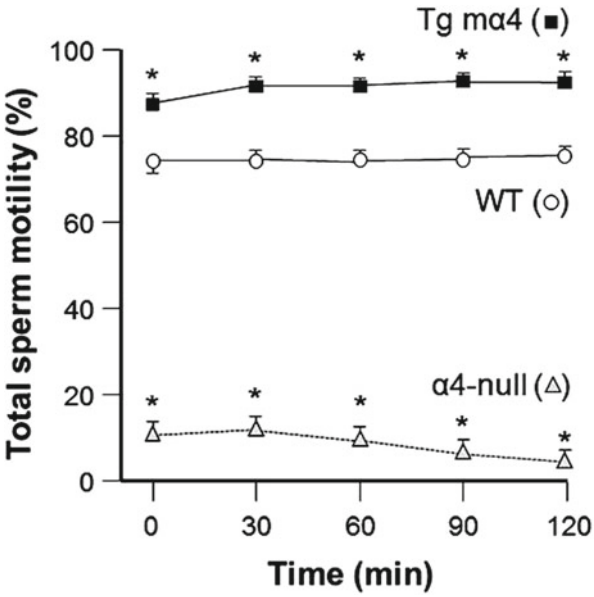

Fig. 5.3 Correlation of sperm motility and expression levels of Na, K-ATPase α4. Sperm total motility was determined by CASA on sperm from wild type (WT), α4-null, and transgenic mice overexpressing α4 (Tg-α4 mice). Sperm motility was measured under non-capacitating conditions for the indicated times. Values are mean ± SEM and *asterisks* show statistically significant differences compared to WT controls

male mice sperm had similar fertility than WT mice, which is expected, since fertility is limited to the female factor. It is clear that one of the main functions of Na, K-ATPase α4 is to support sperm flagellar beat and that changes in its expression influence sperm swimming capacity. Figure 5.3 shows how genetic manipulation of Na, K-ATPase α4 levels in mice results in changes in sperm motility.

9 Conclusions

A great amount of work has been devoted to understanding the role of Na, K-ATPase isoforms in cell biology. It is clear today that the molecular heterogeneity of the Na, K-ATPase is a physiologically relevant event and it represents a strategy that organisms have developed to satisfy cell-specific tasks. The α4 isoform is an example of the exquisite adaptation that the Na, K-ATPase has undergone to serve the very unique motile characteristic that allows sperm to swim. Thanks to Na, K-ATPase α4, sperm can travel the long journey required to find and fertilize the egg. Without doubt, evolution has endowed α4 with properties that allow sperm to undertake the essential mission of preserving life.

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Chapter 6
The Role of the Second Na^+ Pump in Mammals and Parasites

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Abstract The mechanism for active (ATP-dependent) Na^+ extrusion from intracellular compartments, not coupled to K^+ influx and insensitive to ouabain, was discovered 50 years ago by Whittembury using renal cortical tissue, and is commonly denominated "the second Na^+ pump." This Na^+-ATPase, sensitive to furosemide and ethacrynic acid, exists in both polarized and non-polarized cells and transports Na^+ coupled to Cl^- in an electroneutral fashion, so the membrane potential is not changed during Na^+ transport cycles. Cloning of the enzyme revealed proteins of 1039 amino acids in *Trypanosoma cruzi* (TcENA) and 811 amino acids in guinea pig enterocytes (ATNA). They share the main functional catalytic domains, which are highly conserved in the P-type ATPase family, but alignment of the parasite and mammalian enzymes reveals scant homology in terms of residues (Ser and Thr) that are potentially

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phosphorylatable by protein kinases. These differences in primary sequence indicate that selective regulatory mechanisms of the Na^+-ATPases evolved differently to favor adaptation to different environmental challenges (i.e., acquisition of scarce nutrients in parasites). The second pump in mammals (kidney and heart) is regulated by signaling cascades that include angiotensins, angiotensin receptors, protein kinase C, and cyclic AMP-dependent and extracellular-signal-regulated protein kinases. Reactive oxygen species and NO are also important modulators of the Na^+-ATPase. The pump is dysregulated, possibly by abnormal phosphorylations, in diseases and syndromes (frequently associated) such as obesity, chronic undernutrition, hypertension, and cardiac conduction remodeling with increased risk of sudden death.

Keywords Ouabain-resistant furosemide-sensitive Na^+-ATPase • Na^+ transport • ATNA • TcENA • Proximal tubule cells • Enterocytes • *Trypanosoma cruzi* • Kinase-mediated regulation • Angiotensins • Na^+-Coupled cotransport

1 Introduction

For at least six decades during the last century, Na^+ movements across plasma membranes appeared to be linked only to K^+ movements. Except for those that have more recently conquered fresh water, most forms of life evolved in various ways in high Na^+ environments [1], which are considered inadequate for or injurious to the proper cellular functioning [2]. In all eukaryotes, including mammals, the extracellular fluid also contains high concentrations of Na^+—around 150 mM—probably a relic of the aqueous environments in which their ancestors evolved over hundreds of millions of years [3]. Thus, extrusion of Na^+ from the intracellular milieu became a challenge and a requisite for the survival, growth and evolution of almost all species.

At the beginning of the last century it was first postulated that a special mechanism was required in the interface between the extracellular and intracellular compartments of eukaryotes, i.e., the plasma membrane, to maintain a cytosolic Na^+ [4]

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well below the concentration in the external medium. Four decades later the requirement for a conserved metabolism to ensure the asymmetrical distribution of Na^+ ions across the membrane became clear [5], and the term "pump" was soon introduced by Dean [6] when it was understood that the mechanism for Na^+ extrusion must operate against an unfavorable electrochemical gradient for the ion. An important feature of this idea was a tight coupling between Na^+ efflux and K^+ influx, which was explicit in classical studies on the movement of these cations in nerve cells [7] and red blood cells [8]. The progression of this idea toward molecular identification of the pump culminated in two complementary steps when: (1) Skou found that Na^+ and K^+ stimulated the catalysis of ATP hydrolysis by an enzyme (an ATPase) present in membrane fragments of leg nerves from the shore crab [9]; (2) Post and Jolly demonstrated a stoichiometry of $3Na^+$:$2K^+$ for simultaneous, energy-dependent transport of these ions in opposite directions across the red cell membrane [10]. The demonstration that this Na^+-plus-K^+-stimulated ATP hydrolysis was specifically inhibited by ouabain, a cardiac glycoside [11], led to an operational definition of active Na^+ fluxes across the plasma membrane as those mediated by a ouabain-sensitive ATPase. The lines of evidence associating the ouabain-sensitive Na^+-plus-K^+-stimulated ATP hydrolysis "in vitro" with the ATP-dependent Na^+ outward/K^+ inward movements "in vivo" were summarized by Glynn 30 years ago [12].

2 Na^+ Efflux Not Coupled to K^+ Influx

Almost immediately after the seminal discoveries by Skou and Post and their coworkers, several findings from experiments on kidney tubules [13] led over the following years to an emerging though elusive hypothesis: there is a second Na^+ pump independent of K^+. The conclusion of this pioneering work by Whittembury [13], based on the temperature dependence of Na^+ and K^+ fluxes and their correlations with membrane potential, was that *the active Na outflux would be divisible into one fraction coupled to K influx and another that leaves the cell with Cl*. Thus the second Na^+ pump appeared in the world of ion-transporting ATPases. However, until its recent cloning and purification, most people were skeptical about it, despite the clear demonstration that a K^+-independent Na^+ extrusion indeed existed and had specific roles in several physiological and pathological situations, as briefly reviewed below.

Subsequently, Whittembury and Proverbio [14] published another classical study that firmly established and strengthened the basis for the second pump hypothesis. Cells loaded with Na^+ by leaching at 0.6 °C extruded Na^+ with Cl^- after rewarming to 25 °C in the absence of K^+ through a mechanism sensitive to ethacrynic acid, and an additional Na^+ efflux (inhibited by ouabain) was observed when K^+ was added. These findings provided strong functional evidence that two active mechanisms for Na^+ extrusion were co-located in the basolateral membranes of proximal tubule cells: the Na^+-ATPase (which they named pump A) and the (Na^++K^+)ATPase

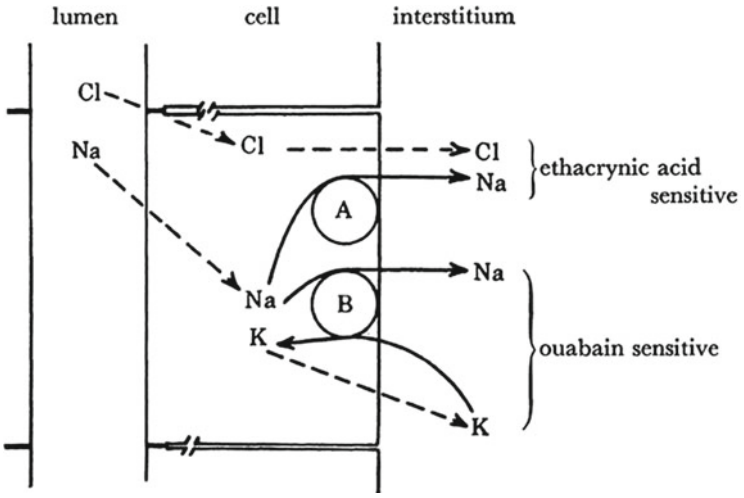

Fig. 6.1 Schematic model of the two proposed active Na^+ transport mechanisms in proximal tubule cells. One is sensitive to ethacrynic acid (and also to furosemide [17]), insensitive to ouabain and associated with Cl^- extrusion (pump A). The other is coupled to K^+ influx (pump B) and sensitive to ouabain. Na^+ enters the cell from the tubular lumen down its electrochemical gradient. Reproduced with permission from Ref. [15]

(named pump B). These pumps are illustrated in the scheme in Fig. 6.1 [14, 15]. Two years later, studies on active Na^+ transport using slices of renal external medulla, which were quantified by measuring oxygen consumption, together with measurements of Na^+ and K^+ contents in the absence or presence of ouabain and ethacrynic acid in different combinations [16], demonstrated that the two Na^+ transport mechanisms are also present in a nephron segment that is crucial for urine concentration and, consequently, for regulating the volume and Na^+ concentration of body fluids.

Despite its usefulness as a pharmacological tool that allowed the second Na^+ pump to be demonstrated, ethacrynic acid also inhibits the (Na^++K^+)ATPase by more than 60 % at a concentration that completely abolishes the ouabain-resistant Na^+-ATPase [17]. Fortunately, the diuretic furosemide, which inhibits the $2Cl^-$:$1Na^+$:$1K^+$ symporter, is also a potent inhibitor of the Na^+-ATPase but has no effect on the (Na^++K^+)ATPase [17]. Since this was demonstrated, "furosemide-sensitive" has become synonymous with "ouabain-resistant", a pharmacological adjective to characterize and label the second Na^+ pump, which has also been identified outside the mammalian kidney. Two examples—certainly there are others not mentioned in this short chapter—illustrate how widely this pump is distributed. In 1979, Preiss et al. demonstrated a Na^+-stimulated and ouabain-insensitive ATPase in muscle cells of the carotid and coronary arteries of different species [18]. On another zoological level, in the world of parasites, Caruso-Neves et al. during the late 1990s described a ouabain-resistant, furosemide-sensitive Na^+-ATPase in *Trypanosoma cruzi* [19], the etiological agent of one of the most widely distributed and serious human parasitic diseases.

3 Towards the Molecular Characterization of the Second Na^+ Pump

Though the early evidence for an ouabain-resistant Na^+-ATPase came from studies on renal epithelia, as far as we know the first successful attempt at molecular cloning and characterization used the *T. cruzi* enzyme. Iizumi et al. [20] reported the cloning of a gene encoding an ATPase similar to the various Na^+-ATPases responsible for expelling intracellular Na^+ in plants and fungi. These entities are called ENA [21], so they termed the pump they had found "TcENA." The deduced amino acid sequence showed, by hydropathy analysis, ten possible transmembrane domains (still an open question, as discussed below) and the highly conserved sequence **D**KTGTL in the catalytic phosphorylation site, which allowed the ouabain-insensitive Na^+-ATPase to be included in the P-ATPase family, i.e., the ion-transporting ATPases that form a phosphorylated intermediate (an acylphosphoprotein) during the catalytic cycle [21, 22]. To complete their pioneering picture, Iizumi et al. [20] purified the enzyme, developed a specific antibody, provided evidence that expression levels are related to Na^+ resistance in culture, demonstrated the plasma membrane distribution of the pump and—perhaps of special functional importance—found that the level of TcENA transcription varies through the developmental stages of the parasite. Figure 6.2 shows the purified membrane-bound enzyme prepared from epimastigote (circulating) forms and its detection on the surfaces of both epimastigotes and amastigotes [20]. Despite this complete picture, it is intriguing that the enzyme is stimulated by K^+ in the presence of ouabain [20].

In 2012, Rocafull et al. carried out a detailed and complete study [23] that constitutes a breakthrough concerning the second Na^+ pump. They purified the enzyme from guinea-pig enterocytes after demonstrating that both Na^+-ATPase and (Na^++K^+)ATPase are present in the basolateral membranes of these cells. Converging with the biochemical data obtained over more than four decades in several laboratories using crude membrane preparations, they [23] showed that the purified Na^+-ATPase had similar Na^+-dependence and furosemide sensitivity (with comparable IC_{50}) and was completely insensitive to K^+ and ouabain. Electrophoretic analysis of the fraction containing furosemide-sensitive Na^+-ATPase activity revealed a main band of 90 kDa (named the α subunit or ATNA) and a duplet band of 50–55 kDa (β subunit), which seemed to form an α/β complex under non-reducing conditions. Mass spectrometry demonstrated that the peptides obtained corresponded to no known protein, and on the basis of the sequences of three trypsin-derived peptides they designed primers that allowed a partial cDNA of Na^+-ATPase to be cloned. They obtained a fragment of 1148-bp, which shared 710-bp with a previously produced pig *atna* partial cDNA encoding a Na^+-ATPase [24]. They succeeded in completing the sequence of *atna* using RNA ligase-mediated rapid amplification of the cDNA ends, and obtained a 2789-bp *atna* mRNA encoding an 811 amino acid sequence containing the nine motifs characteristic of P-ATPases. Modeling of this sequence allowed Rocafull

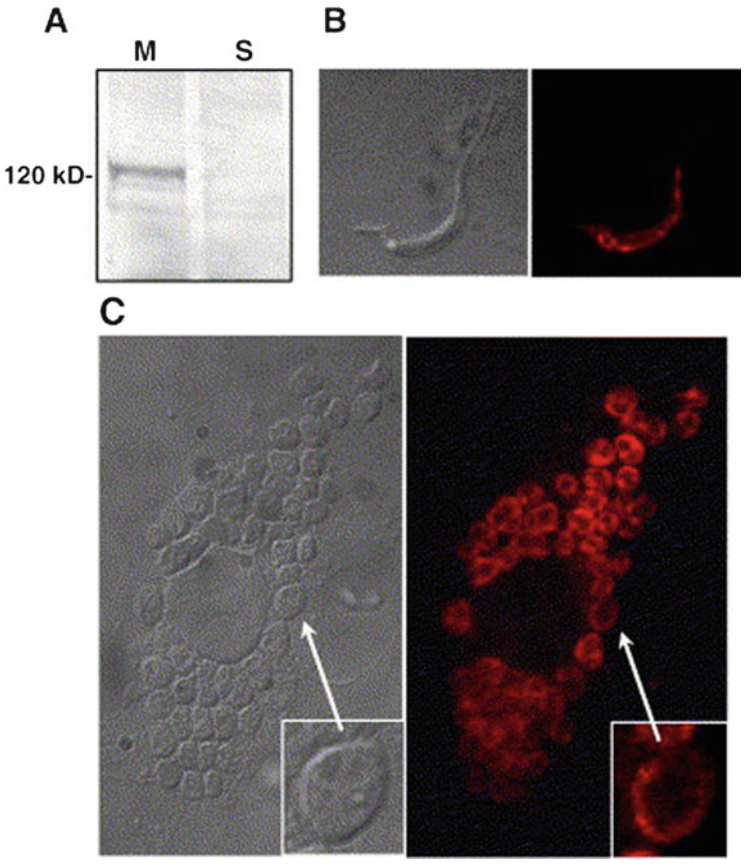

Fig. 6.2 Investigation of the location of TcENA (*T. cruzi* Na^+-ATPase). (**a**) Membrane (M) and soluble (S) fractions were prepared from epimastigotes and probed with TcENA antibody. There is a 120 kDa band in the membrane fraction. (**b**) Detection of TcENA in trypomastigotes. (**c**) Detection of TcENA in intracellular amastigotes. The *arrow* points to a single amastigote, also shown on a larger scale in the *inset*. (**b** and **c**) The *left panels* represent differential interference contrast images; the *right panels* are fluorescence images (FITC-labeled secondary antibody). Reproduced with permission from Ref. [20]

et al. to propose 2D and 3D structures for the whole ATNA subunit and for the active (catalytic phosphorylation) domain, respectively [25]. Figure 6.3 depicts these two structures.

We aligned the reported sequences of TcENA and ATNA [20, 23], searching for regions of homology and/or similarity that could help elucidate their different physiological roles and specific regulatory properties. Identity ranged from 20 % (EMBOSS Needle) to 27 % (ClustalW 2.1) and similarity attained 34 % (EMBOSS Needle). Despite the differences in primary structure, possibly related to their different evolutionary pathways [21], they share three highly conserved motifs (Fig. 6.4). These motifs are characteristics of P-ATPases [21, 26] and are related to

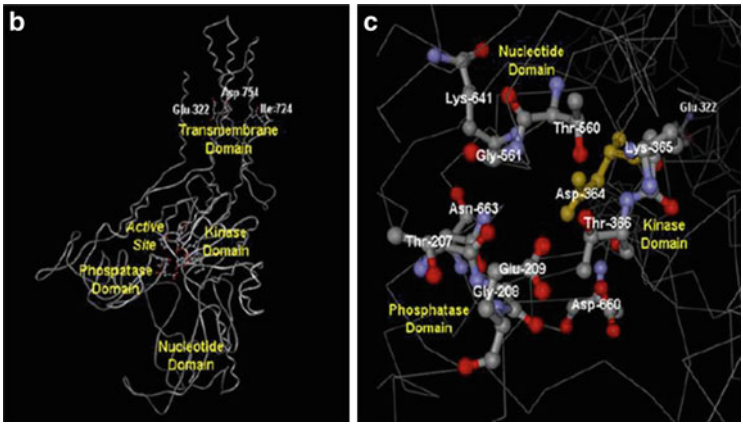

Fig. 6.3 Predicted structure of ATNA, the Na^+-ATPase cloned and purified from guinea pig enterocytes. (**b**) Three-dimensional structure of ATNA, predicted by modeling with CPHmodels-3.0 using rat (Na^++K^+)ATPase as a template. The figure indicates P-type ATPase structural domains and relevant amino acids proposed to be related to Na^+ binding (Glu^{322} and Asp^{754}) and ouabain insensitivity (Ile^{724}). (**c**) A closer view of the ATNA active site. Crucial amino acids for catalytic activity are indicated in ball-and-stick representation. These include the phosphorylatable Asp^{364} (highlighted in *yellow*) and essential residues for nucleotide binding, kinase and phosphatase domains. These panels are part of Fig. 5 in Ref. [25]. Reproduced with permission from Ref. [25]

three key steps in the catalytic cycle of these enzymes: nucleotide binding, phosphorylation, and dephosphorylation [22]. This indicates that, besides catalyzing the same overall reaction, they have energy transduction mechanisms that rely at the molecular level on the same amino acids. With respect to amino acids in the transmembrane domains that are important for Na^+ binding, there is high homology in transmembrane domains M4 and M5 but not M6 (see black squares in Fig. 6.4). It remains to be elucidated whether these differences account for differences in Na^+ affinity and/or transport velocity as adaptive responses to diverse Na^+ environments, and in responses to K^+.

Although the enzymes share the main functional catalytic domains, two other important differences can be seen, though these too present open questions that will be answered only after refined experimental approaches such as NMR are applied to the superexpressed and purified enzymes. One difference is the number of deduced amino acids: 1039 for TcENA [20] and 811 for ATNA [23]; the other is the number of predicted transmembrane domains: ten for TcENA [20] and six for ATNA [23] (enzyme truncated at the C-terminus?). If confirmed, these differences could account for the generation of distinct long-range communications transmitted to conserved catalytic domains, culminating in specific regulatory responses. Another intriguing difference lies in the primary structures, with amino acids with scores $\geq$0.60 for possible participation in regulating TcENA and ATNA through protein kinase C (PKC)- or cyclic AMP-dependent protein kinase (PKA)-mediated mechanisms (see below Sect. 5). Table 6.1 (shaded lines) shows only one potential phosphorylatable amino acid in each

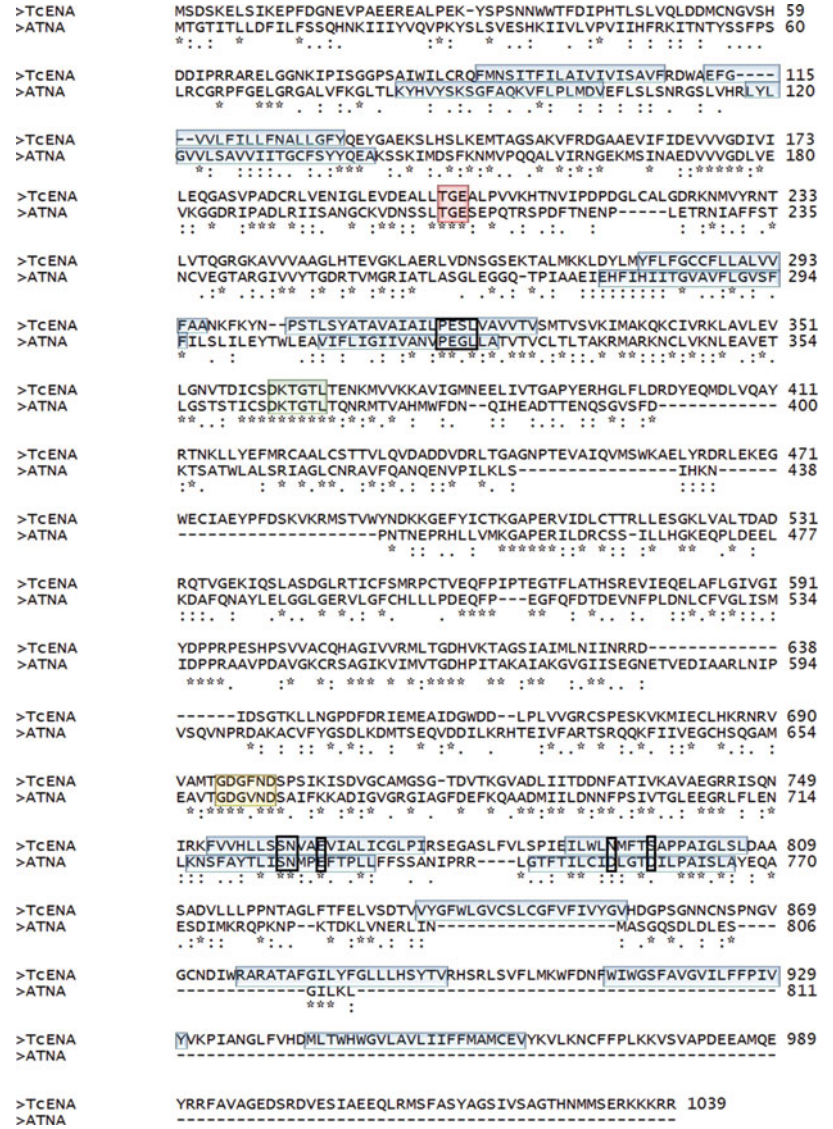

Fig. 6.4 Alignment of the deduced amino acid sequences of TcENA and ATNA. *Blue highlights*: predicted transmembrane regions; *red highlight*: phosphatase motif; *green highlight*: catalytic phosphorylation site; *yellow highlight*: dehalogenase motif of the nucleotide binding domain. *Black squares*: amino acids predicted to be important for Na^+ binding. *Asterisks*: identical amino acids; *one dot*: similar amino acids; *double dot*: very similar amino acids

sequence that aligned with an identical one in the other (Thr^{365} in TcENA and Thr^{368} in ATNA); a second aligned with a highly similar one (Ser^{332} in TcENA and Thr^{335} in ATNA). Interestingly, the first aligned pair is close to a conserved transmembrane sequence presumably involved in Na^+ binding and transport [25];

Table 6.1 Predicted phosphorylation sites with scores ≥0.60 and specific for PKC or PKA in the primary sequences of TcENA and ATNA

TcENA			ATNA		
Amino acid	Score	Kinase	Amino acid	Score	Kinase
			Thr^{4}	0.62	PKA
			Ser^{14}	0.72	PKC
			Ser^{30}	0.62	PKA
			Ser^{32}	0.73	PKC
			Thr^{52}	0.60	PKA
			Thr^{54}	0.86	PKC
			Thr^{81}	0.85	PKC
			Ser^{90}	0.73	PKC
			Ser^{113}	0.73	PKA
Ser^{141}	0.81	PKC			
Ser^{149}	0.79	PKC			
Ser^{236}	0.81	PKC			
			Ser^{298}	0.64	PKA
			Ser^{333}	0.70	PKC
Ser^{332}	0.62	PKC	Thr^{335}	0.65	PKC
			Thr^{360}	0.63	PKC
Thr^{365}	0.73	PKC	Thr^{368}	0.63	PKC
			Thr^{370}	0.66	PKC
			Ser^{398}	0.72	PKC
Thr^{443}	0.75	PKC			
			Ser^{434}	0.71	PKC
Ser^{457}	0.85	PKC			
Ser^{488}	0.66	PKA			
Thr^{489}	0.60	PKC			
Ser^{503}	0.70	PKC			
Ser^{515}	0.64	PKC	Ser^{462}	0.79	PKA
Thr^{534}	0.63	PKC			
Ser^{553}	0.82	PKC			
Ser^{626}	0.61	PKA			
Thr^{643}	0.77	PKC			
Ser^{747}	0.80	PKA			
			Ser^{718}	0.61	PKA
			Thr^{722}	0.78	PKC
			Thr^{747}	0.76	PKA
Ser^{899}	0.61	PKA			
Ser^{902}	0.89	PKC			
Thr^{945}	0.60	PKC			
Ser^{1014}	0.68	PKA			
Ser^{1021}	0.75	PKC			
Ser^{1032}	0.91	PKC			

Analysis of phosphorylatable sites for TcENA (GenBank: AB107891.1) and ATNA (GenBank: EF489487.2) using NetPhosK 1.0 prediction software

the second is located within the conserved motif for catalytic phosphorylation [21, 26]. There is a third case (Thr^{515} in TcENA and Ser^{462} in ATNA). Despite their similarities, the differences in the overall sequence possibly confer, via these residues, specificity for PKC in the *T. cruzi* enzyme and for PKA in the guinea-pig enzyme.

4 Specialized Functions for the Ouabain-Resistant, Potassium-Independent Na^+-ATPase?

Why have several forms of life, from mammals to unicellular parasites, developed a ouabain-resistant, potassium-independent Na^+-ATPase during evolution, together with the (Na^++K^+)ATPase? Several results point to answers that could have a common theme: the evolutionary advantage of a powerful, efficient mechanism for Na^+ extrusion without interference in intracellular K^+ homeostasis. Within the limited space of this chapter we present just a few possible illustrative examples.

Massive Na^+ fluxes occur across the renal and intestinal epithelia of mammals. As stated by Rocafull et al. [25]: *Under these conditions the electroneutral movement of Na^+ and Cl^- by the second sodium pump would eliminate the obligatory regulation of cell potassium concentration to maintain the membrane potential.* The physiological relevance of mediating salt extrusion through leaky epithelia together with a secondary flux of water is clear: it enables the cell volume to be regulated without interference with intracellular K^+. There is also massive absorption and reabsorption of amino acids and glucose, respectively, across the intestinal and proximal tubule epithelia, and H^+ is secreted. These processes are coupled to Na^+ entry across the luminal membrane and, as in the case of salt transport per se, it is clearly convenient from the point of view of cell regulation to have these processes at least partly independent of intracellular K^+ regulation and with no need for simultaneous control of membrane potential [25]. From these considerations it can be concluded that the furosemide-sensitive and ouabain-resistant Na^+-ATPase contributes to the generation of an electrochemical gradient for Na^+, which is the driving force for secondary active fluxes across the kidney proximal tubules and intestine.

An important role of the second Na^+ pump in energizing secondary active transport was also found in parasites. Interestingly, the Na^+-ATPase, but not the (Na^++K^+)ATPase, is coupled in trypanosomatids to the influx of the key nutrient inorganic phosphate (P_i) [27, 28]. Epimastigote forms of both *T. rangeli* and *T. cruzi* have Na^+-dependent and Na^+-independent mechanisms for P_i uptake, the former being dependent on the furosemide-sensitive Na^+-ATPase (Fig. 6.5), which generates the Na^+ gradient utilized by the symporter. Depending on species, the Na^+-ATPase has selective partners that improve P_i uptake globally, probably developed as the result of selective pressure. In *T. cruzi* (Fig. 6.5a), Na^+-independent P_i uptake (possibly coupled to H^+ influx) is fuelled by a (H^++K^+)ATPase with concomitant K^+ cycling. It is possible that Na^+-dependent P_i uptake predominates in extracellular forms, which face high Na^+ environments, while the coupling with (H^++K^+)ATPase favors

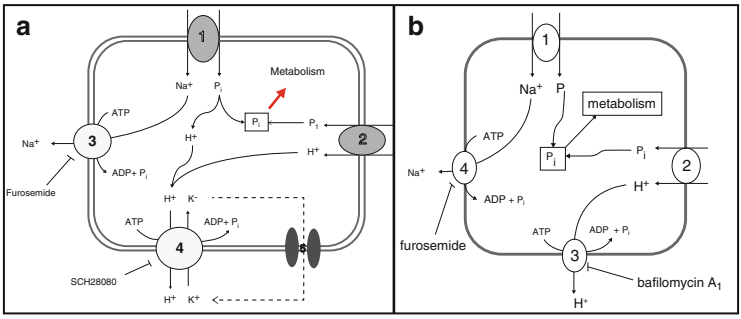

Fig. 6.5 Proposed P_i influx model in epimastigotes of *T. cruzi* (*left panel*) and *T. rangeli* (*right panel*). The furosemide-sensitive Na^+-ATPase (*3*, *left panel*; *4*, *right panel*) is responsible for providing the Na^+-motive force required for P_i uptake through Na^+:P_i cotransporters (*1* in each panel). H^+:P_i cotransporters (*2* in each panel) require a proton motive force provided by (H^++K^+)ATPase in *T. cruzi* (*4*, *left panel*) or H^+-ATPase in *T. rangeli* (*3*, *right panel*). Reproduced with permission from Ref. [27] and [28]

P_i acquisition (by amastigotes) in the intracellular (K^+-rich) milieu. This view is reinforced by the observation that the Na^+-independent mechanism in *T. rangeli* is coupled to a bafilomycin A_1-sensitive H^+-ATPase (Fig. 6.5b) but not to a (H^++K^+) ATPase. This species does not invade the mammalian host cells so it does not face environments rich in K^+ throughout its life cycle. At this point a question emerges: *T. cruzi* also expresses (Na^++K^+)ATPase [29]; so why does ouabain not inhibit Na^+-dependent P_i uptake by *T. cruzi* epimastigotes? The hypothesis of a Na^+ compartment linked to the second Na^+ pump in mammals and parasites is discussed at the end of this chapter (Sect. 6).

Very recently [30], it has been demonstrated that specific stimuli lead to synchronic augmentation of a Na^+-dependent P_i transporter and of the Na^+-ATPase (ENA1) from *Saccharomyces cerevisiae*. P_i starvation and changes in pH induce coordinated transcriptional activation of complex and interacting pathways that culminates in upregulation of both transporters. Besides demonstrating that the Na^+-ATPase can energize secondary active fluxes of solutes in other organisms, these observations support the proposal that specific functional coupling of cotransporters with the second Na^+ pump depends on the regulation of tightly coupled mechanisms of expression [27, 28].

5 Regulation of the Na^+-ATPase

A large body of experimental evidence concerning the regulation of the second Na^+ pump by hormones and autacoids has accumulated during the last two last decades using the kidney-derived enzyme, with the aim of elucidating its involvement in regulating the Na^+ content and volume of body fluid compartments. For this reason, we center our review of this topic on the results of these studies.

Over the past 20 years, Lopes, Caruso-Neves, and coworkers have demonstrated that the renal ouabain-resistant and furosemide-sensitive Na^+-ATPase can be modulated by several hormones. Among these, the peptides of the Renin Angiotensin System (RAS) appear most relevant, especially in the kidney. In 1999 [31] they presented evidence for the participation of G proteins in a signaling network starting with type 1 Ang II receptors (AT_1R), activating in sequence phospholipase C β (PLC-β) and PKC [32–34], and culminating in activation of the Na^+-ATPase, the final target of this regulatory pathway. The demonstration of at least two PKC isoforms in the basolateral membranes of kidney proximal tubules, and of hydroxylamine-resistant phosphorylation activated by a phorbol ester [32], firmly established that the second Na^+ pump is a central player in the ensemble of Ang II-modulated targets responsible for Na^+ and water homeostasis in mammals. The idea that PKC-mediated phosphorylation of Ser and Thr residues is pivotal in regulating the furosemide-sensitive Na^+-ATPase also emerged from these studies, though information about the primary structure of the pump was lacking at that time.

Interesting and apparently paradoxal results were obtained by the same group when the PKA pathway was analyzed. Activation of this route by cAMP, cholera toxin, forskolin or a stable GTP analog (GTPγS) led to a dose-dependent increase in Na^+-ATPase activity [35], together with simultaneous hydroxylamine-resistant phosphorylation of bands of 100 and 200 kDa. Both effects were canceled by the specific PKA catalytic subunit inhibitor, the PKAi peptide. However, the influence of PKA on the Na^+-ATPase is not as simple as it seemed: the PKC pathway was also implicated, ensuring a more refined regulatory mechanism for pump activation. When PKA is activated in basolateral membranes via receptors that bind Ang-(1–7) [36], in a process that includes the participation of a G_s protein, the net result is to counteract the Ang II → AT_1R → PLCβ → PKC-mediated activation [37]. The idea that PKA activation culminates in the decrease of the furosemide-sensitive Na^+-ATPase turnover *previously* enhanced under physiological or pathological conditions when the Ang II → AT_1R pathway is activated, and that the PKC/PKA activity ratio is central to this modulation, received further support from the following observations. First, in chronically undernourished rats, the reaction velocity and Na^+ affinity of the renal Na^+-ATPase are hugely increased in parallel with: (1) more Ang II-positive tubulointerstitial cells neighboring basolateral membranes; (2) more PKC isoforms per PKA [38] and a more than 100 % increase in the PKC/PKA activity ratio [39]. Second, in spontaneously hypertensive rats (SHR), PKC is upregulated and PKA downregulated, and there are opposite changes in the abundances of AT_1R and AT_2R [40], together with increased Na^+-ATPase activity [41]. These results suggest that the wide family of Ser and Thr residues depicted in Table 6.1 are selectively phosphorylated, depending on the activation of different networks that begin with the Ang II receptors—even when the same kinase participates—as seems to be the case for PKA. Perhaps the initial phosphorylation of a specific residue (or a group of them) in a determined physiological or pathological state selectively determines the secondary phosphorylation of others (the "master regulator" residue hypothesis [42]). The different distributions of these residues in the parasite and mammalian Na^+-ATPases with respect to their respective abundances in the C- and N-termini (Table 6.1) could constitute the structural basis for differences in cross kinase-mediated signaling between the two taxa.

Although the focus in this chapter regarding the regulatory mechanisms of Na^+-ATPase has been on PKC and PKA, the field continues to grow and further important features emerge, especially when their potential role in the physiopathology of prevalent diseases is considered. More than a decade ago, Bełtowski et al. presented evidence that reactive oxygen species (ROS) and nitric oxide (NO) have stimulatory and inhibitory effects on renal Na^+-ATPase [43], NADPH and GMP seeming to have pivotal roles. They proposed an interesting mechanism, the relevance of which is now clear: ROS scavenge NO, thus limiting its inhibitory effect. In other words, nitrosylation (by NO) and nitration (by the $ONOO^-$ formed by the reaction between $O_2^{\bullet -}$ and NO) [44] can be important regulators of ion-transporting ATPases.

There is emerging evidence that other kinases, first messengers and receptors participate in regulating the Na^+-ATPase. Recently, Gildea et al. [45] demonstrated that dopamine receptors and AT_2R, which can form heterodimers in basolateral membranes, participate in cooperative inhibition of Na^+ reabsorption in proximal tubule cells. The stimulation of AT_2R recruitment upon activation of the dopaminergic pathway also decreased the Ang II-dependent activation of the extracellular-signal-regulated kinase (pERK1/2), which has a link to AT_1R receptors. In this study, internalization of the ouabain-sensitive (Na^++K^+)ATPase is the mechanism responsible for inhibiting transepithelial Na^+ fluxes [45], although an influence (direct or permissive) on the ouabain-resistant furosemide-sensitive Na^+-ATPase also seems possible, especially when the enzyme is constitutively upregulated. In proximal tubule basolateral membranes isolated from chronically undernourished rats, the upregulated Na^+-ATPase activity "in vitro" returns to normal values when potentially abnormal regulatory phosphorylations of Na^+-ATPase are suppressed by inhibiting the mitogen-activated protein kinase (MAPK) → pERK1/2 pathway and by adding a protein phosphatase [39]. Figure 6.6 depicts the proposed interactions among Ang II receptors, PKC, PKA, cGMP-dependent protein kinase (PKG), ERK1/2 and protein phosphatases (PP) in kidney tubule cells.

Of course, the topics covered in this section regarding the mechanisms by which the second Na^+ pump is regulated are far from complete, but they suffice to illustrate the refined and complex regulation of this ATPase.

6 The Furosemide-Sensitive Na^+-ATPase in Diseases

Altered regulation of the Na^+-ATPase appears to underpin the pathophysiology of important diseases prevalent worldwide. Again, because of space limitations, we consider only a few examples of interrelated metabolic and reno-cardiovascular diseases involving the second Na^+ pump.

Alterations in the ROS/NO balance that affect the renal Na^+-ATPase are associated with hyperleptinemia. The specific upregulation of the renal Na^+-ATPase but not the (Na^++K^+)ATPase by leptin contributes significantly to the Na^+ retention, expansion of the intravascular compartment and arterial hypertension that are encountered in dietary-induced obesity [43, 46]. These early observations by

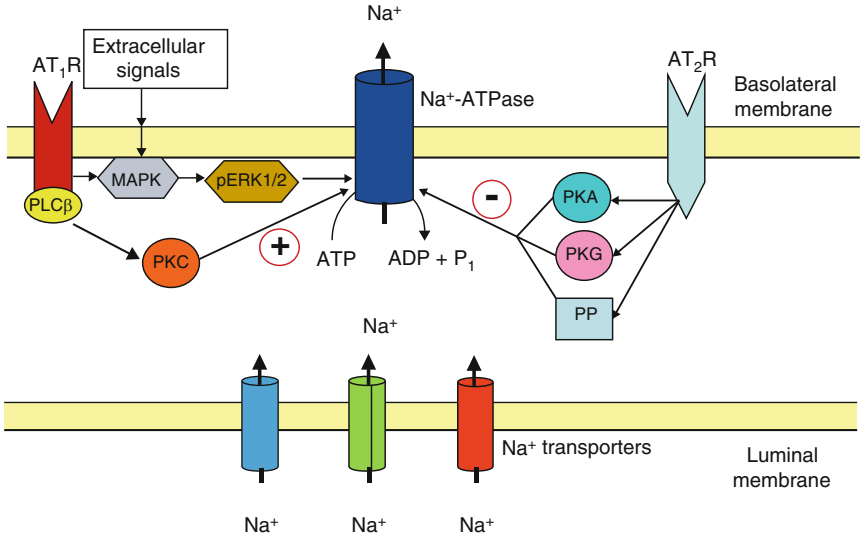

Fig. 6.6 Regulation of Na^+-ATPase in proximal tubule cells. The cartoon depicts the proposed interactions between Ang II receptors (AT_1R and AT_2R) and kinase-mediated signaling pathways that ultimately target the second Na^+ pump. AT_1R signaling (*left side* of the figure) is coupled to activation of the PLCβ → PKC and the MAPK → pERK1/2 pathways, leading to stimulation (+) of Na^+-ATPase. *Right side*: AT_2R participate in the inhibition (−) of Na^+-ATPase by activation of PKA-, PKG- and protein phosphatases (PP)-dependent pathways. These regulatory mechanisms culminate in the fine-tuning regulation of Na^+ extrusion and their disruption is associated with a wide range of life-threatening diseases (see text). Adapted with permission from Ref. [39]

Bełtowski et al. demonstrated that abnormal regulation of renal Na^+-ATPase is one of the most prominent molecular alterations in the metabolic syndrome. Interestingly, Vieira-Filho et al. [47] showed that an altered ROS/NO balance—in which NADPH oxidase again seems to play a central role—relies on constitutive hyperactivity of local renal RAS. They demonstrated an increased number of Ang II-positive tubulointerstitium cells and early unbalanced AT_1R/AT_2R and PKC/PKA, which are likely to be involved in the genesis of hypertension in adults programmed by perinatal undernutrition [47].

If regulatory alterations of the furosemide-sensitive renal Na^+-ATPase participate in the secondary hypertension associated with metabolic syndrome [43, 46], other abnormalities that involve the RAS axis (including unbalanced signaling between PKC and PKA) were apparent in a model of essential hypertension: SHR, as mentioned above [40, 41]. A recent study using SHR [48] also extended the participation of RAS (excluding Ang II) in regulating the second Na^+ pump, implicating a potent short peptide, Ang-(3–4), synthesized in the basolateral membranes of proximal tubule cells by a diverse ensemble of peptidases [49]. The physiological response of Na^+-ATPase to Ang II is lost in SHR; however, the constitutively enhanced activity is inhibited by Ang-(3–4) in a

dose-dependent manner through a mechanism that involves an $AT_2R \rightarrow PKA$ pathway after Ang-(3–4)-induced dissociation of AT_1R/AT_2R heterodimers [48], another example of differences in cross-linking among the same intracellular signaling components that increase the plasticity of regulation of the second Na^+ pump.

Inhibition of the furosemide-sensitive Na^+-ATPase in SHR by Ang-(3–4), but not $(Na^+ + K^+)$ATPase (in normotensive or hypertensive rats) and not Na^+-ATPase in normotensive rats [48], constitutes an example of selective regulation operating via regulatory phosphorylations of different Ser and/or Thr residues depending on the functional state of the pump. In this example, such specific regulation seems potentially significant from a pathophysiological perspective. Inhibition of the SHR Na^+-ATPase "in vitro" correlates with a sustained decrease in arterial pressure and increased 24 h urinary excretion of Na^+—with no change in urinary volume—after a single oral dose of Ang-(3–4) [48]. Again, Ang-(3–4) has no effect on these parameters in normal rats. Some of this information can be seen in Fig. 6.7, which also shows that: (1) the plasma Na^+ concentration is significantly lower in SHR than in normotensive rats; (2) Ang-(3–4) has no effect on plasma Na^+ in either animal group. From this ensemble of results, it can be proposed that Ang-(3–4) mobilizes "spooky" Na^+ [50] accumulated in osmotically inactive compartments represented by negatively charged molecules in skin and muscle [50, 51]. This hypothesis is supported by the observation that more tissue Na^+ is bound to proteoglycans without water retention in humans with refractory arterial hypertension [52]. They also support the view that kidney proximal tubules have two different Na^+-transporting compartments: one mediated by the $(Na^+ + K^+)$ATPase and isosmotically coupled to transepithelial transport of water; the other, hyperosmotic, mediated by the Na^+-ATPase. This type of transport would be facilitated by a selective, osmotically silent, functional and structural interaction between the second Na^+ pump and the neighboring negatively charged interstitium. A similar interaction was proposed for different tissues, without identification of the Na^+ transporter, a decade ago [53]. A comparably tight functional and structural interaction could help to explain why Na^+-dependent P_i transport in *T. cruzi* is coupled to the Na^+-ATPase and not the $(Na^+ + K^+)$ATPase [28].

The abnormalities of the regulatory Na^+-ATPase signaling pathways described above seem to be involved in the genesis of other life-threatening diseases. The increased furosemide-sensitive Na^+-ATPase in basolateral membranes from the proximal tubules of chronically undernourished rats parallels that in cardiomyocytes from the same animals [39]. The administration of Losartan (an AT_1R blocker) suppressed upregulation of the pump in undernourished rats but not the normal activity in control rats, and the PKC/PKA ratio was similarly increased in membranes from kidney and heart. Inhibition of the $MAPK \rightarrow pERK1/2$ pathway also restored Na^+-ATPase activity to normal values without effect on the control activity (Fig. 6.8). In other words, the same altered metabolic status modifies the second Na^+ pump in polarized and non-polarized cells, contributing in different but combined ways to the genesis of cardiovascular syndromes. The expansion of extracellular

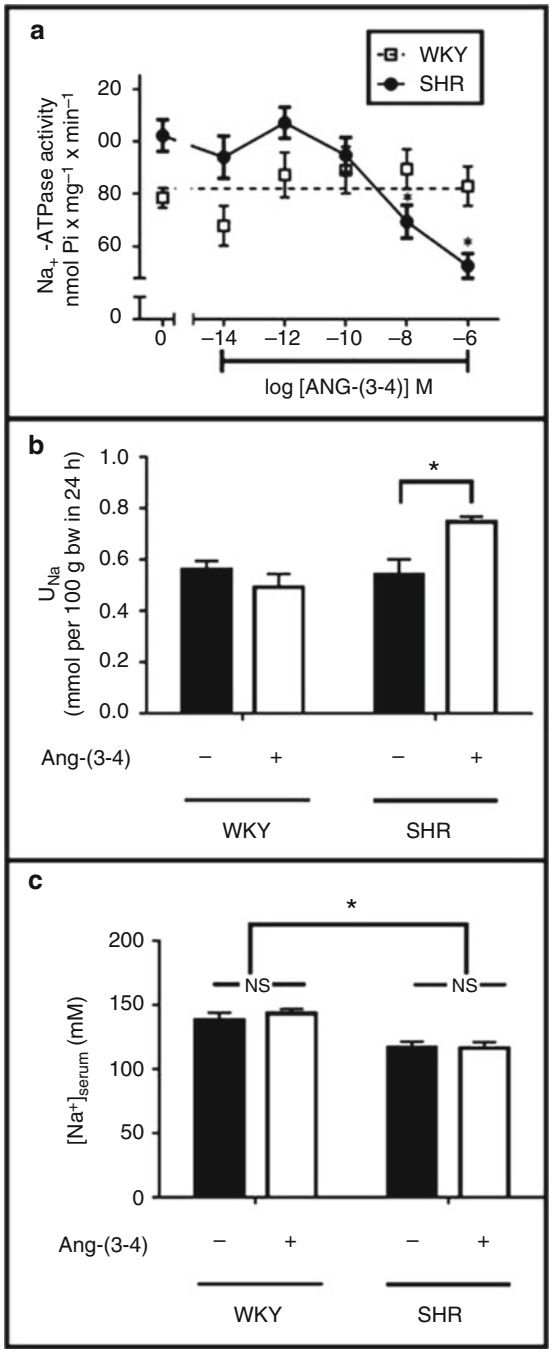

Fig. 6.7 Role of Na^+-ATPase and its modulation by Ang-(3–4) in hypertension. (**a**) The second Na^+ pump is constitutively hyperactive in SHR and is inhibited by Ang-(3–4) in a dose-dependent manner, whereas Ang-(3–4) does not affect the activity of the enzyme from the normotensive control strain (WKY rats). (**b**) Na^+-ATPase inhibition correlates with increased 24 h Na^+ excretion in SHR after administration of a single oral dose of Ang-(3–4). (**c**) Plasma Na^+ concentrations are lower in SHR than the normotensive control strain. Ang-(3–4) has no effect on either group. (**a**) and (**b**) redrawn on the basis of data from Ref. [49] (with permission). *Statistically different from the activity in SHR without Ang-(3–4) in (**a**); *statistically different from U_{Na} without Ang-(3–4) in (**b**); *statistical difference between $[Na^+]_{serum}$ from WKY rats and SHR. NS: no modifications after administration of Ang-(3–4)

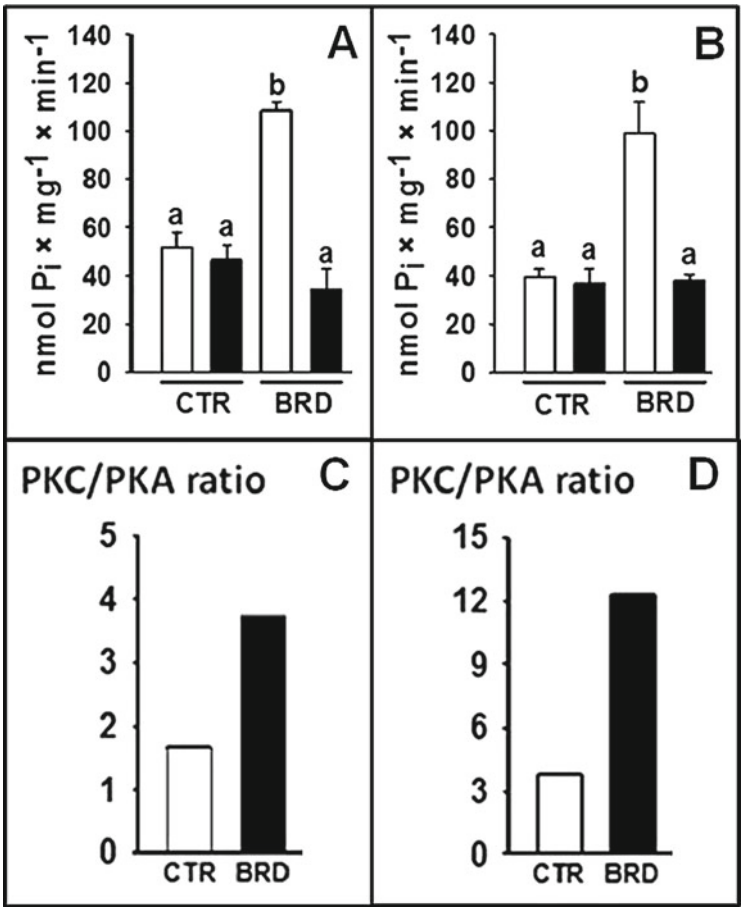

Fig. 6.8 Up-regulated Na^+-ATPase and higher PKC/PKA activity ratio in proximal tubule basolateral membranes from rats chronically undernourished by eating a diet (Basic Regional Diet, BRD) that mimics different deficient diets worldwide. (**a** and **b**) Na^+-ATPase activity in the absence (*empty bars*) or presence (*black bars*) of the MAPK inhibitor, PD098059. The second Na^+ pump is constitutively hyperactive in BRD rats; ATPase activity returns to control levels in the presence of PD098059. (**c** and **d**) Higher PKC/PKA activity ratio in kidney (**c**) and heart (**d**) of BRD rats. Reproduced with permission from Ref. [39]

fluids due to the Na^+-ATPase hyperactivity described above participates in the onset of hypertension. Altered cardiac Na^+-ATPase appears to be linked to simultaneous and serious electrical dysfunctions exclusively seen in the left ventricle: a longer QT interval in the electrocardiogram, longer action potential recorded in cardiomyocytes and triggered activity detected in ventricular tissue strips [39], all of which indicate increased risk of cardiac arrhythmias and sudden death.

7 Conclusions

The ouabain-resistant furosemide-sensitive Na^+-ATPase encountered in different organisms from unicellular parasites to mammals participates in the control of various Na^+ compartments and Na^+-dependent processes. The Na^+-ATPases from different organisms have highly conserved domains related to key steps in catalysis by P-type ATPases, whereas specificity in function and regulation resides largely in the distribution within the primary sequence of Ser and Thr residues that are targets for kinase-mediated phosphorylations. Abnormal regulatory phosphorylations of the Na^+-ATPase mediated by complex and interacting signaling networks are detected in renovascular and cardiac dysfunctions, pointing to the importance of the second Na^+ pump in the genesis of life-threatening diseases.

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Chapter 7
Myocardial Na^+ K^+-ATPase and SERCA: Clinical and Pathological Significance From a Cytological Perspective

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Abstract Structure and functions of Na^+/K^+-ATPase and SERCA are described with details on their subunits, isoforms, and intracellular localization. Main regulatory mechanisms are summarized. Molecular mechanisms of cell death and heart failure are explained with the analysis of the role of Na^+/K^+-ATPase and SERCA in these processes. Facts are considered from a cytological, pathological, and clinical perspective with an accent to new therapeutic strategies. The aim of this contribution is an overview of functional results in a structural context.

Keywords Na^+/K^+-ATPase • SERCA • Cardiomyocytes • Apoptosis • Necrosis • Cell injury • Ischemia • Hypertrophy • Heart failure • Cardiomyopathy

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1 Introduction

Various forms of ATPase have the utmost significance in the understanding of major principles of cardiovascular physiology and molecular mechanisms of cardiovascular diseases. There is ample scope of literature dealing with this problem with new results obtained. This fact promises that our knowledge will be amplified for the benefit of our patients. However, plenty of new results always open new dilemmas and controversies, suggesting that we should recapitulate what we know for a fact and what we have recently learned. Our intention in this chapter is to summarize molecular, cytological, pathological, and clinical aspects of Na^+/K^+-ATPase and SERCA functions. We focus on their role in cardiomyocyte cell death and heart failure. We start with the principles that we have already known and provide new information from a cytological and pathological point of view.

2 Na^+/K^+-ATPase: Structure and Function

Danish researcher, Jeans C. Skou was the first to suggest a link between transport of Na^+ and K^+ across the plasma membrane and ATPase activity in 1950s. For the discovery of the Na^+/K^+-ATPase, Jeans C. Skou was awarded by the Nobel Prize in Chemistry 1997.

2.1 Structure of Na^+/K^+-ATPase

Na^+/K^+-ATPase is a membrane-embedded protein complex, a hetero-oligomer composed of α and β subunits, in a 1:1 ratio [1, 2]. The large catalytic α-subunit (~110 kDa) contains binding sites for Na^+ and K^+ ions, ATP and for cardiac glycoside ouabain [1, 2]. Catalytic subunit has conserved aspartate^{369} residue where terminal phosphate of ATP can be attached [3, 4]. The α-subunit has ten transmembrane

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domains and two large intracellular loops. The ATP binding site is located in the larger cytoplasmic loop [2]. Both amino and carboxyl ends of this molecule are located intracellularly [4].

The smaller and highly glycosylated β-subunit (~35–55 kDa) acts as a chaperone required for proper folding and localization of Na^+/K^+-ATPase subunits to the plasma membrane [1, 2, 5]. Beta subunit is composed from one transmembrane segment, short cytoplasmic tail and large glycosylated extracellular segment [6, 7]. In vitro studies suggest that separation of α and β subunits results in a lack of measurable enzyme activity [8].

2.1.1 Isoforms of α Subunit

Four isoforms of α-subunit have been described (α1, α2, α3, and α4) [1, 9, 10]. While the α1-isoform is expressed ubiquitously and is a housekeeping form, α2 is expressed largely in the brain, muscle, adult heart, and number of other tissues [2, 11]. The α3-isoform is found in ovaries, neurons, fetal and adult hearts, and white blood cells [1, 11, 12]. The α4-isoform is localized in the testis, and this isoform is specifically synthesized at the spermatogonia stage. The main role of α4-isoform is in the sperm motility [13]. Various α isoforms are tissue-specific, and they share a high degree of sequence identity (ca. ~85 % identity) [1, 12, 14]. Different α subunit isoforms could be localized in different regions of the same cell [15] and are capable of carrying out specific functions.

2.1.2 Isoforms of β Subunit

There are three isoforms of the β-subunit (β1, β2, and β3) [1, 2]. Beta1 isoform is, like α1, ubiquitously expressed suggesting a housekeeping role for the α1-β1 Na^+/K^+-ATPase in most cells [16]. Beta 2 isoform is expressed mainly in the brain and muscle [17], while the β3 isoform is predominantly expressed in the skeletal muscle, brain, lung, testis, and liver [16, 18]. In human heart, α1, α2, and α3 are expressed together with β1 and very low levels of β2 in a region-specific manner [19, 20]. Alpha and β subunit isoforms of Na^+/K^+-ATPase have been encoded by different genes. They are synthesized independently of each other in the endoplasmic reticulum and assembly very soon after the synthesis [21–24]. Isoforms combine to form a number of Na^+/K^+-ATPase isoenzymes expressed in a tissue- and cell-specific manner [24].

2.1.3 The Role of FXYD Proteins

FXYD proteins (also referred to as γ-subunits) have functions in stabilization or attenuation of Na^+/K^+-ATPase [25] and form an auxiliary subunit of Na^+/K^+-ATPase. The FXYD proteins are a family of seven small regulatory proteins (FXYD1–7). They are transmembrane proteins and have conserved FXYD amino acid motif

located in their extracellular domain [25, 26]. N-terminal part of FXYD is extracellular, while C-terminus is cytoplasmic [25]. FXYD proteins are expressed in a tissue specific fashion. FXYD1 (phospholemman) is expressed in the heart and the skeletal muscle [27–29].

2.2 Functions of Na^+/K^+-ATPase

Na^+/K^+-ATPase is universally expressed in all animal cells. The primary function of the Na^+/K^+-ATPase is generation and maintenance of electrochemical Na^+ and K^+ gradients across the cell membrane. The Na^+/K^+-ATPase is responsible for the low intracellular Na^+ and high intracellular K^+ concentrations required for normal cellular functions. Activity of this enzyme occurs in several steps and is dependent on ATP hydrolysis [1, 2]. Following binding of ATP to the enzyme, three Na^+ ions from the cytoplasm associate with the active site of Na^+/K^+-ATPase. Phosphorylation of the Na^+/K^+-ATPase (at aspartate residue) results in its conformational change. As a consequence of this change, three bound Na^+ ions are released out of the cell. Thereafter, two extracellular K^+ ions bind along with dephosphorylation process and are transported into the cell [1, 2, 30].

The Na^+/K^+-ATPase is the specific target for the action of ouabain, digitalis and endogenous cardiac glycosides. They regulate cardiac contractility by indirect way (positive inotropy) [31, 32]. By binding to the extracellular part of Na^+/K^+-ATPase, cardiac glycosides inhibit its activity [2, 32] and increase Na^+ concentration. These actions precede increased intracellular Ca^{2+} concentration [31, 32] which, on the other hand, enhances heart contraction [31, 33]. This mechanism is the basis for the usage of cardiac glycosides (digoxin) in the therapy of congestive heart failure. Number of Na^+/K^+-ATPase molecules decreases in heart failure, but also in some other disorders as for example, obesity [34, 35]. Cardiomyocytes of patients with heart failure are more sensitive to effects of cardiac glycosides [34, 36–39]. Rathore et al. [40] reported that higher serum digoxin concentrations are associated with increased mortality in patients diagnosed with heart failure. That is why, as explained in further discussion, cardiac glycosides are known for their narrow therapeutic window.

2.3 Regulation of Na^+/K^+-ATPase Function in Physiological and Pathophysiological Conditions

Na^+/K^+-ATPase activity can be regulated by hormones and environmental factors through: gene expression, trafficking of the newly synthesized enzyme subunits, and phosphorylation. The first mechanism affects de novo Na^+/K^+-ATPase synthesis or degradation through regulation of gene transcription [1, 41–45]. Second critically important step in regulatory process is the synthesis of subunits of Na^+/K^+-ATPase and their translocation to the plasma membrane from intracellular stores [22, 46, 47].

Regulation of Na^+/K^+-ATPase activity can be achieved as well through direct effects on the kinetic behavior of the enzyme located in the membrane. This is a short-term regulation, accomplished within minutes to hours. It is realized via protein kinase A (PKA), protein kinase C (PKC), protein kinase B (PKB), or cGMP-dependent protein kinase (PKG) phosphorylation [1, 39, 48–50]. Activation of PKA and PKC leads to modulation of Na^+/K^+-ATPase activity in a tissue- and species-specific manner. PKA of the cardiomyocytes is one of the principle molecules involved in sympathetic innervations. After β adrenergic receptors activation, signal is transduced through cardiac-specific isoform of phosphoinositide 3-kinase (PI-3 kinase), p100γ [49]. PKA is associated with its intracellular domain. It breaks down cAMP molecule and controls the activity of Na^+/K^+-ATPase through phospholemman phosphorylation. However, regulation of Na^+/K^+-ATPase through this third mechanism is an issue of intensive research with, currently, very controversial results, conclusions and opinions [38, 39, 50, 51].

Phospholemman (FXYD1 protein) is regulated through activity of PKA and PKC. Dynamic interaction between protein kinases, phospholemman, and Na^+/K^+-ATPase controls the intracellular concentration of Na^+. Unphosphorylated phospholemman inhibits Na^+/K^+-ATPase, while phosphorylated phospholemman relieves this inhibition. It is an immediate response, activated by sympathetic innervations, the fact fully coherent with previously mentioned detail that PKA is involved in short-term regulation. Hyperphosphorylation of phospholemman through PKA activity enhances the activity of Na^+/K^+-ATPase and prevents Na^+ overload during normal, physiological response to increased heart rate, as for example in stress [52]. On the other hand, it was shown that blockade of β adrenergic receptor has beneficial effect for patients, owing to reduction in PKA activity. One of the supposed regulatory mechanisms is almost completely opposite to previously described one. Namely, it is suggested, by using animal models and isolated cardiomyocytes that Na^+/K^+-ATPase can be regulated by glutathionylation. It is a form of a reversible oxidative modification in which the glutathione (GSH) forms a disulfide bond with β1 subunit of Na^+/K^+-ATPase and subsequently inhibits it [53]. This is caused by PKA activation and "mediated by the activation of PKC and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in a downstream pathway shared with angiotensin II (Ang II)" [53]. Blockage of β adrenergic receptors, decreases the activity of PKA in this model, reduces glutathionylation and stimulates the activity of Na^+/K^+-ATPase [53].

2.4 Na^+/K^+-ATPase and Intracellular Signaling

Although Na^+/K^+-ATPase is generally considered a non-receptor membrane molecule, it can serve and act in certain pathways as a signal transducer [54]. Lower doses of ouabain (non-inhibitory doses) after binding to Na^+/K^+-ATPase trigger signaling that involves inositol 1,4,5-trisphosphate receptor (IP3R) [54–56]. Activation of IP3R results in increase of intracellular Ca^{2+} [54, 55] and activates the pleiotropic transcriptional factor nuclear factor kappa B (NF-κB) [56]. NF-κB has dual

potential effect on cardiomyocytes. As a part of TNF-α pathway, it mediates detrimental effects of TNF-α on cardiomyocytes. On the other hand, data on pharmacologic inhibition of NF-κB indicate that it could be involved in cardioprotective mechanisms during ischemic preconditioning of the myocardium [57]. Another common intracellular pathway activated by ouabain-related inhibition of Na^+/K^+-ATPase is PI3K. PI3K phosphorylates membrane phospholipid and generates the Akt kinase (protein kinase B). PI3K/Akt signaling pathway has antiapoptotic activity in cardiomyocytes. However, the net effect of proapoptotic and antiapoptotic properties of ouabain remains to be elucidated.

From the pathological and clinical point of view, control of Na^+/K^+-ATPase is of essential importance for the therapy of heart failure, one of the leading causes of death in modern world. Inhibition of Na^+/K^+-ATPase exerts the positive inotropic effect, but at the same time is associated with known mechanisms of cardiomyocytes cell death, which is the major concern in the application of cardiac glycosides. New studies offer promising and exciting results. It was shown that inhibition of Na^+/K^+-ATPase could be achieved simultaneously with the activation of another ATPase important for cardiomyocytes preservation and contractility, SERCA (isoform SERCA2a) [58]; SERCA structure, function, and role of Na^+/K^+-ATPase and SERCA in heart failure are explained in the following sections.

3 SERCA Proteins: Structure, Isoforms, and Function

3.1 Structure of SERCA

Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a 110 kDa integral membrane protein and belongs to P-type family of ion pumps, which also includes plasma membrane Ca^{2+}-ATPase (PMCA), Na^+/K^+-ATPase, H^+-ATPase, and K^+-ATPase [59, 60]. It is the only active transporting pump located on the membranes of the sarcoplasmic reticulum (SR) [61]. In vertebrates there are three main different forms of SERCA pumps (SERCA1, 2, and 3), encoded by ATP2A1, ATP2A2, and ATP2A3 genes, located on three different chromosomes (chromosome 16, 12, and 17). These genes produce more than ten isoforms by alternative splicing [60, 62–64]. These isoforms are highly conserved in structure, with 75 % or more homology between them [60]. Besides its role in coding the SERCA2 protein, the ATP2A2 gene is associated with certain conditions such as Darier's disease and Acrokeratosis verruciformis [65].

3.2 Isoforms of SERCA

SERCA1 has two main isoforms, SERCA1a and 1b, expressed in adult fast-twitch skeletal muscles (1a) and fetal tissues (1b). The main form of SERCA in cardiac muscle cells is the SERCA2 which has four isoforms (a, b, c, and d) [62]. SERCA2a,

also known as "muscle specific isoform", is expressed in cardiac muscle cells, slow-twitch skeletal muscles and smooth muscle cells. SERCA2b is a ubiquitous form present in muscle, but also in non-muscle cells [60]. The existence of SERCA2c protein was demonstrated in the heart [66] and its mRNA was also found in hematopoietic cells [67]. In humans, SERCA3 includes five isoforms (SERCA3b, 3c, 3d, 3e, and 3f) in addition to the species-unspecific SERCA3a, making a total of six isoforms. These isoforms are mostly expressed in non-muscle cells [60, 62], but recent evidence suggest cardiac expression of certain isoforms, such as SERCA3d and SERCA 3f, which are only present in humans [62, 68]. In summary, in human cardiomyocytes six isoforms have been detected so far: SERCA2a, SERCA2b, and SERCA2c, as well as SERCA3a, SERCA3d, and SERCA3f with specific intracellular localization [61].

3.3 Functions of SERCA

Contraction is the main characteristic of muscle cells and it is mediated by calcium ions. The main intracellular depot of Ca^{2+} is the sarcoplasmic reticulum (SR), a membranous network present in muscle cells, able to sequester and store millimolar amounts of calcium [60]. By releasing Ca^{2+} from its cisternae into the cytosol, it initiates the process of muscle cell contraction. Most of the Ca^{2+} responsible for muscle contraction comes from the SR and it is released during cardiac systole by the process of Ca^{2+} induced Ca^{2+} release, where an increase of cytoplasmic Ca^{2+} concentration produced by the L-type Ca^{2+} current is the main trigger for Ca^{2+} from SR [69]. The ryanodine receptor (RyR) located on SR, serves as a Ca^{2+} release channel through which Ca^{2+} enters the cytosol. A key factor that controls the level of SR Ca^{2+} levels is the activity of SERCA. By reuptaking the Ca^{2+} back to SR, it lowers the cytosolic calcium levels and replenishes the Ca^{2+} stores in SR, thus enabling the relaxation and new contraction of muscle cells in a repeated contraction-relaxation cycles [60]. SERCA and the role of Ca^{2+} ions in the muscle contraction was firstly described almost simultaneously by two different laboratories in two different articles: that of Ebashi and Lipmann in 1962 and Hasselbach and Makinose in 1961 [70, 71].

3.4 Regulation of SERCA Function in Physiological and Pathophysiological Conditions

The activity of SERCA pumps is regulated by certain proteins such as sarcolipin and phospholamban. Sarcolipin, which is mainly localized in the atrium, inhibits the expression of SERCA1a and SERCA2b, while phospholamban suppresses the function of SERCA protein and is highly expressed in ventricular muscle. Thus, these two intrinsic membrane proteins expressed in the SR, lower the SERCA affinity for Ca^{2+} [64, 72]. Phospholamban is in dynamic interaction to SERCA2a.

It is a 52 amino acid protein of the sarcoplasmic reticulum and exists in monomeric and pentameric form. The monomeric form is a key regulator of the SERCA activity.

The SERCA pumps represent key elements that are necessary for the normal contractility of the human myocardium. Contractile dysfunction which is present in certain conditions such as cardiac hypertrophy and heart failure can be attributed to the reductions in SERCA activity [73]. Reduced expression of SERCA2a (predominant cardiac isoform) contributes to the abnormal contractility of the myocardium and is present in several cardiac diseases including ischemic heart disease and certain forms of cardiomyopathies, which tend to progress to heart failure [72]. Patients with end stage heart failure of different etiologies show decreased levels of SERCA2a mRNA and SERCA2a protein, but certain studies reported unchanged levels of SERCA, while others only reported alterations in phospholamban status.

The important role of SERCA pumps has been studied extensively in numerous studies on transgenic animals that overexpress SERCA or are deficient in SERCA pump isoforms in cardiomyocytes [60]. Overexpression of the predominant cardiac SERCA isoform (SERCA2a) in experimental transgenic animals resulted in increased maximal rates of contraction and relaxation of the heart, without any structural or functional abnormalities and with a normal life span.

4 Cardiomyocytes Ultrastructure and Localization of ATP-ases

The further discussion on ATPases and their functional, clinical, and pathological significance should be preceded by brief resume of cardiomyocytes structure. Cardiomyocytes are principal, contractile cells of the myocardium and working force of the cardiac pump. On a light microscopy level, cardiomyocytes resemble striated muscle with alteration of dark (A) and light (I) bands. Cardiomyocytes consist of even smaller subunits called myofibrils which consist, further, of series of basic structural, organizational and functional units—sarcomeres (Fig. 7.1). Each sarcomere contains thick myofilaments made of myosin II in the central region, and thin F-actin myofilaments associated with Z lines, at the end of sarcomeres (Fig. 7.1). Besides F-actin, thin filaments contain troponin and tropomyosin. Organization of thick and thin filaments within sarcomeres and association of sarcomeres into myofibrils form a morphological pattern of cardiac muscle as a cross-striated muscle. A bands are formed of myosin filaments with portions of actin filaments in-between, while I bands contain actin myofilaments. Running through the midlines of I bands are Z lines. The position of myofilaments is controlled and preserved during each myocardial cycle by a network of cytoskeletal and cytoskeleton-associated proteins. These proteins form supportive mesh that protects sarcomeres from mechanical stress.

Accessory proteins, components of this network are titin, nebulin, α actinin, myomesin, desmin, αB-kristalin, plectin, dystrophin, tropomodulin, and ankyrin.

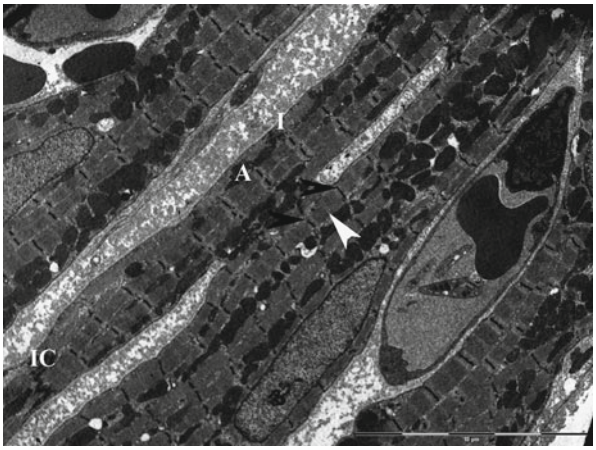

Fig. 7.1 Structure of cardiomyocytes (Transmission Electron Microscopy (TEM); original magnification 3500×, bar = 10 μm). *Dark bands*—A bands, contain myosin and actin (A); *light bands*—I bands with actin myofilaments (I); Z lines (*black arrowheads*); H band and M line—*white arrowhead* (H band is a space between ends of actin filaments inserted in between myosin filaments and it contains creatine kinase; M line contains myomesin that links myosin filaments); *IC* intercalated disks (Courtesy of Aleksandar Djordjevic, Department of Chemistry, Faculty of Sciences, University of Novi Sad and NanoBiomedicine team)

Desmin is an intermediate filament that forms lattice surrounding the Z lines of sarcomeres. It is linked to Z lines via plectin and stabilizes myofibrils and anchors them to sarcolemma. Ankyrin, αB-kristalin, dystrophin, dystroglycan complex, and sarcoglycan complex form the attachment area of actin filaments to sarcolemma and laminin of the external (basal) lamina at the specific structural unit called costamere [74]. Association of costameric proteins with Na^+/K^+-ATPase is important for survival of cardiomyocytes as explained in following sections.

Myofibrils are surrounded with cisternae of smooth endoplasmic reticulum—sarcoplasmic reticulum. Sarcoplasmic reticulum is a network of longitudinal and transversal cisternae. Longitudinal cisternae parallel myofibrils, while transversally oriented cisternae (terminal cisternae) are in a form of membranous sacs in close proximity and laterally to T tubules. T tubules are invaginations of the plasma membrane—sarcolemma. Association of terminal cisternae and T tubules form diads. Diads are located at the level of Z lines.

Cardiomyocytes are interconnected with intercalated disks, junctional complexes that consist of fasciae adherentes, desmosomes, and gap junctions. Intercalated disks are located at the level of Z lines. Z lines consist of α-actinin which anchors actin to Z lines. Z lines also contain desmin, nebulin, titin, and plectin. Sarcoglycan, dystroglycan, and dystrophin are also components of intercalated disks. For more details on cardiomyocytes structure, readers are referred to excellent literature [75–81].

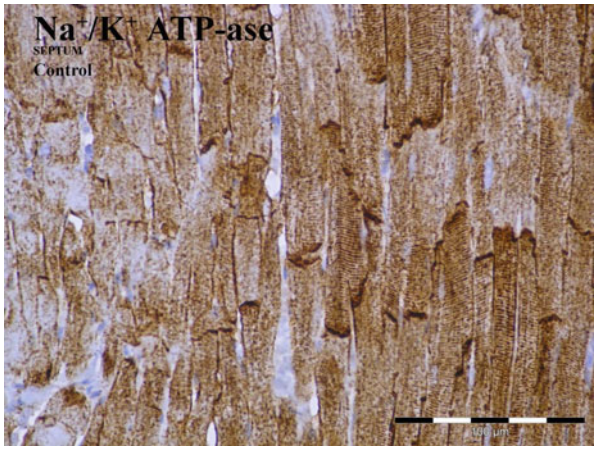

Fig. 7.2 Na^+/K^+-ATPase in cardiomyocytes of interventricular septum (immunohistochemical staining for Na^+/K^+-ATPase, original magnification 400×, bar = 100 μm)

4.1 Localization of Na^+/K^+-ATPase and SERCA

Na^+/K^+-ATPase is a transmembrane protein, and hence its primary localization is sarcolemma (Fig. 7.2). However, the distribution of Na^+/K^+-pump isoforms in cardiomyocytes is not equal: α2- and α3-subunits are located mainly in T tubules (in close proximity to sarcoplasmic reticulum), while α1-subunits are more uniformly distributed throughout the sarcolemma [76, 77]. Alpha1, α2 and β1 subunit of Na^+/K^+-ATPase are present at the level of intercalated disks [76, 78]. In muscle cells, SERCA pumps are localized in the SR (SERCA1, SERCA2a). Immunohistochemical studies on other cells showed that SERCA2 and SERCA3 are mostly localized inside the network of endoplasmic reticulum, but also in the outer membrane of the nuclear envelope [79]. Additional and more detailed studies on human cardiomyocytes showed that SERCA2a and SERCA2b have a uniform pattern of distribution in the sarcoplasmic reticulum, although certain differences between them still exist. For example, SERCA2a is in the regions located close to the T-tubules and to longitudinal sarcoplasmic reticulum, while SERCA2b appears to be mostly localized in the regions close to T-tubules only. SERCA2c isoform is in close proximity to the sarcolemma and in intercalated disks, as well as SERCA3a. SERCA3d and SERCA3f proteins are located in perinuclear and subplasmalemmal regions of human cardiomyocytes [62].

5 ATP-ases and Mechanisms of Cardiomyocyte Cell Death

The insufficiency of membrane ionic transport systems is associated with the increased rate of cell death. The first reason for the Na^+/K^+-ATPase and SERCA insufficiency is the lack of ATP and derangement of the aerobic respiration or

mitochondrial oxidative phosphorylation. The most frequent cause of cardiomyocytes injury due to a lack aerobic metabolism and ATP deficiency is ischemic heart disease with its main entities: angina pectoris, acute myocardial infarction, chronic ischemic heart disease and sudden cardiac death. Disruption of the oxidative phosphorylation itself provokes series of mutually connected intracellular events leading to disturbance of other three processes vital for the preservation of the cell, namely: protein synthesis, cell membrane integrity and genetic material preservation. The oxidative phosphorylation blockage with the decreased ATP reserves and insufficiency of Na^+/K^+-ATPase leads to changes in the concentration gradient of Na^+ and K^+ ions. There is an efflux of K^+ ions, and influx of Na^+ ions. Since water isoosmotically follows potassium, intracellular edema and hydropic swelling are progredient.

These changes are reversible up to a certain point. Hydropic swelling of the cell is a reversible change [80]. Dilatation of cisternae of sarcoplasmic reticulum and mitochondria due to lack of ionic and osmotic disbalance are main morphological characteristic of reversible injury and illustrate the fact that the disruption of the structural integrity and influx of water is not an exclusive characteristic of the plasma membrane, but membranes of organelles are affected as well, hence the dilatation of sarcoplasmic reticulum and mitochondria.

Reduced cellular respiration is associated with the decrease of intracellular ATP, but also with the increase of AMP and the activation of anaerobic glycolysis and enzymes phosphofructokinase and phosphorylase. Lactic acid, the side product of this metabolic pathway, is formed. A presence of lactic acid leads to intracellular acidosis. Low pH values interfere with the normal functioning of intracellular enzymes essential for the synthesis of structural proteins and phospholipids, which ultimately leads to further disruption of cell membranes and membranes of the organelles.

The control of Ca^{2+} ions influx is affected by disruption of membranes and energy depletion. Namely, the Ca^{2+} concentrations inside cardiomyocytes cytoplasm are regulated at very persistent level of 30–100 nM during the diastole. This is achieved by the activity of sarcolemmal Ca^{2+}-ATP-ase and Na^+/Ca^{2+} exchanger as well as the activity of SERCA and Ca^{2+} buffering molecules [81, 82]. Decreased amount of ATP is followed by insufficiency of the Ca^{2+}–Mg^{2+}-ATP-ase and sarcolemmal Na^+/Ca^{2+} exchanger (NCX).

Namely, metabolic acidosis initiates Na^+/H^+ exchange also, with transport of H^+ out of the cell. H^+ is exchanged for Na^+, transported into the cell [80]. High concentration of sodium activates NCX, contributing to increase in intracellular Ca^{2+}. Calcium ions are also released from sarcoplasmic reticulum. We have already described this process in the section on SERCA structure and function. Namely, the ryanodine receptor (RyR) located on SR, serves as a Ca^{2+} release channel through which Ca^{2+} enters the cytosol.

Recently, a new specific form of transient intracytoplasmic influx of Ca^{2+} was described, called Ca^{2+} sparks. Ca^{2+} sparks occur when RyR opens spontaneously and release a small amount of Ca^{2+} locally [82, 83]. Ca^{2+} sparks are present in both systole and diastole. During diastole Ca^{2+} sparks are independent from Ca^{2+} influx

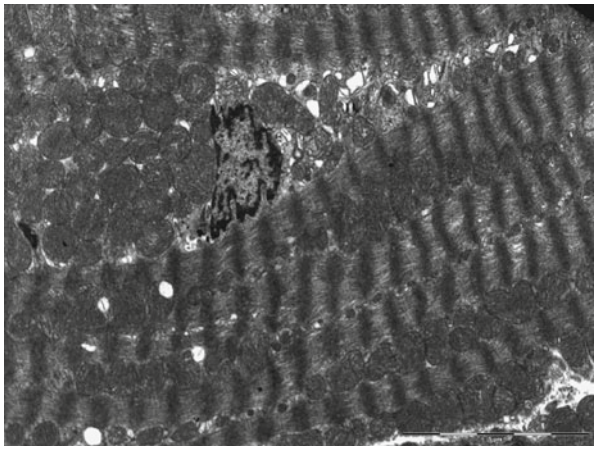

Fig. 7.3 Structure of cardiomyocytes (Transmission Electron Microscopy (TEM); original magnification 5600×, bar = 5 μm). Hypercontraction of sarcomeres (Courtesy of Aleksandar Djordjevic, Department of Chemistry, Faculty of Sciences, University of Novi Sad and NanoBiomedicine team)

through sarcolemma associated molecules [82]. During excitation-contraction coupling and systole, openings of L-type Ca^{2+} channel induces release of Ca^{2+} into cytosol which resemble thousands of Ca^{2+} sparks triggered by this process [82].

Insufficiency of Na^+/K^+-ATPase and NCX provoke ample and spontaneous diastolic Ca^{2+} sparks and cause arrhythmias and sudden cardiac death [84]. On the other hand, control of calcium ions through SERCA mechanism and their transport back to lumens of the SR cisternae is insufficient due to the lack of ATP.

Increase of the free cytosolic calcium ions activate enzymes leaking through the damaged organelles membranes. Activated phospholipases (phospholipase A2) and proteases further damage the membrane and cytoskeleton. Costameric ankyrin dissociates from its attachment [84] with severe disturbance of cytoskeleton and degeneration of myofibrils. At the same time, α1 and α2 subunits of Na^+/K^+-ATPase are detached from cytoskeleton, namely ankyrin-B. These events are directly provoked by influx of Ca^{2+} and activation of calpain in the setting of complex intracellular changes and metabolic acidosis [85–87] and are proved to augment Na^+/K^+-ATPase insufficiency.

Influx of calcium ions, also, leads to irregular and dissociated contraction of the cardiomyocytes with hypercontraction of sarcomeres and contraction band necrosis (Fig. 7.3). This phenomenon called the calcium paradox is a consequence of uncontrolled influx of calcium ions into energy-depleted cardiomyocytes. It is associated with the ischemic damage of the cell. That is why it could be found at infarction area and at the periphery of infarcted areas where it is a part of the ischemia-reperfusion sequence [85–88].

Nevertheless, it is not unique for the ischemic damage, but other forms of injury agents provoke it too by mechanisms similar to aforementioned mechanisms. It is also observed in the donors' hearts [89]. It is observed in damages activated through ROS generation, for example lipid peroxidation associated with doxorubicin application [90].

In summary, defects in oxidative phosphorylation and ATP deprivation induce complex intracellular alterations with ionic and osmotic disbalance, hydropic

swelling of the cell, and dissociated contraction. Balances of sodium, potassium and calcium ions are interconnected and lead to a same result.

Such observations are recently confirmed in a sophisticated model of a knock-in mouse in which the phospholemman residues phosphorylated by PKC and PKA have been mutated with reduction of Na^+/K^+-ATPase. This exacerbated Na^+ overload and resulted in profound contractile dysfunction [52].

Disruption of lysosome membranes leads to leakage of lysosomal enzymes and their activation in the conditions of low intracellular pH and results in tissue digestion and necrosis.

Mitochondrial damage leads to further reduction or blockage of the oxidative phosphorylation, switch from aerobic into anaerobic metabolism and ceased synthesis of phospholipids, which subsequently afflicts integrity of all membranes. During ischemia there is an increase of reactive oxygen species (ROS). They cause lipid peroxidation of cardiolipin and damage the electronic transport chain, harming further oxidative phosphorylation.

During the mitochondrial injury, cytochrome c is released from the disrupted inner membrane of mitochondria, through mitochondrial permeability transition pores, which resembles potential for the activation of the inner apoptotic pathway. Endonucleases are also activated by uncontrolled calcium ions influx during the cell injury. In concordance with this observation is the fact, that all these changes are followed with the translocation of annexin A5 to the sarcolemma [91] and activation of caspases 3 and 8 [80, 92] which is consistent with apoptotic cell death. Mitochondrial injury has a potential to activate autophagy [93]. Regardless of a specific form of cell death, all these changes are followed by nuclear degeneration: karyolysis, karyopiknosis, and karyorrhexis, indicating irreversible changes. These changes are observed in endothelial cells as well, so the myocardial tissue is deprived of oxygen and glycose with progression of defects and absence of protective mechanisms. Once started, cellular injury is obviously a vicious circle that could hardly be stopped. After this summary, the first question is what type of cell death is actually provoked with this sequence of processes?

According to contemporary opinions on cardiac muscle cell death [89, 90, 94], two concurrent mechanisms of myocardial cell death persist in different models: apoptosis and necrosis. However, considering the presented mechanism of cellular injury, it is clear that each cellular damage has the potential for both necrosis and apoptosis, and intermediate forms of cell death could also be expected.

There are at least two more aspects that deserve to be mentioned. Reduction of the content of Na^+/K^+-ATPase itself is proved to have similar effect as ATP depletion. Decrease in synthesis, expression of mRNA, quantity or activity of Na^+/K^+-ATPase or its subunits is observed in cardiovascular diseases including heart failure, various forms of cardiomyopathies, hypertension, hypertrophy or obesity. Nevertheless, in the analysis of this particular question, we should always keep in mind that the decrease may be primary, but also a consequence of serial changes in the synthetic potential of the already injured cell, where the injury is provoked by hypoxia, ischemia, volume- or pressure-overload, reactive oxygen species (ROS), or complex genetic disorders.

Reduction of α1 subunit in an animal model of hypertension induced increased rate of myocardial cell death. However, the number of cardiomyocytes was higher. It was confirmed that although the reduction of α1 subunit induced increase in cell death, it stimulates expression of proliferative marker Ki67 in cardiomyocytes as well as increase in number of c-kit positive progenitor cells [95]. High glucose level is associated with reduced Na^+/K^+-ATPase activity and increased proapoptotic machinery: raised caspase-3 activity and Bax, as well as down-regulated Bcl-2 expression [96]. On the opposite, insulin has a protective effect and prevents apoptosis of cardiomyocytes as confirmed in the setting of digoxin treated heart failure [97].

Resveratrol, active principle of grape and red wine, has a cardioprotective effect in the model of ischemia-reperfusion injury. It increases the activity of both Na^+/K^+-ATPase and Ca^{2+}-ATPase, increasing at the same time viability of cardiomyocytes. It reduces apoptotic cell death by increase in Bcl2 and decrease of Bax and caspase-3, reduces intracellular calcium and balance the activity of reactive oxygen species (ROS) [98]. Stable expression of Na^+/K^+-ATPase is essential for the survival of cardiomyocytes after ischemia-reperfusion injury in ouabain-preconditioning model [99].

Third question is relation of ouabain and ouabain-like cardiac glycosides, including digoxin, to cell death phenomenon. Cardiac glycosides have been used for the treatment of heart failure because of their capabilities of inhibiting Na^+/K^+ ATPase. This inhibition raises intracellular Na^+ and attenuates Ca^{2+} extrusion via the Na^+/Ca^{2+} exchanger, causing intracellular Ca^{2+} elevation and empowering contractile strength of the heart [100]. The question is if the therapeutic doses of ouabain and digoxin induce and increase cardiomyocyte cell death by inhibition of Na^+/K^+-ATPase in addition to positive inotropic effect?

It was shown that ouabain induces increased frequency of cardiomyocyte cell death by both apoptosis and necrosis, and reduced cell viability. It simultaneously activates antiapoptotic mechanism of PI3K/Akt, which is, however, insufficient to block effects of proapoptotic ouabain activity [101]. In addition to positive inotropic effect and elevated oxygen consumption, inhibition of Na^+/K^+-ATPase by glycosides, impair mitochondrial energetics and cause oxidative stress especially in conditions of increased workload.

The studies on guinea pig cardiomyocytes showed that elevated intracellular Na^+ induced by glycosides treatment caused mitochondrial Ca^{2+} deficiency by activating the mitochondrial Na^+/Ca^{2+} exchanger, the major mitochondrial Ca^{2+} efflux pathway. Furthermore, it is associated with significantly decreased NADH level and increased reactive oxygen species (ROS) accumulation [102–105].

Li et al. proposed that Na^+/K^+-ATPase inhibition such as by cardiac glycosides causes mitochondrial oxidative stress and increased ROS production through two separate pathways. In first way, Na^+/K^+-ATPase inhibition blunt mitochondrial Ca^{2+} accumulation, that reduces NADH production and therefore ROS removal. And in second way, Na^+/K^+-ATPase inhibition increasing intracellular Ca^{2+} and ATP hydrolysis, which produces a large amount of ADP that stimulates mitochondrial respiration and therefore ROS production [100].

The interrelation of described processes causes that many different sources of cell injury produces similar changes in myocardial tissue. Intracellular edema,

swelling, vacuolization and hypercontraction of sarcomeres are common signs of injury in ischemic heart disease. They are present in reversible form during extracorporeal long-term preservation of donors' hearts in the procedure of heart transplantation [94]. Lipid peroxidation of membranes in doxorubicin toxicity model has the same effect [90]. Damage of intracellular membranes by advanced glycation end products and ROS, due to NADH oxidase activity, in diabetes has also the same effect. As resumed in following section, hypertrophy and heart failure of different origin include the same sequence of processes.

6 Molecular Mechanisms of Heart Failure

Heart failure (HF) remains a major cause of morbidity and mortality in the developed world. In the population under the age of 65, HF prevalence approaches 1 % [106]. Over the last decade, important progress has been made in understanding of various intracellular and molecular mechanisms of HF. Heart failure (congestive heart failure—CHF) is a complex, chronic and progressive disorder, although acute forms of heart failure persist in association with sudden volume overload, acute myocardial infarction, valvular dysfunction or compromised ventricular filling and retention of blood in peripheral circulation. It is a common endpoint of several pathophysiological pathways included in different forms of cardiomyopathies, ischemic heart disease, hypertension or valvular heart disease.

Heart failure includes forward and backward component, systolic and diastolic insufficiency. Systolic dysfunction comes from reduced myocardial contractility most frequently due to ischemic heart disease, hypertension or dilated cardiomyopathy. Diastolic dysfunction is caused by inability of ventricles to relax and accept sufficient volume of blood during diastole in conditions like hypertrophy of left ventricle, myocardial fibrosis or constrictive pericarditis. The end result of systolic and diastolic dysfunction is reduced cardiac output, forward component of heart failure, as well as retention of blood in venous circulation—backward component.

Heart failure due to conditions with pressure or volume overload is preceded by hypertrophy. Hypertrophy is a compensatory mechanism aimed to enhance contractile strength of the myocardium. On a molecular level, hypertrophy is achieved trough intensive synthesis of new myofilaments and division of mitochondria, as well as accumulation of other organelles. Extensive synthesis of myofilaments' proteins is followed with their intensive association into new sarcomeres, and hence the size of cardiomyocytes is raised. Two forms of hypertrophy exist. Concentric hypertrophy develops with pressure overload (systemic hypertension, chronic pulmonary hypertension or valvular disease, i.e., aortic stenosis). In this form of hypertrophy, new sarcomeres are arranged parallel, in a fashion that augments transversal cardiomyocyte diameter [107].

Volume overload stimulates another form of hypertrophy with deposition of newly synthesized sarcomeres along the longitudinal axis of cardiomyocytes and dilatation of ventricles. The gene expression in hypertrophy is changed towards activation of c-myc, c-fos, jun, and EGR1 as well as towards the activations of

fetal programs. For example, β-myosin heavy chains are synthesized instead adult forms of α-myosin heavy chains [57]. Atrial natriuretic peptide (factor) (ANP) is synthesized in ventricular cardiomyocytes in addition to physiological synthesis in atrial myoendocrine cells. These changes have compensatory effect up to a certain point. For example, Ca^{2+}/calmodulin-dependent protein phosphatase (calcineurin) is shown to have a role in inducing hypertrophy. It is activated to dephosphorylate the transcription factor NF-A3, enabling its translocation to the nucleus and enhance hypertrophic remodeling [108]. However, calcineurin, in this setting, activates apoptotic signaling pathways at the same time and is responsible for the increased rate of cardiomyocytes' cell death.

More important, the enlargement of cardiomyocytes is not followed by the proper extension of capillary network. The capillary-to-cardiomyocyte ratio, which should be at least 1:1 in normal myocardium, is reduced, while the distance between capillaries and cardiomyocytes is increased. Hypertrophic myocardium is in increased need for oxygen consumption and there is a profound discrepancy between the oxygen and nutrients available and oxygen and nutrients needed. That is why most cardiomyocytes stay without nutritive support and enter some form of reversible injury or cell death as described above through the aforementioned mechanisms. This is followed by the decrease in the number of functional cardiomyocytes and fibrosis, since the regeneration of cardiomyocytes from circulating and cardiac progenitor cells or dividing cardiomyocytes is suppressed and cell death prevailed [57, 90, 109]. With the development of this irreversible damage of cardiomyocytes and fibrosis, cardiac hypertrophy loses its compensatory potential, sliding to heart failure with contractile dysfunction and dilatation of chambers.

In viable cardiomyocytes, synthesis of all proteins including contractile proteins and enzymes is altered, leading to reduced synthesis, synthesis of dysfunctional proteins, structurally anomalous proteins or nonspecific and less functional isoforms of proteins [110, 111]. Besides, duration of heart failure in each patient, nature and severity of the injury that caused heart failure, and dynamics of heart failure development could affect findings of molecular and cytological research. These facts should be kept in mind in the interpretation of results on Na^+/K^+-ATPase and SERCA expression, presented in the following chapters.

7 NA^+/K^+-ATP-ase and Heart Failure

Before the consideration on a linkage between Na^+/K^+-ATPase and heart failure, we will summarize briefly functions of Na^+/K^+-ATPase. Na^+/K^+-ATPase (Na^+/K^+-pump) is an enzyme located in the cell membrane which transports three sodium ions outside of the cell and two potassium ions into the cell, generating ion gradients necessary for the maintenance of the membrane potential [112]. Na^+/K^+-pump has been found in almost all animal tissues, including human myocardium. For its proper functioning, this enzyme uses the energy of the hydrolysis of ATP molecule. Na^+/K^+-ATPase indirectly modulates the myocardial contractility by controlling the

function and driving force for Na^+/Ca^{2+} exchanger (NCX) [113]. NCX functions as a membrane transporter for extrusion of Ca^{2+} outside of the cardiomyocytes, at the same time transferring three Na^+ into the cell [114]. Activity of Na^+/Ca^{2+} exchanger greatly depends on intracellular Na^+ concentration, and thus Na^+/K^+-ATPase activity: even a slightly elevated Na^+ concentration limits the function of NCX which extrudes less Ca^{2+}, resulting in higher intracellular Ca^{2+} concentration. Cardiac glycosides, which are used in the treatment of heart failure, act as Na^+/K^+-ATPase inhibitors by the aforementioned mechanism to evince their inotropic effect [115].

Na^+/K^+-ATPase is composed of three subunits: α, β and γ-subunit. Na^+/K^+-ATPase α-subunit contains the binding sites for ions (Na^+ and K^+), ATP and cardiac glycosides, and also has catalytic ability for ATP molecule. Smaller, β-subunit is important for the transport of synthesized Na^+/K^+-ATPase to the plasma membrane, while it also modulates ATPase activity [29]. Na^+/K^+-ATPase γ-subunit (also called FXYD) is the latest subunit discovered. FXYD represents a family of proteins associated with Na^+/K^+-ATPase which modulates the function of this enzyme [26]. Cardiomyocytes contain only one form of FXYD protein called phospholemman, which regulates the function of Na^+/K^+-ATPase. Namely, in unphosphorylated state, phospholemman inhibits Na^+/K^+-ATPase by reducing the affinity for intracellular Na^+, while phosphorylated phospholemman disinhibits Na^+/K^+-ATPase, returning it to its active state [116, 117].

Cardiomyocytes contain three α (α1–α3) and two β (β1–β2) isoforms of Na^+/K^+-ATPase subunits. Measurement showed that Na^+/K^+-ATPase α1-subunit is predominantly present in cardiomyocytes, while Na^+/K^+-ATPase α2- and α3-subunits are expressed to a lesser extent [118]. Early measurements of Na^+/K^+-ATPase quantity showed that the concentration of Na^+/K^+-pump in normal human ventricular myocardium was approximately 700 pmol/g wet weight [119]. Heart failure is characterized by the elevation of Na^+ concentration in human cardiomyocytes but also in animal models [120, 121]. There are two possible explanations for this finding: reduced Na^+ extrusion (which implies changes in Na^+/K^+-ATPase) or larger Na^+ influx (implying changes in other Na-pumps, such as Na^+/H^+ exchanger) [122].

Different studies have shown that the reduced Na^+ extrusion could be the result of Na^+/K^+-ATPase alterations [20, 34]. It was demonstrated that the expression of certain Na^+/K^+-ATPase subunits was diminished in cardiomyocytes obtained from human heart failure material. Particularly, α1-subunit expression was found to be lower by 38 %, α3-subunit by 30 %, β1-subunit by 30 % and overall Na^+/K^+-ATPase activity was lower by 42 % in heart failure cardiomyocytes, with unchanged levels of mRNA [20, 34].

Animal heart failure models showed different results when observing expression and activity of Na^+/K^+-ATPase subunits. Rabbit heart failure model pointed to similar results as human heart failure cardiomyocytes, with all α-subunits having lower protein expression [123], whereas in rat heart failure model Na^+/K^+-ATPase α1-isoform expression was unchanged, with α2-subunit expression reduced and α3-subunit expression increased [124]. Different structural or functional mechanisms underlie the lower Na^+/K^+-pump activity. Therefore, heart failure in rabbit was characterized only by reduced Na^+/K^+-pump expression with normal Na^+/K^+-ATPase pumping

ability [125], while certain rat heart failure models showed decreased Na^+/K^+-ATPase pumping ability with normal Na^+ affinity [124].

Nevertheless, it is still unclear whether Na^+/K^+-ATPase dysfunction is one of the reasons for heart failure, or perhaps compensatory mechanism functioning similarly to cardiac glycosides. In their animal model of heart failure, Zahler at al. [126] indicated that it is more probable that Na^+/K^+-ATPase expression is reduced during early development of myocardial dysfunction and asymptomatic phase of heart failure, and not being its compensatory mechanism. Since heart failure is a condition principally characterized by reduced left ventricle ejection fraction (LVEF), Norgaard et al. [35] showed correlation between decreased LVEF and decreased Na^+/K^+-ATPase concentration in cardiomyocytes.

Cardiac glycosides have been in use for the treatment of heart failure for more than two centuries. Due to serious toxic effect and narrow therapeutic window, number of indications for the use of cardiac glycosides has been minimized. In patients with heart failure and atrial fibrillation, cardiac glycosides are recommended for the control of ventricular rate in patients intolerable to beta-blocker therapy [127]. Cardiac glycosides, such as digoxin, exert their positive inotropic effect by inhibiting Na^+/K^+-ATPase and increasing Na^+ intracellular concentration, which in turn stimulates Na^+/Ca^{2+} exchanger to transport Ca^{2+} inside the cell, elevating Ca^{2+} availability for muscle contraction and thus improving cardiomyocyte contraction force [128].

During therapeutic digitalization, not all Na^+/K^+-ATPase receptors are occupied by digoxin; it is estimated that the percentage of occupied Na^+/K^+-ATPase receptors is 24–35 % in the human heart [119, 129]. Cardiac glycosides bind to Na^+/K^+-ATPase α-subunit consequently blocking this enzyme. It was generally believed that cardiac glycosides had similar affinity for different Na^+/K^+-ATPase α-subunits. However, studies comparing ouabain's affinity for Na^+/K^+-pump showed that its affinity for α2β1-isoform was two times greater than for α1β1- or α3β1-isoforms [130, 131]. Moreover, these studies showed different Na^+ affinity for Na^+/K^+-ATPase isoforms; in one study Na^+ affinity was similar for all enzyme isoforms [131], while the other study showed that the highest affinity was for α1β1-isoform and the lowest for α3β1 Na^+/K^+-ATPase heterodimer [130].

Cardiac glycosides may have additional useful roles in the treatment of heart failure, which do not include Na^+/K^+-ATPase inhibition, yet their neurohumoral effects. Except inhibiting Na^+/K^+-ATPase in cardiomyocytes, cardiac glycosides also inhibit extracardiac Na^+/K^+-ATPase, thus possibly improving overall hemodynamic and restoring bar receptor activity in heart failure patients [132, 133]. Also, in recent years it has been proposed that cardiac glycosides may have hormone-like functions, by acting on different Na^+/K^+-ATPase isoforms which in that case may have receptor-like functions [134]. Such presumption has been supported by the evidence of potential antitumorous effect of cardiac glycosides [135]. Also, it has been shown that cardiac glycosides act by inhibiting cell growth and division, stimulate programmed cell death and release endothelin-1 from endothelial cells [134].

During the ongoing search for new effective, well tolerable and less toxic inotropic drug, an antibody was developed against the L7/8 extracellular domain of the Na^+/K^+-ATPase α-subunit. This antibody showed not only the inhibition of

Na^+/K^+-pump, but also several cardioprotective effects against ischemia and reperfusion through PI3K/Akt signaling cascade [136]. This finding may open new therapeutic approach in heart failure patients, and also shed a new light on Na^+/K^+-ATPase physiologic function.

8 Downregulation of SERCA in Heart Failure

One of the key abnormalities in both human heart failure and experimental models of heart failure is abnormal intracellular calcium ion (Ca^{2+}) handling. Before further consideration, we will summarize briefly facts on SERCA function.

SERCA plays a pivotal role in both myocardial contraction and relaxation. The predominant SERCA isoform in the heart is SERCA2a (97.5 %), although low levels of SERCA2b (2.5 %) are also found [137].

SERCA functions to pump Ca^{2+} into the sarcoplasmic reticulum (SR). It returns the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to resting levels causing relaxation of the cardiomyocyte and replenishment of the SR Ca^{2+} store for the next cycle [138]. Alterations in SERCA level affect Ca^{2+} homeostasis and cardiac contractility by influencing Ca^{2+} reuptake as well as the expression and activity of other Ca^{2+} handling proteins [139].

The reduced function of SERCA in heart failure is caused by reduced synthesis, activity or gene expression. Reduced SERCA-to-phospholamban ratio has the same effect. Phospholamban as a major regulator of SERCA2a activity is directly involved in development of cardiac disease, including heart failure [140].

SERCA2 activity is reversibly regulated by phospholamban through cAMP dependent phosphorylation [137, 140–143]. Dephosphorylated phospholamban is an inhibitor of SERCA, while phosphorylation of phospholamban relieves this inhibition.

Phospholamban activity, itself, is regulated by two phosphoproteins, the inhibitor-1 of protein phosphatase 1 (PP1) and the small heat shock protein 20. PKA and PKC are molecules actively involved in phosphorylation of phospholamban as well. In general, the whole Ca^{2+} transporting machinery including L-type Ca channels and the ryanodine receptor (RyR) is under control of PKA and PKC [49, 53].

Recently, two new molecules have been identified as regulators of SERCA activity: SUMO, S100 and the histidine-rich Ca^{2+}-binding protein [140] and regucalcin [108]. It is also established that previously described Ca^{2+} sparks or precisely controlled diastolic sparks are essential for the normal balance of SERCA activity [82, 83].

On experimental heart failure model, it was established that out of six isoforms of SERCA expressed in heart, there is decreased expression of transcripts of SERCA2a, SERCA3b and 3c, while the expression of SERCA2b and 3a transcripts remains unchanged. Although SERCA2a is downregulated in heart failure, it is still predominant isoform in cardiomyocytes.

In heart failure in humans, there is a reduction of SERCA2a and an increase of SERCA3f. SERCA3f is proved to induce protein synthesis anomalies, endoplasmic reticulum stress, and apoptosis in cell cultures [60, 61].

Disturbances in the regulatory function of SERCA/phospholamban have also been implicated as important contributors to heart failure pathogenesis. Phospholamban is less phosphorylated in heart failure due to increase in global phosphatase expression in cardiomyocytes of patients with heart failure, resulting in even greater SERCA inhibition.

Interesting also is the observation that functional PLB-null genotype in humans causes heart failure, whereas the gene-targeted PLB-knockout mice have no apparent cardiac problems [144].

Molecules that control phospholamban activity are implicated in heart failure as well. Heart failure and downregulation of adrenergic receptors are associated with reduced cAMP-dependent protein kinase (PKA). This mechanism leads to the inactivation of inhibitor-1 with consequently increased activity of PP1. Hyperactive PP1 leads to the dephosphorylation of phospholamban and inhibition of SERCA2a thus reducing the calcium uptake [145].

Inhibition of SERCA activity leads to profound disturbance in concentration of calcium ions which along with other molecular mechanisms of heart failure described previously deepen the morphological and physiological disturbances. In concordance with this is the finding that preserved SERCA activity and controlled calcium ions turnover inhibits calcineurin associated apoptotic pathway [61].

8.1 Potentials for New Therapeutic Approaches

Kranias et al. recognized SERCA/phospholamban complex and its role in cardiac contractility, and indentified the potential for new therapeutic approach by targeting this complex [140].

Normalization of SERCA2a function has been shown to increase contractility in failing human cardiomyocytes and to improve hemodynamics along with survival in rodent and large animal models of heart failure [146–148].

The overexpression of SERCA2a has also been found to restore energetic supply and to decrease ventricular arrhythmias in a model of ischemia/reperfusion injury [149–151]. Therefore, SERCA2a is one of the most promising targets for the treatment of HF.

Ferrandi et al. [152] showed that istaroxime represents the first example of a small molecule that exerts a luso-inotropic effect in the failing human heart through the stimulation of SERCA2a activity and the enhancement of Ca^{2+} uptake into the SR by relieving the phospholamban inhibitory effect on SERCA2a in a cAMP/PKA independent way. We previously mentioned that istaroxime inhibits Na^+/K^+-ATPase with positive inotropic effect simultaneously with the activation of SERCA2a [58]. In such a way, it brings together positive properties of Na^+/K^+-ATPase inhibition with better control of calcium ions concentration. The consequence of this potential

therapeutic strategy is achievement of enhanced contractility (inotropy) with facilitated relaxation (lusotropy).

Advances in the understanding of the molecular basis of myocardial dysfunction together with the evolution of gene transfer technology has placed congestive heart failure as a separate task within reach of gene-based therapy [61, 153–155]. Lipskaia et al. focused on gene therapy using SERCA2a or molecules regulating SERCA2a activity to treat heart failure. New data show that SERCA2a gene transfer improves contractile function and restores electric stability of the failing cardiomyocytes [156].

9 Downregulation of SERCA in Aging Heart

Aging is associated with alteration in cardiac structure and function, while the most prominent feature is increased left ventricular mass (left ventricular hypertrophy), impaired diastolic function and preservation of systolic function [157]. The characteristic cellular changes in aging myocardium include myocyte hypertrophy, interstitial fibrosis, and impaired myocyte relaxation. Many studies suggest that calcium dysregulation contributes to impaired function of cardiomyocytes in the aging process [158–166]. The intracellular calcium transient is regulated by a family of proteins including sarcoplasmic reticulum (SR) calcium ATPase (SERCA), its inhibitory protein phospholamban (PLB), the calcium storage protein, calsequestrin, and the SR calcium release channel (ryanodine receptor) [159]. SERCA plays a particularly important role in maintaining intracellular calcium through its ability to pump cytosolic calcium into SR during myocardial relaxation [166]. Several studies have shown that SERCA activity is decreased in aging heart [159, 165]. In some cases, this decrease in activity has been related to a decrease in SERCA protein level or a decrease in the ratio of SERCA to PLB [159]. Other studies have also demonstrated age-associated decreases in the amount of calcium/calmodulin-dependent protein kinase (CaMK), endogenous CaMK-mediated phosphorylation of SERCA and PLB, and the phosphorylation-dependent stimulation of SR calcium sequestration [159]. Besides changes in amounts and isoforms of calcium regulation proteins in heart tissue, recent studies suggest that alterations in the function of SERCA can be regulated by means of oxidative posttranslational modifications [167, 168]. It is well known that reactive oxygen species (ROS) and oxidative stress are increased in aging myocardium [169–171].

In cardiac myocytes in vitro, it has been shown that oxidants (e.g., nitroxyl or peroxynitrite) in low, "physiologic" levels cause reversible S-glutathiolation of SERCA at cysteine 674 (C674) leading to its activation. In contrast, higher levels of oxidants (e.g., H_2O_2 or peroxynitrite) that may be associated with pathologic conditions lead to irreversible oxidation of SERCA at one or more sites, including sulfonation at C674. Irreversible oxidation of C674 may inhibit basal enzyme activity and further prevent activation via S-glutathiolation. Studies in aging myocardium have further demonstrated irreversible oxidation of SERCA cysteines and nitration of tyrosines [172]. It was also observed that myocardial levels of 3-nitrotyrosine and

4-HNE indicative of oxidative stress and sulfonation of SERCA at C674 are markedly increased in aging hearts and that these increases are prevented in transgenic mice with catalase overexpression. Furthermore, catalase overexpression prevents decreased SERCA activity, and impaired diastolic function in myocytes from aging hearts [172, 173]. These studies suggest that reactive oxygen species such as H_2O_2 contribute to impaired diastolic function in cardiac aging, at least in part via oxidative modification of SERCA, and in particular, via sulfonation at C674 [172–174]. The recent study in male animal aging model indicates that aging reduces cell shortening, which is associated with a decrease in the amplitude of the systolic Ca^{2+} transient. This may be occurring due to a decrease in peak L-type Ca^{2+} current. The same study has shown that SR Ca^{2+} load appears to be maintained during normal aging but evidence suggests that SR function is disrupted, such that the rate of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-mediated Ca^{2+} removal is reduced and the properties of SR Ca^{2+} release in terms of Ca^{2+} sparks are altered [175]. Besides, it was shown that there are male–female differences in the way the heart ages at the cellular level. The data on aging and gender-base differences have important clinical implications. Although aging is a physiological process, many of its aspects, including alterations in Ca^{2+} homeostasis, make the myocardium prone to disease [175].

Improved comprehension of cellular mechanisms of aging will help us to understand susceptibility to different cardiovascular diseases during aging. Ultimately, we will be able to identify new targets for intervention in the treatment of these diseases.

10 ATP-ases and Cardiomyopathy

By definition, cardiomyopathy is a primary, intrinsic defect of the myocardium. Before the advance in genetics, cardiomyopathies were mostly characterized as idiopathic. Due to progress in molecular biology techniques, numerous gene abnormalities were identified as specific causes of cardiomyopathies.

Ischemic cardiomyopathy is a term used in clinical practice to describe clinical and morphological findings in patients with heart failure caused by ischemic heart disease and by strict definition it should not be a part of cardiomyopathy entity.

Cardiomyopathies are classified as dilated, hypertrophic, and restrictive. Characteristic of dilated cardiomyopathy is progressive cardiac dilatation and systolic dysfunction, while hypertrophic cardiomyopathy is defined by myocardial hypertrophy and mostly diastolic dysfunction [111].

Ischemic heart disease and consequent heart failure resemble dilated cardiomyopathy, while hypertensive heart disease bears a resemblance to dilated or hypertrophic cardiomyopathy depending on a phase in the disease progression.

There are changes in ATPases activities in different forms of cardiomyopathy. As showed in the study of Norgaard et al., among 24 patients with idiopathic dilated cardiomyopathy, 19 had impaired LV function and lower Na^+/K^+-ATPase concentration whereas 5 patients had normal LV function and higher Na^+/K^+-ATPase concentrate [35].

Table 7.1 Summary of studies dealing with SERCA2a, RyR, and phospholamban in diabetic cardiomyopathy

	SERCA2a	RyR	Phospholamban	References
Protein content	No changes	Decrease	Decrease	[179–181]
mRNA	Decrease	Decrease	No changes	[179]
	No changes	Decrease	Increase	[180]

Results of studies suggest decrease in regulatory molecules of SERCA2a

In previous discussion we interpreted results applicable mostly on ischemic cardiomyopathy. Semb et al. studied expression of Na^+/K^+-ATPase subunits in the post-infraction rat model of congestive heart failure (CHF) [176] and found that expression of α2 and α3 isoforms was affected whereas expression of the α1- and β1-subunits (mRNA and protein) was not significantly different than in controls. Expression of α2 isoform at the level of mRNA and protein were lower in CHF hearts and the α3 isoform mRNA was higher.

We will review changes of ATPases expression and activity in cardiomyocytes in *diabetes mellitus*. Diabetic cardiomyopathy is the term used for cytological and pathological cardiac alterations developed through the course of diabetes, with clinical manifestations. Myocardium is affected due to effects of several mechanisms, including metabolic disturbances: hyperglycemia and advanced glycation end products (AGEs) generation, as well as prolonged activation of PKC. Enhanced atherosclerosis, especially of coronary arteries, and diabetic microangiopathy provoke further ischemic injury of cardiomyocytes [177].

Overall analysis of the available literature showed that ionic balance due to activity of Na^+/K^+-ATPase and SERCA is disturbed in cardiomyocytes during diabetic cardiomyopathy. At the level of sarcoplasmic reticulum, there is a deficiency of both Ca^{2+} release as well as Ca^{2+} uptake [178]. SERCA2a, RyR and phospholamban molecules are equally affected and although there are some discrepancies among different studies (Table 7.1), conclusions suggest disruption of Ca^{2+} transport and increase of intracellular Ca^{2+} [179–181]. Phosphorylation of phospholamban is also reduced mostly due to activity of protein phosphatase 1 [182]. The changes of SERCA2a and its regulatory proteins are associated with Na^+/K^+-ATPase activity decrease along with increased activity of Na^+/H^+ and Na^+/Ca^{2+} exchanger as explained in previous sections. Complex cell injury ensues with deepening of metabolic disturbances and increased intracellular Ca^{2+}. One of the consequences is activation of calpain induced apoptosis of cardiomyocytes as previously referred.

11 Conclusions

Na^+/K^+-ATPase and SERCA insufficiency and their interrelation with multiple intracellular functions start the cascade of events that represent almost a universal model of injury associated with heart failure of different origin [90, 183]. The analysis of Na^+/K^+-ATPase and SERCA is even more complex because they are direct targets

for the orthodox therapy of heart failure, as well as for new therapeutical strategies [136, 152, 156]. That is why it is of essential importance to know their roles in all aspects of normal and pathological functioning of cardiomyocytes, to be able to understand future findings yet to come.

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Chapter 8
Understanding the Dysfunction of Na^+/K^+-ATPase in Rapid-Onset Dystonia Parkinsonism and Amyotrophic Lateral Sclerosis

Biswarup Ghosh, Angelo Lepore, and George M. Smith

Abstract The Na^+/K^+-ATPase is a membrane bound P-type ATPase that exchanges Na^+ and K^+ ions across the plasma membrane in expense of ATP hydrolysis. It maintains for electrical excitability, neurotransmitter transport, volume regulation, and other vital cellular functions. The four isoforms of the Na^+/K+-ATPase α-subunit have a cell-specific and developmentally regulated expression pattern. There are different isoforms of the Na^+/K+-ATPase expressed in neurons and glial cells. Alterations of Na^+/K^+-ATPase activity due to global loss or isoform specific mutational effect are evident in different neurological disorders. Mutations in the ATP1A3 gene which encodes Na^+/K^+-ATPase α3 subunits, cause Rapid-Onset of Dystonia Parkinsonism (RDP), a rare movement disorder characterized by sudden onset of dystonic spasms and slowness of movement. It is evident that Na^+/K^+-ATPase α2 subunits are upregulated in spinal cord of sporadic and familial amyotrophic lateral sclerosis (ALS) patients. The α2-Na^+/K^+-ATPases are also enriched in astrocytes expressing mutant superoxide dismutase 1 (SOD1), which causes familial ALS. Here we focused to understand the physiological and molecular fundamentals associated with dysfunction of Na^+/K^+-ATPase in RDP and ALS.

Keywords Na^+/K^+-ATPase • Isoforms • RDP • ALS

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1 Introduction

In 1997, the Nobel Prize in Chemistry was shared by Danish researcher Jens C Skou for his discovery of the Na^+/K^+-ATPase. Skou was the first to suggest, in 1957, a link between transport of Na^+ and K^+ across the plasma membrane and a Na^+ and K^+ activated ATPase activity [1]. The basic function of Na^+/K^+-ATPase, or Na^+ pump, is to maintain the high Na^+ and K^+ gradients across the plasma membrane of animal cells. The enzyme pumps $3Na^+$ and $2K^+$ ions against their concentration gradient, at the expense of an ATP molecule [2]. The Na^+/K^+-ATPase maintains the $Na^+$and K^+ gradient that is of fundamental importance for maintenance of neuronal excitability and conduction of the action potential and for secondary transport systems involved in synaptic uptake of neurotransmitters and regulation of cell volume, pH, and calcium (Ca^{2+}) concentrations. Mitochondrial dysfunction due to hypoxia, ischemia, inflammation, and other conditions reduces ATP availability and impairs Na^+/K^+-ATPase activity triggering neuronal depolarization, cytotoxic edema, and neuronal, axonal, and glial injury. The Na^+/K^+-ATPase forms macromolecular complexes with other membrane proteins and also triggers intracellular transduction signals involved in synaptic plasticity. There are different isoforms of the Na^+/K^+-ATPase expressed in neurons and glial cells [3, 4]. The elucidation of the crystal structure for the Na^+/K^+-ATPase catalytic subunit and the functional consequences of specific mutations led to identification of specific amino acid sequences critical for normal function of the enzyme. Mutations affecting the Na^+/K^+-ATPase α2 subunit which is expressed in astrocytes, is linked to familial hemiplegic migraine type 2 (FHM2); mutations affecting Na^+/K^+-ATPase α3 subunit, which is expressed in neurons, are linked to Rapid-Onset Dystonia and Parkinsonism (RDP) [5]. Impaired activity of Na^+/K^+-ATPase α2 subunit is also associated with amyotrophic lateral sclerosis (ALS) patients [6, 7].

2 Subunits of Na^+/K^+-ATPase

The Na^+ pump molecule is a heterooligomer composed of α and β subunits and both of the subunits are required for enzymatic activity. Four isoforms of the α-subunit (α1, α2, α3, and α4) [8–12] and three isoforms of the β subunit (β1, β2, and β3) [13–15] are encoded by different genes in vertebrates. The α subunits are composed of ~1018 residue (~110 kDa). The β subunits are smaller compared to α, consisting of ~300 residues (~55 kDa). Alpha is the catalytic subunit that contains the binding sites for cardiac glycosides, ions, and ATP and the transient phosphorylation site (an aspartate residue, D369) [16] In mammalian, each of the 4 alpha subunits are independently expressed under the control by its own promoter, which drives expression in a cell and tissue specific manner. The α1 subunit seems to be ubiquitously expressed and found in all tissues investigated so far [17]. Alternative splicing of the α1 subunit results in the polypeptide, α-1 T in canine vascular smooth muscle cells [18]. Whether this truncated form functions in vivo remains to be determined. The α2 isoform is expressed in skeletal muscle, adipocytes and brain, and in small

Table 8.1 Human Na^+/K^+-ATPase isoforms (Data from the genomic database of National Centre for Biotechnological Information)

Isoform	Gene	Human chromosome	Locus	Specific expression
α_1	ATP1A1	1 p13-11	476	Constitutive, ubiquitous, dominant in epithelia of kidney, intestine, and glands
α_2	ATP1A2	1 q21-23		Muscle, heart, brain
α_3	ATP1A3	19q12-13.1		CNS, brain
α_4	ATP1A4			Testis, spermatozoa
β_1	ATP1B1	1 q22-25	481	Ubiquitous, like α1 subunit
β_2	ATP1B2	17p	482	Muscle, adhesion molecule of glial cells (AMOG) in brain
β_3	ATP1B3	3 q22-23	483	Mostly in neural tissue
γ	ATP1G	11q23		Kidney

amounts in heart [19]. The α3 isoform is found mainly in nerves and brain but also in heart tissue [19]. The α4 isoform is found only in testis. Across species the degree of homology for the α1 and α2 isoforms is ~92 % and is over 95 % for α3 [9]. There is also a high degree of homology (87 %) among the α1, α2, and α3 isoforms [12].

The sensitivity of Na^+/K^+-ATPase for Na^+ and K^+ cations is known to change depending on the isoform of the β subunit included in the α–β complex [20] because the β subunit affects the activation of Na^+/K^+-ATPase based on the extracellular K^+ concentration [21]. The β subunit, which does not participate in the process of catalysis directly is nevertheless tightly bound to the α-subunit and required for the delivery of both subunits to the plasma membrane and insertion of the catalytic subunit [22]. Regulation of gene expression for each isoform and formation of various combinations of α–β complexes are tissue specific [23]. Synthesis of both corresponding mRNAs and the isoforms encoded by these mRNAs is under the control of various hormones [24, 25]. The summary of gene expression for each isoform is given in Table 8.1.

The Na^+/K^+-ATPase is often associated with a tissue-specific regulatory subunit that belongs to a protein family containing the FXYD (phenylalanine-X-tyrosine-aspartate) amino acid sequence [26, 27]. The FXYD component modulates the affinity of the enzyme for Na^+ and K^+ and is an important target for phosphorylation [28]. For example, FXYD1 interacts with α1 isoforms in the skeletal muscle and heart and with α2 (glial) and α3(neuronal) isoforms in the cerebellum; FXYD7, which is exclusively expressed in the brain, interacts with α1 isoforms in neurons and glial cells.

3 Physiological Relevance of Na^+ Pump Isozymes in Glial and Neuronal Cells

The unique expression, function, and regulation of the Na^+ pump isozymes strongly suggest their physiologic importance. There are 3 isoforms (α1, α2, and α3) and three β isoforms (β1, β2, and β3) in the central nervous system [12, 25] which have

different kinetic properties and regulation [12]. Generally, neurons predominantly express the α3 isoform and astrocytes predominantly express the α2 isoform, whereas both neurons and glia can express the α1 isoform. Some large neurons within the dorsal horn of the spinal cord express both α2 and α3 isoforms, whereas in the ventral horn some motor neurons express α1 and α3 isoforms, while the rest just express the α3 isoform [29–31]. The most striking is the presence of apparent complementary difference in predominant isoforms between dorsal and ventral horn within spinal cord.

In neurons the rapid changes in Na^+ and K^+ concentrations accompanying nerve activity may require the fast adjustments provided by isozymes different from α1β1. This may be the role of the neuronal α3-containing isozymes. Because of the low apparent affinity of α3 for Na^+, this isoform operates at slow rates in cells at rest. When the ion gradients are dissipated after depolarization, intracellular Na^+ levels increase and α3 becomes activated. In this manner, isozymes composed of α3 function as spare pumps to help the ubiquitous α1β pumps restore the resting membrane potential of the cells. Also, the high affinity of α3 for ATP allows it to function at the low nucleotide concentrations occurring near the cell membrane during intense neuronal activity.

The properties of α2 suggest that it is important for the function of glial and neuronal cells. The high affinity for ATP and Na^+ provides α2 with a steady working capability. This allows the isoform to effectively clear higher K^+ concentrations within the extracellular space after depolarization, even at decreased cytosolic concentrations of Na^+ and ATP. The α2 is important in preventing K^+-induced depolarization to maintain neuronal excitability. The role of α2 has been shown in mice in which the expression of the isoform was knocked out. These mice exhibited akinesia and died soon after birth because of irregular breathing caused by an abnormal rhythmic firing of the neurons of the respiratory center [32]. Another important role of α2 derives from its ability to regulate intracellular Ca^{2+} levels. This has been shown by gene targeting studies in excitable tissues of mice.

The hypercontractility induced by α2 in the heterozygous knockout animals [33] has been attributed to larger cellular Ca^{2+} transients, caused primarily by increasing in cytoplasmic Na^+ levels, which consequently augments Ca^{2+} levels via the Na^+/Ca^{2+} exchanger. In the α2 deficient mice, this mechanism is supported by the abnormally increased intracellular Ca^{2+} levels in astrocytes from these animals [34, 35]. Interestingly, Juhaszova and Blaustein [19] found that in glial cells α2 colocalizes with the Na^+/Ca^{2+} exchanger and the underlying endoplasmic reticulum, suggesting that these components act as a functional unit to regulate cytoplasmic Na^+ and Ca^{2+}. In this manner, regulation of cations can be limited to microdomains preventing global cell ionic changes. Beyond the requirement of the β subunits for Na^+/K^+-ATPase maturation and modulation of activity, an unforeseen function has been reported for the β2 isoform. This isoform is present in glia and acts as a recognition molecule that mediates neuron-glia interactions important for cell adhesion, neuronal migration, and neurite outgrowth. These properties for the β2 isoform are supported by observations uncoordinated motor function, tremors and paralysis

in animals in which the β2 gene was deleted [36], and by the ability of β2 isoforms to promote cell adhesion and reduce the invasive characteristics of glioma cells [37]. Although there are many exceptions to the rule, neurons predominantly have the α3β1 isozyme and astrocytes predominantly express the α2β2 isozyme, whereas both neurons and glia can express the α1 subunit.

4 Alterations of Na^+/K^+-ATPase in Pathological Conditions

A central role for the Na^+/K^+-ATPase in pathogenesis has been widely implicated, particularly in cardiovascular, neurological, renal, and metabolic diseases [38–40]. In general, a downregulation of Na^+/K^+-ATPase is found under these conditions. The Na^+/K^+-ATPase activity was 34 % lower in ischemic cortex and 40 % lower in ischemic basal ganglia after 30 min ischemia; after 60 min ischemia, both Na^+/K^+-ATPase activity and K^+ concentration were decreased in the ischemic hemisphere [41, 42]. This is consistent with the observations that ischemia or hypoxia increase the production of ROS [43–46] and release endogenous inhibitors of Na^+/K^+-ATPase [47]. The functional subunits α2 and α3 were the ones that were mostly affected by a focal cerebral ischemia [42]. An inhibition of Na^+/K^+-ATPase secondary to cellular energy depletion might contribute to delayed membrane depolarization of cortical neurons after traumatic brain injury [48]. The Na^+/K^+-ATPase activity was reduced or insufficient to maintain ionic balances during and immediately after episodes of ischemia, hypoglycemia, epilepsy and after administration of glutamate agonists. It was proposed that a reduction and/or inhibition of Na^+/K^+-ATPase contributed to the central neuropathy found in those disorders [49]. Dysfunction or deficiency of Na^+/K^+-ATPase has been identified in chronic neurodegenerative diseases as well; for example, the α3 subunit mRNA was ~30–45 % lower in Alzheimer's brain relative to controls [50]. The possibility that a deficiency in the Na^+/K^+-ATPase activity might be a common pathogenesis of central nervous system disorders was tested in patients of CNS glioma, multiple sclerosis, systemic lupus erythromatosis and several other pathological conditions. In short, it appears factual that reduced Na^+/K^+-ATPase activity is a common event in a number of neural degenerative and metabolic diseases.

Two movement disorders are caused by different missense mutations in the ATP1A3 gene encoding the α3 subunit of Na^+/K^+-ATPase: Alternating Hemiplegia of Childhood (AHC), characterized by episodes of transient hemiplegia/hemiparesis, dystonia and choreoathetosis [51–53], and Rapid-Onset Dystonia Parkinsonism (RDP), characterized by abrupt onset of dystonia with parkinsonism after a stressful event, typically in late adolescence or early adulthood [5, 54, 55]. The pathophysiology of RDP differs from dystonia and Parkinson's disease since L-DOPA treatment [55] and deep brain stimulation of the basal ganglia [56] have little or no therapeutic effect. Poor responses to these therapies suggest that dysfunction in motor circuits outside the basal ganglia contribute to RDP symptoms.

Upregulation of Na^+/K^+-ATPase α2 subunit is evident in central nervous system of ALS patient and rodent animal model of ALS [7]. Global loss of Na^+/K^+-ATPase in CNS is also a hallmark of ALS pathology [6].

5 Rapid-Onset Dystonia Parkinsonism (RDP) and Na^+/K^+-ATPase

RDP is a hereditary form of dystonia and is characterized by the abrupt onset of slowness of movement (parkinsonism) and dystonic symptoms. Parkinsonism includes tremors, unusually slow movement (bradykinesia), rigidity, an inability to hold the body upright and balanced (postural instability), and a shuffling walk that can cause recurrent falls. Dystonia is a condition characterized by involuntary, sustained muscle contractions. RDP causes movement abnormalities that make difficulty to walk, talk, and carry out other activities of daily life. It affects the arms and legs, causing muscle cramping and spasms. Facial muscles are often affected which results in problems with speech and swallowing. The sudden onset of symptoms over hours to a few weeks, often associates with physical or emotional stress, suggests a trigger initiating a nervous system insult resulting in permanent neurologic disability. Functional studies and structural analysis of the protein suggest that these mutations impair Na^+/K^+-ATPase activity or stability. It implicates the Na^+/K^+-ATPase, a crucial protein responsible for the electrochemical gradient across the cell membrane, in dystonia and parkinsonism.

The pathophysiologic mechanisms underlying RDP are poorly understood. Nine different RDP mutations have thus far been identified in the α3 gene [55, 57–59]. With a single exception, all mutations are located in the cation binding transmembrane domains or in closely associated regions, resulting in reduced affinity for intracellular Na^+. For example the mutation replacing Asp923 by an asparagine (D923N) results in a ~ 200-fold reduction of Na^+ affinity for activation of phosphorylation from ATP, reflecting a defective interaction of the E1 form with intracellular Na^+ [60]. During the normal functional cycle, Na^+/K^+-ATPase undergo large conformational changes between E1 and E2 forms (Na^+- and K^+-forms, respectively).

To achieve a better understanding of the pathophysiology of the disease RDP, regional and cellular distribution of Na^+/K^+-ATPase α3 subunit may contribute insight. Na^+/K^+-ATPase α3 subunit is widely expressed in neuronal populations but mainly in GABAergic neurons in areas and nuclei related to movement control. In rodent brain, GABAergic neurons in all nuclei of the basal ganglia (striatum, globus pallidus, subthalamic nucleus, and substantia nigra) express high levels of the α3 subunit [61]. The basal ganglia are one of the key circuits in the brain involved in fine motor control. Several thalamic nuclei structures harboring connections to and from the cortex expressed high levels of the Na^+/K^+-ATPase α3 isoform. Other structures with high expression of Na^+/K^+-ATPase α3 included cerebellum, red nucleus, and several areas of the pons (reticulotegmental nucleus of pons). High expression of the Na^+/K^+-ATPase α3 isoform is also found in projections and cell bodies of the

hippocampus; most of these Na^+/K^+-ATPase α3-positive cell bodies colocalize to GABAergic neurons. It is interesting that Na^+/K^+-ATPase α3 expression was not significantly high in the dopaminergic cells of substantia nigra.

Physiologically, Na^+ affinity is impaired in RDP. In general, the Na^+/K^+-ATPases are ion pumps of fundamental importance in maintaining the electrochemical gradient essential for neuronal survival and function. Functional analysis demonstrated a drastic reduction in Na^+ affinity due to mutations in Na^+/K^+-ATPase α3 subunit. A reduced Na^+ affinity has been described for certain RDP mutants [62–64]. In general the ubiquitously expressed α1-subunit mediates the active transport of Na^+-K^+ in the resting membrane. During neural excitation, the α2-subunit contributes to the initial fast uptake of extracellular K^+, whereas α3 is involved in slower post-stimulus recovery [65]. In RDP, Na^+ affinity seems to be mainly impaired without significant reduction in K^+ affinity [62]. Consequently, changes in intracellular Na^+ can result in secondary changes in Ca^{2+} via the Na^+/Ca^{2+} exchange system for signaling cascades. It might be possible that disturbance of the Na^+ gradient along with the driving force for uptake of key neurotransmitters could impair dopamine uptake. However, positron emission tomography imaging in RDP compared with idiopathic Parkinson's disease shows that RDP patients do not have impairment of the dopamine re-uptake system [66]. Alternatively, Na^+/Ca^{2+} misbalance may affect other neurotransmitters and cause abnormal firing of specific neurons. Irregular and spontaneous bursts of neuronal discharges occur in the medial globus pallidus in patients with dystonia [67, 68], altering the basal ganglia output signal to the motor cortex [69]. An increased cortical motor excitability and/or disruption of basal ganglia inhibitory control due primarily to a misbalance in Na^+ concentration may result in the dystonic, bradykinetic movement disorder observed in RDP without significant involvement of the dopaminergic system.

6 Amyotrophic Lateral Sclerosis and Na^+/K^+-ATPase

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disease characterized by degeneration and death of motor neurons of the brain and the spinal cord. It is clinically characterized by progressive paralysis and eventual death from respiratory failure within 2–5 years of onset. No effective treatment is currently available beyond supportive care and riluzole, a putative glutamate release blocker linked to modestly prolonged survival. The pathology of ALS is characterized by the loss of pyramidal Betz cells in the motor cortex as well as loss and degeneration of the large anterior horn cells of the spinal cord and lower cranial motor nuclei of the brainstem [70]. The disease starts with limb weakness, often preceded by cramps, and with bulbar weakness causing dysarthria and dysphagia.

Although the majority of ALS cases are sporadic(sALS),with no family history, 10 % are familial and are caused by mutations in the superoxide dismutase1 (SOD1),TAR DNA Binding Protein (TARDBP) and Fused In Sarcoma (FUS) genes. Recent studies have identified expanded repeats in noncoding region of chromosome

9 open reading frame 72(C9orf72) as the most frequent genetic cause of ALS [71]. Mutations in SOD1 account for 20 % of familial ALS and 5 % of apparently sporadic disease. Mutations in TARDBP account for 5–10 % of familial ALS and mutations in FUS for about 5 %.

The mechanisms underlying neurodegeneration in ALS are multifactorial and operate through inter-related molecular and genetic pathways. Specifically, neurodegeneration in ALS might result from a complex interaction of glutamate excitotoxicity, generation of free radicals, cytoplasmic protein aggregates, SOD1 enzymes, combined with mitochondrial dysfunction, and disruption of axonal transport processes through accumulation of neurofilament intracellular aggregates. Mutations in TARDBP and FUS result in formation of intracellular aggregates, which are harmful to neurons. Activation of microglia results in secretion of proinflammatory cytokines, resulting in further toxicity. Ultimately, motor neuron degeneration occurs through activation of calcium-dependent enzymatic pathways. Currently it is evident that Na^+ pump dysfunction is also an important cause in ALS pathogenesis [6, 7].

The Na^+/K^+-ATPase consumes 50 % of the energy supply in the CNS [72]. Its catalytic subunit is sensitive to damage by free radicals and other oxidative stressors [73–75]. This leads the oxidized Na^+/K^+-ATPase subunit to be degraded by calpain, proteasomal, and lysosomal pathways [76, 77]. It thus may be one of the links between alterations in free radical homeostasis and ALS pathology. In other circumstances, the inhibition of Na^+/K^+-ATPase increases the sensitivity of neurons to glutamate excitotoxicity because of complementary effects on neurons (enhancing glutamate effects and Ca^{2+} accumulation) and astrocytes (reducing the driving force for Na^+-dependent glutamate clearance) [78–83, 91]. Furthermore, the free radical nitric oxide (NO) normally regulates the Na^+/K^+-ATPase via the activation of soluble guanylate cyclase and cGMP [84], a pathway that is shared by glutamate and oxygen free radicals in the CNS. This pathway forms a convergence point for the action of several intercellular and intracellular molecular messengers that have been implicated in neuronal viability under stress [85, 86]. Together, these studies suggest that either loss or excessive inhibition of Na^+/K^+-ATPase could contribute to motor neuron death via direct oxidative damage or via the enhancement of NO and other free radical effects.

SOD1 normally converts superoxide, a by-product of mitochondrial metabolism, to water and hydrogen peroxide. Simple loss of SOD1 activity has been ruled out as a cause of the disease. Global Loss of Na^+/K^+-ATPase activity or isoform content was found in a Transgenic Mouse Model of Amyotrophic Lateral Sclerosis [6]. Ouabain-sensitive Na^+/K^+-ATPase activity was decreased in transgenic mutant SOD1 mice. Losses in Na^+/K^+-ATPase activity could be attributable to enzyme inactivation, protein degradation, changes in gene expression, failure to transport newly synthesized protein to the axon, loss of neurons, or a combination of these. The loss of NO regulation in transgenic mutant SOD1 mice might have been predicted, considering the severity of the illness.

Recent research found that a protein complex of α2-Na^+/K^+-ATPase and α-adducin was enriched in astrocytes expressing mutant superoxide dismutase 1

(SOD1), which causes familial amyotrophic lateral sclerosis (ALS) [7]. The levels of α2-Na^+/K^+-ATPase is elevated in spinal cord of familial ALS patients harboring distinct SOD1 mutations as well as sporadic ALS. Knockdown of α2-Na^+/K^+-ATPase in mutant SOD1 astrocytes protects motor neurons from degeneration, including mutant SOD1 mice in vivo. Higher α2-Na^+/K^+-ATPase activity contribute to ALS pathology as heterozygous disruption of the α2-Na^+/K^+-ATPase gene suppresses degeneration in vivo and increases the lifespan of mutant SOD1 mice. The pharmacological agent digoxin, which inhibits Na^+/K^+-ATPase activity, protects motor neurons from mutant SOD1 astrocyte–induced degeneration. Upregulation of Na^+/K^+-ATPase stimulates mitochondrial respiration and expression of inflammatory genes in SOD1 mutant astrocytes which may in turn induce motor neuron degeneration. An increase in the levels and activity of Na^+/K^+-ATPase would alter the demand for cellular metabolism and ATP. Mitochondria generate the majority of the cellular ATP. Mitochondrial respiration may increase reactive oxygen species (ROS) in mutant SOD1 astrocytes, which in turn activate inflammatory factors leading to non–cell autonomous degeneration of motor neurons. Increased ROS level is evident in familial amyotrophic lateral sclerosis with mutations in SOD1 [87]. Notably, both ROS and inflammation have been linked to the pathogenesis of ALS [88, 89].

The levels of Na^+/K^+-ATPase isoforms have been reported to be downregulated in mutant SOD1 spinal cord [6]. However, α2-Na^+/K^+-ATPase was upregulated in the spinal cord of symptomatic mutant SOD1 mice and this upregulation occurred specifically in glia in these mice [7]. Interestingly, the levels of α2-Na^+/K^+-ATPase were elevated in spinal cord of familial ALS patients harboring distinct SOD1 mutations as well as sporadic ALS. The finding that chronic activation of α2-Na^+/K^+-ATPase in astrocytes is critical for neurodegeneration suggests that α2-Na^+/K^+-ATPase might represent an attractive target for the identification of new therapies for neurodegenerative diseases. It was found that the therapeutic drug digoxin, which is widely used to treat heart failure [90], protects motor neurons against degeneration [7]. Cardiac glycosides appear to be neuroprotective in models of ischemic stroke, prevent polyglutamine-induced cell death, and inhibit SOD1 and TDP-43 aggregation in cells [91–94]. The outcomes of Na^+/K^+-ATPase inhibitors in the treatment of heart disease should prove useful in the development of inhibitors selective for the α2 isoform of Na^+/K^+-ATPase in glial-dependent neurodegeneration.

7 Conclusions

Different neurological disorders are associated with loss of Na^+/K^+-ATPase activity or isoform specific mutational changes. Mutations in Na^+/K^+-ATPase α3 subunit are associated with Dystonia Parkinsonism. On the other hand, Na^+/K^+-ATPase α2 subunit is upregulated in spinal cord of sporadic and familial amyotrophic lateral sclerosis.

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Chapter 9
Activity of Membrane ATPases in Human Erythrocytes Under the Influence of Highly Hydroxylated Fullerenol

Anita Krokosz and Jacek Grebowski

Abstract Incubation of erythrocyte membranes with highly hydroxylated fullerenol $C60(OH)_x$, $x > 30$ led to decreases in Na,K-ATPase, Ca-ATPase and Mg-ATPase activities.

The inhibition of the activities of erythrocyte ATPases caused by fullerenol could be the result of its direct and/or indirect (via membrane fluidity changes or influencing other erythrocyte proteins) interaction with the enzymes. Fullerenol affected also cytoskeletal transmembrane proteins, particularly the band 3 protein. Despite the inhibition of Na,K-ATPase by fullerenol, an increase in potassium ion leakage was not observed. Blocking the leakage can result from physical "blockages" of potassium channels by fullerenol molecules.

In the intact human erythrocytes fullerenol can associate mostly with the surface of the plasma membrane; however, it could also migrate deeper inside the membrane increasing its fluidity. Fullerenol has more than 30 -OH groups on the surface, and therefore can interact with the functional groups of amino acids of membrane proteins and the heads of membrane phospholipids via hydrogen bonds. Both, fullerenol and plasma membrane have negative overall charge, however, fullerenol can adsorb to the protein domains of the plasma membrane by van der Waals or dipolar interactions.

Keywords Fullerenol • Nanoparticles • Erythrocytes • Na • K-ATPase • Membrane proteins

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1 Introduction

Human erythrocyte Na,K-ATPase (EC 3.6.1.37) is responsible for the active transport of K^+ inside and Na^+ outside the erythrocytes. Human erythrocyte comprises several hundreds, between 230 and 470, of the protein molecules [1]. Na,K-ATPase is a heterodimer consisting of two α subunits (1020 amino acids, 112 kDa) and two β subunits (302 amino acids, 55kD). The α subunits are the catalytic subunits of the enzyme. They penetrate from 7 to 8 times the lipid bilayer and have binding sites modifying transporter activity. The β subunits only once pass through the membrane lipid bilayer and are highly glycosylated. The β subunits are crucial for the appropriate location of the enzyme in the membrane and for its proper functioning, as they regulate the activity of the α subunits [2]. Probably the β subunits are responsible for maintaining the proper conformation of the α subunits in the membrane and their stabilization. The ATPase protein is phosphorylated by ATP in the presence of Mg^{2+} and Na^+, and then dephosphorylated in the presence of K^+. The Na,K-ATPase extrudes three Na^+ ions in exchange for two K^+ ions, hydrolyzing one molecule of ATP [3, 4].

Membrane proteins, which are responsible for enabling asymmetric concentrations of ions across the membrane play a very important role in cell functioning. Na^+ and K^+ gradients generated by Na,K-ATPase are used by gradient-driven systems to move ions across the erythrocyte plasma membrane. These systems may respond to changes in cell volume, pH or membrane integrity. The chloride content Cl^- of the human erythrocytes depend on the cation content as the Na, K, Cl cotransport is regulated by sodium and potassium gradients. Impaired Na,K-ATPase function can be linked to very severe effects [5]. This impairment in Na,K-ATPase activity in erythrocytes plays probably a role in the development of diabetic complications [6, 7] and hypertension [8–10].

2 Fullerenols

The unique physicochemical properties of nanocompounds attract a great deal of attention in recent years [11–13]. A very promising group among these is the fullerenols (Fig. 9.1). Fullerenols are water-soluble polyhydroxylated fullerenes C60 derivatives. The carbon cage of fullerenol is built of rings, each consisting of 5–6 carbon atoms, bound by coupled π bonds. Fullerenols have become a major point of interest in biomedical nanotechnology, thanks to their low toxicity compared to nano-C60 suspensions. Fullerenols could play multiple functions reviewed in Refs. [11, 12, 14].

In vivo studies showed that fullerenol can scavenge the free radicals that are massively induced during ischemia/reperfusion injury of the small intestine in dogs [15]. Fullerenol $C_{60}(OH)_{24}$ derivatives protect mice against oxidative stress in ischemia reperfused lung models [16]. Fullerenols prevent toxicity to healthy organs

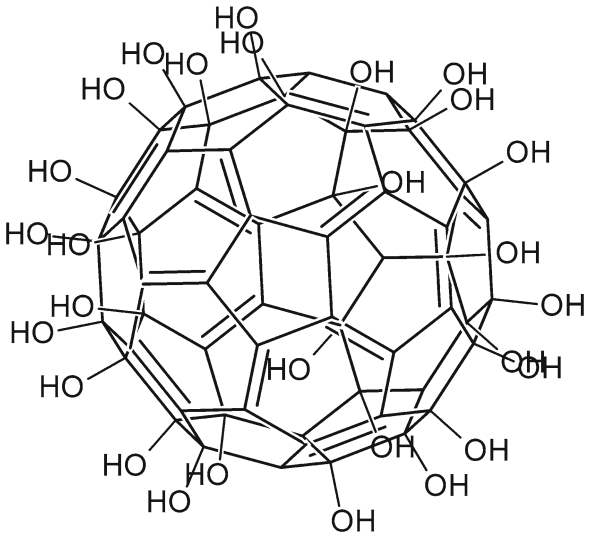

Fig. 9.1 The structure of fullerenol

during chemo and radiation therapy. They exert hepatoprotective effects in doxorubicin-induced hepatotoxicity in rats with mammary carcinomas [17], prevent acute doxorubicin induced pulmotoxicity [18] and cardiotoxicity [19] in rats with malignant neoplasm, and protect mice from lesions to multiple organs induced by ionizing radiations [11]. Their protective effects were shown to depend on the direct scavenging of ROS produced by cancer therapy [11]; the same study however reports an additional cell-protective effect, showing that fullerenols induce a significant overexpression of antiapoptotic Bcl-2 and Bcl-xL proteins as well as the transactivation of the cytoprotective GSTA4, MnSOD, NOS, CAT, and HO-1 genes [11] (with permission from [12])].

Fullerenols could act as effective free radical scavengers. High concentrations of fullerenol (0.71–0.88 mmol/L) are more effective at scavenging hydroxyl radicals (above 50 %) than DPPH (1,1-diphenyl-2-picrylhydrazyl radical) (up to 50 %). There are three possible reactions responsible for the decay of OH radicals (Fig. 9.2). Fullerenol may function as an antioxidant by the donation of the hydrogen atom, abstracted from the hydroxyl group, which was confirmed by EPR (Electron Paramagnetic Resonance) detection of a fullerenol radical $C_{60}(OH)_{23}O^{\bullet}$. The second reaction which may occur between a hydroxyl radical and fullerenol is the addition of $^{\bullet}OH$ radicals to the olefinic double bonds between carbon atoms constituting the fullerene core. The last possibility is one electron oxidation of fullerenol to radical cation. These three mechanisms are not mutually exclusive [20, 21]. Moreover, it was suggested that the most probable mechanism of the reaction of OH with fullerenol was the addition of $^{\bullet}OH$ radicals to fullerenol carbon cage via the formation of a π-complex. This complex could dissociate to reform the reactants or rearrange to the σ-complex [21]. Fullerenols are good radioprotectants; they possess double

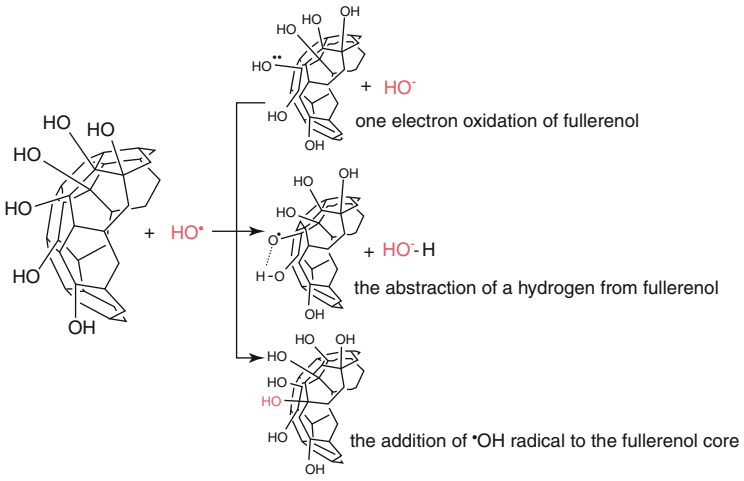

Fig. 9.2 Possible reactions between •OH radicals and $C_{60}(OH)_{36}$ fullerenol "Reproduced with permission" [21]

bonds (C=C), high electron affinity, ease of radical binding, and reactivity towards nucleophilic substituents [22]. The radioprotective properties of fullerenol have been confirmed by in vitro and in vivo studies [23–25].

It has been shown in vivo that fullerenol prevented the oxidation of glutathione (GSH), decreased lipid peroxidation (the level of MDA, malondialdehyde), and protected superoxide dismutase (SOD), which were attributed to the ability of $C_{60}(OH)_{24}$ to scavenge lipid radicals and reactive oxygen species (ROS). Moreover, fullerenol protected mitochondrial proteins against oxidation, maintained the mitochondrial membrane potential, and inhibited apoptosis induced by ionizing radiation. These data indicate that fullerenol possesses radioprotective properties; however, the protective nature of fullerenol might be concentration dependent [11, 25]. Due to the presence of hydroxyl groups on the surface of fullerenol molecules, it is possible for them to take part in many interactions, e.g. creating hydrogen bonds with biomolecules. Hydrogen bonds are relatively weak interactions compared to covalent bonds; nevertheless, they play an important role due to their frequent occurrence in biological systems and their roles in biochemistry and cell maintenance [26, 27]. The many hydroxyl groups on the surface of fullerenol molecules make it possible for them to form hydrogen bonds between different biomolecules. In effect, fullerenol can adsorb on the heads of membrane phospholipids and interact with membrane proteins, thus influencing their functions. Membrane proteins play crucial roles in maintaining plasma membrane function. They are responsible for selective transport, the shape and architecture of the cell, and signal transduction [28]. Moreover, the high aqueous solubility and neutral pH of highly hydroxylated fullerenes and their accessibility to further modification make them promising agents for drug delivery to particular locations in the cell, as well as potential anti-cancer agents to kill tumor

cells [29–31]. The hydroxyl groups increase hydrophilicity, which can be useful for delivering sparingly water-soluble or hydrophobic cytotoxic agents (with permission from [32]).

3 The Influence of Fullerenol on Erythrocyte Ion-Dependent ATPases

Membrane proteins with ATPase activity, which are responsible for enabling asymmetric concentrations of cations across the membrane at the expense of ATP hydrolysis, play a very important role in cell functioning. Among the transport ATPases worth mentioning are Na,K-ATPase, which transports Na^+ outside and K^+ ions inside the cell [3] and Ca-ATPase, which is responsible for the transport of Ca^{2+} to the extracellular space [33]. Both enzymes require Mg^{2+} ions for the active transport of cations [4]. Impaired Na,K-ATPase function can be linked to very severe effects. It has been shown that a decrease in the activity of Na,K-ATPase may result in either apoptotic or "mixed" cell death. Depleting intracellular K^+ triggers an increase in intracellular Ca^{2+}, an event perceived as a trigger for excitotoxicity leading to necrotic cell death [5]. Bearing this in mind, it is reasonable to investigate the interactions of fullerenols not only with the lipid bilayer of the plasma membrane, but also with the proteins anchored to it. It is known, for example, that the activity of ATPases can be influenced either by a direct interaction with other molecules or by changes in the fluidity of the lipid bilayer [34] (with permission from [32]).

Incubation of erythrocyte membranes with fullerenol at concentrations of 50, 100, and 150 μg/mL at 37 °C for 1 h led to a decrease in the Na^+/K^+-ATPase activity proportional to the fullerenol concentration (Fig. 9.3). Fullerenol inhibited the Na^+/K^+-ATPase activity by 51 % at 50 μg/mL of fullerenol and by 77 % at 150 μg/mL of fullerenol. The decreases in the ATPase activity were still observed when fullerenol was removed from the membrane suspensions by washing three times with PBS. In this case, the inhibition of the Na^+/K^+-ATPase activity was smaller, by about 10–14 %, in comparison with the samples containing fullerenol during the ATPase activity assessment. Analogous experiments were made for Mg^{2+}-ATPase and Ca^{2+}-ATPase. ATPases were inhibited with increasing fullerenol concentration either in the presence of fullerenol or after its removal by washing with PBS. The decrease in the activity of Ca^{2+}-ATPase after removing fullerenol was lower by about 7–8 % compared with samples in which fullerenol was present during the Ca^{2+}-ATPase activity assessment. Mg^{2+}-ATPase activity was inhibited by fullerenol to the same extent for samples either with fullerenol present or absent during the assessment (with permission from [32]).

We checked that fullerenol molecules can associate with the erythrocyte plasma membrane. After incubation of suspensions of erythrocyte membranes with different concentrations of membrane proteins (0.5, 1.0 and 1.5 mg/mL) with fullerenol (50–150 μg/mL) for 1 h at 37 °C, the amount of fullerenol bound to the membrane increased proportionally to the fullerenol concentration in the sample. The amount

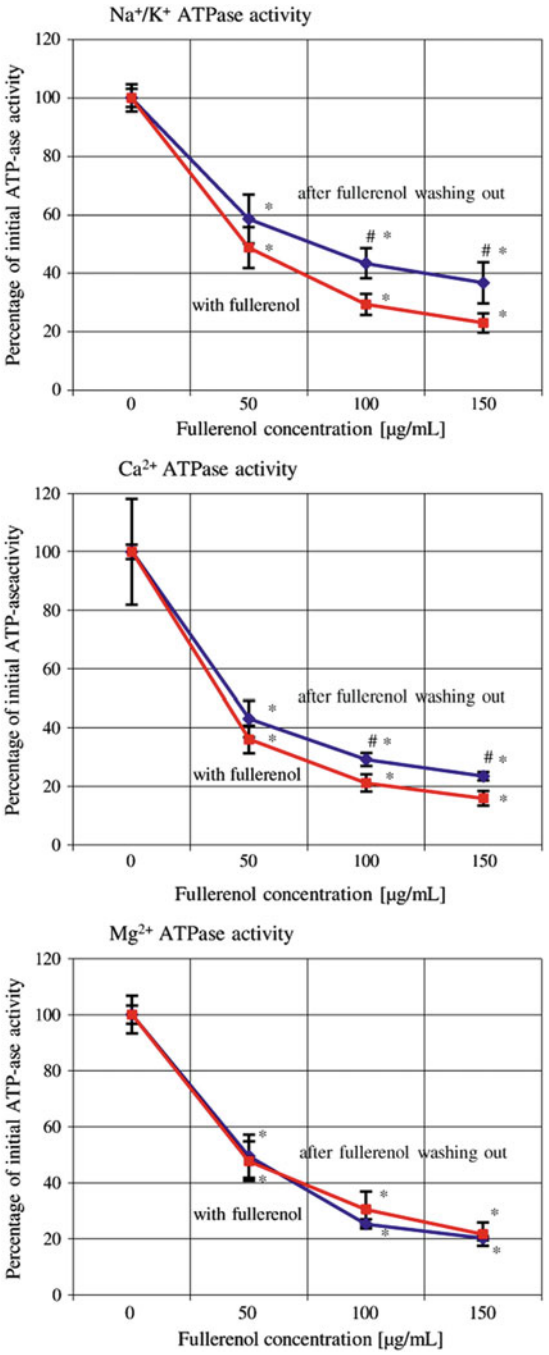

Fig. 9.3 The inhibition of erythrocyte membrane ATPases by fullerenol. Initial activities for Na,K-ATPase, Ca-ATPase, and basal Mg dependent ATPase activity, expressed as nmol Pi/(mg protein × h), were: 143.3 ± 6.6, 226.0 ± 5.6, and 115.5 ± 7.8, respectively. Results are expressed as the percentage of the initial ATPase activity with standard deviation shown as error bars. *Asterisks* are used to mark values statistically different in comparison with control (*$p < 0.001$); *hashes*—difference between samples containing fullerenol and after its removal by washing with PBS (#$p < 0.05$) "Reproduced with permission" [32]

of fullerenol incorporated into the erythrocyte membrane varied from 2.50 μg/mgMPr for 50 μg/mL of $C_{60}(OH)_{\sim 30}$ to 6.76 μg/mgMPr (MPr—membrane protein) for 150 μg/mL of fullerenol in samples in which the protein concentration was 1 mg/mL. Fullerenol also increased the fluidity of the membrane at concentrations between 50 and 150 μg/mL. The largest decrease in the value of the anisotropy coefficient of fluorescent label was observed for the hydrophilic surface of phospholipid heads providing information about the organization of the membrane at its surface. For fluorescent labels used to monitor the fluidity in the hydrophobic-hydrophilic region of the membrane and the hydrophobic region between the two leaflets of the membrane bilayer, significant changes were observed only at fullerenol concentration of 150 μg/mL. It can be assumed that fullerenol binds mostly to the surface of the membrane, but at relatively high concentrations can migrate deeper inside the membrane [32].

The leakage of potassium ions from the cells is related to disruption of active membrane transport and a result of the decrease in ATP levels, which can occur during prolonged incubation of erythrocytes [35]. As shown in our previous work, fullerenol inhibits the activity of ion-dependent ATPases as a result of both direct interaction with the enzyme and the influence of fullerenol on membrane fluidity [32]. Inhibition of Na^+,K^+-ATPase in erythrocytes causes distortion of the ion balance and leads to osmotic hemolysis [36, 37].

Despite the inhibition of Na^+,K^+-ATPase by fullerenol, an increase in hemolysis and potassium ion leakage was not observed. Blocking the leakage can result from physical "blockages" of potassium channels by fullerenol molecules. A similar effect was observed for blocking potassium ion channels by fullerene and nanotubes, where the blockage effect was dependent on the size and shape of the nanocompounds [38]. The ion channels can be flanked by the nanocompounds as the result of electrostatic interaction or plain adsorption, which influences ion exchange and, therefore, cellular metabolism (with permission from [39]).

Functional changes in Na^+/K^+-ATPase under the influence of fullerenol could be associated with two effects. The first is the interaction of fullerenol molecules with subunits of the enzyme.

Molecules of fullerenol penetrating the membrane could presumably interact with β subunits and through changes in their structure influence the action of the whole enzyme. These postulates are supported by the results of Calvaresi and Zerbetto in 2010 [40], who proved using computational methods the possibility of blocking the catalytic subunits of ATPases via interaction with fullerenols. As models they used rat Na^+/K^+-ATPase (1MO8 in PDB), which through ATP hydrolysis maintains an appropriate gradient of Na^+ and K^+ ions on both sides of the membrane, and bovine mitochondrial F1-ATPase (1EFR), participating in oxidative phosphorylation providing ATP synthesis. In both models the interaction of C60 with the enzymes can be described as the inhibition of the catalytic mechanism. Within Na^+/K^+-ATPase, C_{60} binds to the very mobile α1 subunit region delimited by Gln396-Ala416 [41, 42]. On the other hand, in F1-ATPase, C_{60} interacts with both the α and β subunits [42], hindering the rotational movement necessary for the functioning of the protein.

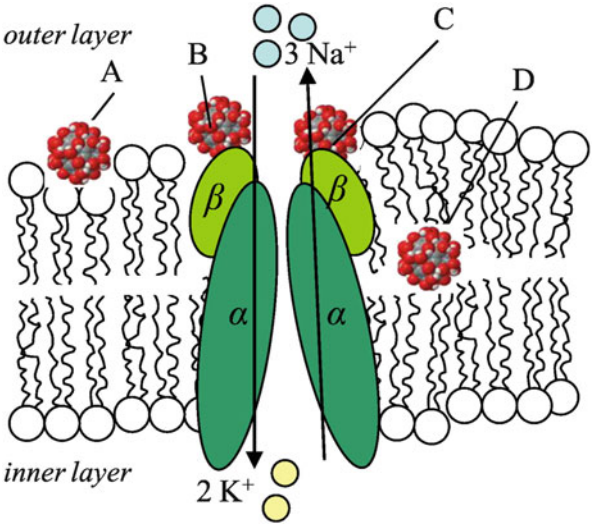

Fig. 9.4 Fullerenol interactions with Na,K-ATPase subunits spanning a lipid bilayer. (**a**) Attachment of fullerenol to the outer layer of the lipid bilayer; (**b**) fullerenol interactions with β subunits of Na^+/K^+-ATPase; (**c**) simultaneous fullerenol interactions with an enzyme and a lipid bilayer; (**d**) incorporation of fullerenol into the hydrophobic, inner region of the lipid bilayer of the plasma membrane "Reproduced with permission" [32]

Another explanation for the attenuation of Na^+/K^+-ATPase activity could be the influence of fullerenol molecules on the lipids of the plasma membrane (Fig. 9.4) and, in consequence, changes in its fluidity. Such changes have an impact on the ATPase activity [34]. For example, cholesterol is a compound playing a major role in the regulation of the structure and dynamics of the lipid bilayer. It can moderate the activities of various membrane transporters such as Ca^{2+} channels, Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase in different cells, including erythrocytes and endothelial cells [43].

Therefore, it is possible to conclude that the observed decrease in ATPase activity is a consequence of changes in the microviscosity of the erythrocyte membrane, caused by the incorporation of $C_{60}(OH)_{\sim 30}$ into its interior (with permission from [32]). In whole erythrocytes, fullerenol can indirectly interact with other cytoplasmic, peripheral and transmembrane proteins.

4 The Influence of Fullerenol on the Other Erythrocyte Membrane Proteins

In order to investigate the influence of fullerenol on the membrane proteins, erythrocyte ghosts incubated with fullerenol for 3 and 48 h at 37 °C were separated by SDS-PAGE electrophoresis in reductive and non-reductive conditions. As presented in Fig. 9.5, fractions identified after staining with Coomassie Brilliant Blue contained spectrins α and β, ankyrin, band 3 (AE1), 4.1 (EPB41), 4.2 (EPB42), and 4.9 (EPB49) proteins, actin, low-molecular-weight proteins, and hemoglobin. There were no statistically significant differences between control samples and samples containing

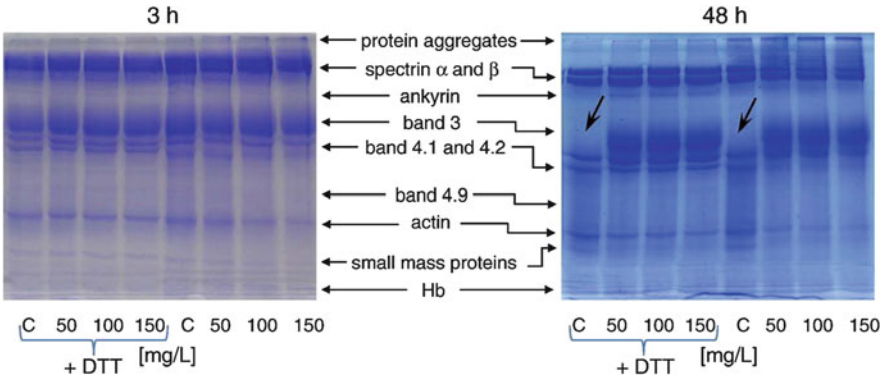

Fig. 9.5 SDS-PAGE of proteins of erythrocyte membranes incubated with fullerenol for 3 and 48 h at 37 °C under non-reductive or reductive conditions (DTT at 0.25 mM). *Arrows* indicate a complete disappearance of the band 3 protein "Reproduced with permission" [39]

membranes incubated with fullerenol up to 100 mg/L for 3 h. Interestingly, for the samples containing 150 mg/L of fullerenol incubated in the same conditions, there was a significant decrease in the band identified as actin with a simultaneous increase in the band 3 protein fraction. The decline of the actin band when the highest concentration of fullerenol was used can suggest that the nanocompound causes association of this protein. The enrichment in the band 3 protein fraction, which has a molecular weight comparable to two molecules of actin connected by a molecule of fullerenol, suggests a possibility of interactions between fullerenol and erythrocyte cytoskeletal proteins. A prolonged, 48-h incubation resulted in the disappearance of the band 3 fraction and enrichment in the broad band of low molecular mass proteins (smearing) in control erythrocyte ghosts. This is a result of substantial fragmentation of band 3 protein, which could originate either from proteolytic or ROS-induced cleavage [44]. The presence of fullerenol in all used concentrations prevented the degradation of band 3 protein. The presented data indicate that fullerenol preferentially binds to band 3 protein and prevents its degradation. A large number of hydroxyl groups on the surface of the fullerenol carbon cage provide possibilities for interaction and attachment of other molecules, e.g., drugs [30]. At the same time, functional -OH groups can adsorb to cytoskeletal erythrocyte proteins (with permission from [39]).

The results obtained with the use of isolated erythrocyte ghosts are consistent with the influence of fullerenol on peripheral and transmembrane proteins, such as ATPases [32] and band 3 protein. The influence of fullerenol on transmembrane proteins is clearly reflected by the changes in activity of Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase, which can originate from both direct interaction with the proteins and indirect influence on the fluidity of the membrane [32]. Band 3 protein plays a central role in cytoskeleton formation, therefore, conformational changes induced by various factors in this molecule result in echinocyte formation [45] (with permission from [39]).

It has to be noted that a 48-h incubation of erythrocytes led to aggregation of the membrane proteins. The use of reducing agents prevents these events, indicating that aggregation is promoted by the creation of –S-S- bridges [46]. Fullerenol, when used in concentrations up to 100 mg/L did not influence aggregate formation. An increase in concentration to 150 mg/L, however, promoted protein aggregation. Aggregates formed in the presence of fullerenol in these conditions could not be reduced by DDT, which confirms fullerenol–protein association. The influence of fullerenol on the proteins is not limited to association or networking. This nanocompound can induce deformation of the protein in the binding residues. Low concentrations of $C_{60}(OH)_{20}$ (15–30 mg/L) inhibit microtubule polymerization by binding to tubulin in the ratio 9:1 [47]. Fullerenol $C_{60}(OH)_{36}$, by association with band 3 protein, not only prevented its degradation but also influenced the binding sites of spectrin, band 4.1 and 4.2 proteins or actin, leading to changes in the cytoskeleton affecting erythrocyte morphology [48–50] (with permission from [39]). The morphological changes of erythrocytes induced by fullerenol were investigated with flow cytometry and phase contrast microscopy (Fig. 9.6 and 9.7). Changes in the shape of the cells observed by flow cytometry are in accordance with microscopic observations of erythrocytes exposed to fullerenol. Fullerenol seemed to slightly enhance the formation of dendrites, typical for echinocyte forms at a concentration of 150 mg/L.

Formation of echinocytes is caused by a number of factors, which, among other things, affect the conformation of band 3 protein, the main foundation of the cytoskeleton [45]. Moreover, it is a transmembrane anion exchanger protein responsible for chloride and bicarbonate transport, which is correlated with potassium and sodium active transport by Na+/K+-ATPase. Fullerenol, by inhibiting the function of Mg^{2+}-dependent membrane ATPases [32], may be able to disturb the distribution of lipids in the inner and outer membrane layers, thus, triggering the collapse of the discoidal shape of erythrocytes [51]. Further, the altered morphology of the cells can be explained by the formation of hydrogen bonds between the nanoparticles and the lipid head groups. The presence of each nanoparticle engaged a number of lipids to reduce their areas per lipid molecule. It is not impossible that a synergistic effect of fullerenol on membrane ATPases, band 3 protein and lipids is responsible for part of the echinocytic transformation.

Our data show that fullerenol can slightly alter the morphology and, therefore, the inner-organization of the cells through association with cytoskeletal proteins. These results confirm that fullerenol is able to interact with transmembrane cytoskeletal proteins, in particular band 3 protein and ATPases (with permission from [39]).

5 Conclusions

Incubation of erythrocyte membranes with fullerenol led to decreases in Na^+,K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase activities. The inhibition of the activities of erythrocyte ATPases caused by fullerenol could be the result of its direct and/or indirect (via membrane fluidity changes or influencing other erythrocyte proteins)

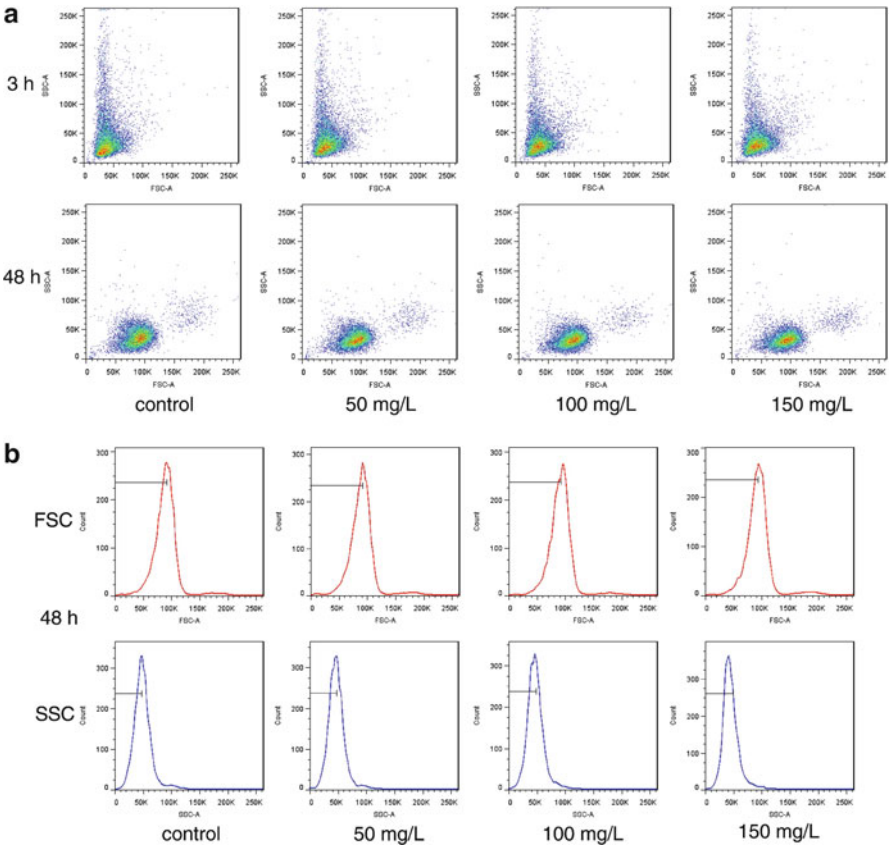

Fig. 9.6 Flow cytometry analysis of fullerenol-induced changes in erythrocyte morphology. (**a**) Scattering diagrams of human control erythrocytes and erythrocytes incubated for 3 and 48 h with fullerenol in concentrations ranging from 50 to 150 mg/L. The FSC-A/SSC-A diagram is a dual parameter contour plot proportional to the total cell diversity. (**b**) The FSC-A histograms represent the light scattered near the forward direction (proportional to the volume of the particles). The SSC-A histograms represent scattering at the right angle (dependent on cell shape and internal properties) "Reproduced with permission" [39]

interaction with the enzymes. Fullerenol affected cytoskeletal transmembrane proteins, particularly the band 3 protein. Despite the inhibition of Na^+,K^+-ATPase by fullerenol, an increase in potassium ion leakage was not observed. Blocking the leakage can result from physical "blockages" of potassium channels by fullerenol molecules. In the intact erythrocytes fullerenol can associate mostly with the surface of the plasma membrane; however, it could also migrate deeper inside the membrane increasing its fluidity. Fullerenol has more than 30 -OH groups on the surface, and therefore can interact with the functional groups of amino acids of membrane proteins and the heads of membrane phospholipids via hydrogen bonds.

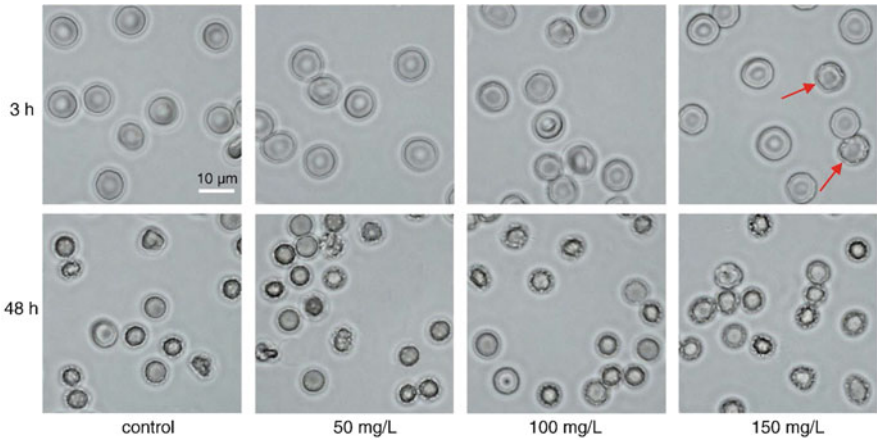

Fig. 9.7 Microscopic images of erythrocytes exposed to fullerenol. The cells were incubated in the presence of fullerenol at 50–150 mg/L for 3 and 48 h at 37 °C. Echinocyte formation for 150 mg/L of $C_{60}(OH)_{36}$—indicated by *arrows*. All images are at the same scale (scale bar: 10 µm) "Reproduced with permission" [39]

Both fullerenol and plasma membrane have negative overall charge; however, fullerenol can adsorb to the protein domains of the plasma membrane by van der Waals or dipolar interactions [39, 52].

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Chapter 10
Xenobiotics-Mediated Modulation of ATPases and Biomedical Implications

Bechan Sharma, Shweta Singh, Sunil Kumar Jaiswal, and Nikhat Jamal Siddiqi

Abstract A xenobiotic is a chemical compound found in an organism but normally not produced or expected to be present in it. Xenobiotics are the substances foreign to any biological system. Mostly, these are artificial or synthetic substances such as drugs including antibiotics, which did not exist in nature. Natural compounds can also become xenobiotics if they are taken up by another organism. Pollutants such as dioxins, polychlorinated biphenyls, pesticides, and dyes also belong to this category. A compound that is normal to one organism may be a xenobiotic to another; for example the sewage for a fish. When an animal produces a toxin as a defense mechanism against predators, these toxins can be thought of as xenobiotics to the predator. However, predators can also evolve defenses against these xenobiotics. The term xenobiotic is also used to refer to organs transplanted from one species to another. Most of the xenobiotics evoke response in an individual by acting at their specific targets. Some of them actively interact with different ATPases and are capable to efficiently modulate their structures and functions. This chapter illustrates an updated account of interactions of different xenobiotics with varied ATPases, xenobiotics induced modulations in the structures and functions of different ATPases and their implications in design and development of newer potential anticancer agents. In addition, the possible ameliorative strategies to encounter adverse effects generated by such xenobiotics are also discussed.

Keywords Xenobiotics • ATPases • Synthetic chemicals • Oxidative stress • Drugs • Expression • Amelioration • Cancer

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1 Introduction

A xenobiotic is a chemical substance foreign to an organism. The term xenobiotic is derived from the Greek words ξένος (xenos) = foreigner, stranger and βίος (bios, vios) = life, plus the Greek suffix for adjectives -τικός, -ή, -ό (tic). Most of the environmental xenobiotics possess abiological activity acting as pollutants in the environment. Several xenobiotics are the substances lipophilic in nature. Specifically, drugs such as antibiotics are xenobiotics in humans because the human body does not produce them naturally, or consume them in normal diets, nor they are part of a normal food. Natural compounds can also become xenobiotics if they are taken up by another organism, such as the uptake of natural human hormones by fish or the chemical defenses produced by some organisms as protection against predators. Thus, a compound that is natural to one organism may be a xenobiotic to another [1]. Xenobiotics can also be defined as substances that are present in higher-than-normal concentrations, or ones that are entirely artificial. The term xenobiotics may also refer to pollutants such as dioxins and polychlorinated biphenyls. Xenobiotics are the substances foreign to any biological system. They are artificial or man-made substances, which did not exist in nature. Organs transplanted across the species are also included in the list of xenobiotics as the transplantation of pig hearts and other organs to humans could be made. Kidneys are currently the most commonly transplanted organ. When an animal produces a toxin as a defense mechanism against predators, these toxins can also be thought of as xenobiotics from the point of view of the predator [2].

Xenobiotics are eliminated from the body through xenobiotic metabolism, in which the xenobiotics are deactivated and secreted away from the body. The liver is typically the primary focus of xenobiotic metabolism, and excretion can occur through urine, feces, breath, and sweat. Hepatic enzymes metabolize major amount of xenobiotics present in the body. The xenobiotic metabolism occurs in basically two different phases: I and II. In Phase I the xenobiotics undergo a series of redox reactions, hydrolytic, epoxidation, and transfer reactions where as the phase II reactions are basically associated with the conjugation reactions. The ultimate aim of a battery of these reactions is to convert highly hydrophobic and more toxic xenobiotics into more hydrophilic chemical species and less toxic substances. Sometimes these chemical pathways and their reactions can be fatal, as is the case with poisoning deaths and harmful drug interactions. This process is called bioactivation, the reverse phenomena of phase I and phase II biotransformation reactions, in which the less toxic parent compounds are converted into more toxic products. The metabolic pathways that human body employs to process xenobiotics have long been a subject of interest on the part of medical science. It is largely through studying these pathways that new drugs are proposed and developed, especially in the case of chemotherapeutics used in cancer treatment. They are also important in studying the potential effects of pollutants on an environment, to see whether the chemical will be broken down, or remain in the environment and cause harm. Thus, an

understanding of xenobiotic metabolism is critical for the pharmaceutical industry because they are responsible for the breakdown of drugs [2].

The effect of different xenobiotics is mediated via their interactions with specific targets in living systems; for example many organocarbamates and organophosphates act on neurotransmission system and block it primarily by drastically inactivating acetylcholinesterase (AChE). Some of these chemicals/materials actively interact with and modulate the activities of different adenosine triphosphatases (ATPases). However, the level of interactions between a xenobiotic material and ATPases varies. For many of such compounds the mechanism of interaction is not yet known. This chapter presents a recent account of xenobiotics-mediated modulation in ATPases and the biochemical/molecular consequences induced by such chemicals in biological systems. The possible amelioration in order to protect the living systems from xenobiotics induced toxicities is also included.

2 Adenosine 5′-Triphosphatases (ATPases): Classification, Structures, and Functions

These enzymes are basically hydrolases but catalyze both the synthesis and hydrolysis of ATP. Normally, the ATPases (EC 3.6.1.3, adenylpyrophosphatase, ATP monophosphatase, triphosphatase, SV40 T-antigen, adenosine 5′-triphosphatase, ATP hydrolase, complex V (mitochondrial electron transport), (Ca^{2+}+Mg^{2+})-ATPase, HCO_3^--ATPase, adenosine triphosphatase) are recognized as a class of enzymes that catalyze the decomposition of ATP into ADP and a free phosphate ion by using one molecule of H_2O [3–8] . This dephosphorylation reaction or breaking of a gamma-phosphodiester bond releases energy, which the enzyme (in most cases) harnesses to drive other chemical reactions that would not otherwise occur. This process is widely used in all known forms of life. Some of these enzymes are transmembrane proteins (i.e., bound to the plasma membrane) and they help mediate the movement of solutes across the membrane, typically against their concentration gradient. These are called transmembrane ATPases. Some of the known ATPases are (1) P-type ATPase (E1E2-ATPases; found in bacteria, fungi and eukaryotic plasma membranes and organelles and function to transport a variety of different ions across the membranes), (2) F-type ATPase (F1FO-ATPases) found in mitochondria, chloroplasts, and bacterial plasma membrane, primary producers of ATP using the proton gradient generated by oxidative phosphorylation (mitochondria) or photosynthesis (chloroplasts), (3) Vacuolar-type ATPase (V-ATPase or V1VO-ATPases; primarily found in eukaryotic vacuoles, responsible for catalysis of ATP hydrolysis to transport solutes and lower pH in organelles like proton pump of lysosomes), (4) A-type ATPase (A1AO-ATPases; found in Archaea and function like F-ATPases), (5) E-type ATPase (cell surface enzymes that hydrolyse a range of NTPs, including extracellular ATP), (6) ATP Synthase, (7) sodium–potassium

adenosine triphosphatase (Na^+/K^+-ATPase), (8) Proton ATPase, (9) Calcium ATPase (Ca^{2+}-ATPase), (10) hydrogen potassium ATPase (H^+/K^+-ATPase), and (11) Magnesium ATPase (Mg^{2+}-ATPase) [9].

In fact, these ATPases have been categorized based on their structure (such as F-, V-, and A-ATPases with rotary motors), function (such as synthesis or degradation of ATP) and the nature of ions that they transport. P-ATPases (E1-E2 ATPases) are found in bacteria, fungi and in eukaryotic plasma membranes and organelles, and function to transport different ions across their plasma membranes. Its name is due to the short time attachment of inorganic phosphate at the aspartate residue at the time of activation. The function of P-ATPase includes the transport of various compounds, like ions and phospholipids across a plasma membrane at the expense of energy derived from hydrolysis of ATP. There are many different classes of P-ATPases, which transport a specific type of ion. P-ATPases may be composed of one or two polypeptides, and can exist primarily into two chief conformations, E1 and E2.

The F-ATPases (F1FO-ATPases) located in mitochondria, chloroplasts, and bacterial plasma membranes are the prime producers of ATP, using the proton gradient generated by oxidative phosphorylation (mitochondria) or photosynthesis (chloroplasts). The V-ATPases (V1VO-ATPases) are primarily found in eukaryotic vacuoles, catalyzing ATP hydrolysis to transport solutes and lower pH in organelles like proton pump of lysosome. The A-ATPases (A1AO-ATPases) are found in Archaea and function like F-ATPases whereas E-ATPases are cell-surface enzymes that hydrolyse a range of NTPs, including extracellular ATP.

The ATP synthase of mitochondria and chloroplasts is an anabolic enzyme that harnesses the energy of a transmembrane proton gradient as an energy source for adding an inorganic phosphate (Pi) group to a molecule of adenosine diphosphate (ADP) to form a molecule of adenosine triphosphate (ATP) ($ADP + Pi \rightarrow ATP$). This enzyme works when a proton moves down the concentration gradient, giving the enzyme a spinning motion. This unique spinning motion bonds ADP and Pi together to create ATP. ATP synthase can also function in reverse, that is, use of energy released by ATP hydrolysis ($ATP \rightarrow ADP + Pi$) to pump protons against their electrochemical gradient.

The transmembrane ATPases such as Na^+/K^+-ATPase are known to import many metabolites necessary for cell metabolism and export toxins, wastes, and solutes that can hinder cellular processes. An important example is the sodium–potassium exchanger or Na^+/K^+-ATPase that is responsible to maintain the cell membrane potential. The coupling between ATP hydrolysis and transport is a stringent chemical reaction, in which a fixed number of solute molecules are transported for each ATP molecule hydrolyzed; for example, 3 Na^+ ions out of the cell and 2 K^+ ions inward per ATP hydrolyzed, for the Na^+/K^+-ATPase. Transmembrane ATPases harness the chemical potential energy of ATP, because they perform mechanical work: they transport solutes in a direction opposite to their thermodynamically preferred direction of movement—that is, from the side

of the membrane where they are in low concentration to the side where they are in high concentration. This process is considered active transport. For example, the blocking of the vesicular H^+-ATPAses would increase the pH inside vesicles and decrease the pH of the cytoplasm.

The H^+/K^+-ATPases are involved in the acidification of the contents of the stomach. Besides exchangers, other categories of transmembrane ATPase include co-transporters and pumps (however, some exchangers also act as pumps). Some of these such as Na^+/K^+-ATPase, cause a net flow of charge, but others do not. These are called "electrogenic" and "nonelectrogenic" transporters, respectively. Some xenobiotics such as cardiac glycosides and their derivatives (digitoxin, digoxin, etc.) may exert their toxicity by selectively interfering and blocking the sodium–potassium pump, a ubiquitous and crucial ion transporter. The manifestations of digitoxin-induced toxicity include malfunctions of cardiac, gastrointestinal, and neuronal systems.

3 ATPases: Interactions with Xenobiotics and Biomedical Implications

Many xenobiotics including pesticides are known to interact with ATPases and thereby adversely influence the activities of these enzymes; though the mechanisms of actions of some of them are not well understood. In an in vivo study, Jaiswal e al have demonstrated that carbofuran, a carbamate pesticide which is known for inhibiting activity of AChE, could significantly inhibit activity of Na^+-K^+-ATPase in rat erythrocyte membrane [10]. These workers have explained that Na^+-K^+-ATPase, a membrane-bound sulfhydryl containing integral membrane protein, maintains resting cell membrane potential by pumping sodium and potassium ions against the electrochemical gradient across the cell membrane [11]. The maximum activity of Na^+-K^+-ATPase present at nerve endings maintains an ionic gradient across the membrane by utilizing 40–50 % of ATP produced in the brain for neuronal excitability [12, 13]. Any alteration in Na^+-K^+-ATPase activity may cause neuropsychiatric disorders [14, 15]. The diminished activity of Na^+-K^+-ATPase due to carbofuran appears to be associated with the peroxidation of unsaturated lipids of neuronal membrane as the thiol (−SH) group of Na^+-K^+-ATPase is known to be highly susceptible to oxidative stress [16]. It has been demonstrated that carbofuran mediated modulation of enzyme activity could be efficiently ameliorated by pretreatment of rats with vitamin E [10]. It appears that the mitigation of carbofuran-induced oxidative stress by vitamin E treatment would be crucial in the recovery of Na^+-K^+-ATPase [17]. Thus, Na^+-K^+-ATPase appears to serve as a potential biomarker against any xenobiotics mediated toxicity in biological systems. This hypothesis has been validated by another group of workers who have explained that the xenobiotics-induced lipid peroxidation alters the functions of membrane-bound motives of transporters of

glutamate and glucose, and ion-motive ATPases (Na^+-K^+-ATPase and Ca^{2+}−ATPase), thereby disrupting cellular homeostasis [18]. The impact of organophosphates and organochlorines on blood brain barrier, ion channels/transporters, and membrane lipid contents has been reported to alter the brain function, which could be ameliorated by the application of vitamins and melatonin [19, 20].

Cascorbi and Forêt [21] have investigated the interaction of individual and combined xenobiotics on functional properties of the glucose-transport system and the Na+/K(+)-ATPase located on the plasma membrane of human skin fibroblasts. They evaluated the effects of both the hydrophobic and hydrophilic xenobiotics in single and combinatorial forms and observed considerable inhibition in the activity of Na+/K(+)-ATPase. The results of their studies have indicated that the inhibition of integral functional proteins is based on the accumulation of xenobiotics in the plasma membrane, probably due to the enhanced membrane fluidity. However, the physicochemical properties of the xenobiotics are equally important in bioaccumulation.

The cell surface molecule ABCC10 is a broad-acting transporter of xenobiotics, including cancer drugs such as taxanes, epothilone B, and modulators of the estrogen pathway. Malofeeva et al. [22] have shown that *Abcc10*$^{-/-}$ mice exhibit increased tissue sensitivity and lethality resulting from paclitaxel exposure compared to wild-type counterparts, arguing that ABCC10 functions as a major determinant of taxane sensitivity in mice. These researchers have proposed the mechanistic basis of ABCC10 action by characterizing the biochemical and vectorial transport properties of this protein. These workers have demonstrated that a number of cytotoxic substrates, including docetaxel, paclitaxel and Ara-C, increased the ABCC10 basal ATPase activity. They further observed that the clinically valuable multi-kinase inhibitor sorafenib, and a natural alkaloid, cepharanthine, interacted with this protein and inhibited ABCC10 docetaxel transport activity [22]. The exact mechanism of action, however, is not yet known. The cell surface molecule ABCC10 is a broad-acting transporter of xenobiotics, including cancer drugs such as taxanes, epothilone B, and modulators of the estrogen pathway. *Abcc10*$^{-/-}$ mice exhibit increased tissue sensitivity and lethality resulting from paclitaxel exposure compared to wild-type counterparts, arguing that ABCC10 functions as a major determinant of taxane sensitivity in mice. Cytotoxic substrates like docetaxel, paclitaxel and Ara-C increase ABCC10 basal activity. Multi-kinase inhibitor sorafenib and a natural alkaloid, cepharanthine inhibit ABCC10 docetaxel transport activity.

4 Phosphoglycoprotein (P-gp): Interactions with Xenobiotics and Implications in Development of Anticancer Agents

Leslie et al. [23] have indicated that the 190-kDa phosphoglycoprotein (P-gp) multidrug resistance protein 1 (MRP1) (ABCC1) confers resistance to a broad spectrum of anticancer drugs and also actively transports certain xenobiotics with

reduced glutathione (GSH) (co-transport) as well as conjugated organic anions such as leukotriene C(4) (LTC(4)). They have screened a series of bioflavonoids for their ability to influence different aspects of MRP1 function and found that most flavonoids inhibited MRP1-mediated LTC(4) transport in membrane vesicles. They also investigated that the inhibition by several flavonoids was enhanced by GSH. According to these workers, the flavonoids such as naringenin and apigenin markedly stimulated GSH transport by MRP1, which suggested that they might be cotransported with this tripeptide. Quercetin inhibited the ATPase activity of purified reconstituted MRP1 but stimulated vanadate-induced trapping of 8-azido-alpha-[(32)P]ADP by MRP1 ATPase. In contrast, other flavonoids such as kaempferol and naringenin stimulated both MRP1 ATPase activity and trapping of ADP. By conducting several concerned experiments using intact MRP1-overexpressing cells, these authors have concluded that dietary flavonoids may modulate the organic anion and GSH transport, ATPase, and/or drug resistance-conferring properties of MRP1. They further suggested that at least some of these compounds, i.e., flavonoids may interact with different sites on the MRP1 molecule and modulate its function (Leslie et al., 2001). The presence and functions of P-gp have also been established in aquatic organisms that mediates multixenobiotic resistance (MXR) defense in them [24].

The structure and function of P-glycoprotein (P-gp, ABCB1) drug pump has been exhaustively worked out. It is known to protect us from toxic compounds and it confers multidrug resistance. Each of the homologous halves of P-gp is composed of a transmembrane domain (TMD) with 6 TM segments followed by a nucleotide-binding domain (NBD). The predicted drug- and ATP-binding sites reside at the interface between the TMDs and NBDs, respectively. The P-glycoprotein multidrug transporter is a 170-kDa efflux pump which exports a diverse group of natural products, chemotherapeutic drugs, and hydrophobic peptides across the plasma membrane, driven by ATP hydrolysis. The transporter has been proposed to interact with its drug substrates within the membrane environment; however, its nature and number of the drug binding site(s) were not well known. Sharom et al. [25] have reported that the two nucleotide binding domains of P-glycoprotein were responsible for ATP binding and hydrolysis, which was coupled to drug movement across the membrane. In 2012, another group of workers have displayed that the ATPase activity of the P-glycoprotein drug pump is highly activated when the N-terminal and central regions of the nucleotide-binding domains are linked closely together [26]. These authors have explained it in the light of the crystal structures and EM projection images of P-glycoprotein, which suggest that the two halves of P-gp are separated by a central cavity that closes upon binding of nucleotide. Binding of drug substrates may induce further structural rearrangements because they stimulate ATPase activity. They used disulfide cross-linking with short (8 Å) or long (22 Å) cross-linkers to identify domain-domain interactions that activate ATPase activity over ten fold. The results of a series of experiments conducted by them suggested that trapping P-gp in a conformation in which the NBDs are closely associated

likely mimics the structural rearrangements caused by binding of drug substrates that stimulate ATPase activity [26].

Bessadok et al. [27] have shown that two multispecific ABC proteins such as ABCC8/9 (a sulfonylurea receptor) and ABCB1 (a multidrug resistance transporter P-glycoprotein) share common structural features. These authors have explained that ATP-sensitive K(+) (K(ATP)) channels are the target of a number of pharmacological agents, blockers like hypoglycemic sulfonylureas and openers like the hypotensive cromakalim and diazoxide. These agents act on the channel regulatory subunit, the sulfonylurea receptor (SUR), which is an ABC protein with homologies to P-glycoprotein (P-gp). P-gp is a multidrug transporter expressed in tumor cells and in some healthy tissues. Both of these two ABC proteins exhibit multispecific recognition properties. These workers have shown that SUR ligands could be substrates of P-gp while monitoring ATPase activity of P-gp-enriched vesicles. They observed that the blockers glibenclamide, tolbutamide, and meglitinide increased ATPase activity, with a rank order of potencies that correlated with their capacity to block K(ATP) channels. P-gp ATPase activity was also increased by the openers SR47063 (a cromakalim analog), P1075 (a pinacidil analog), and diazoxide, thereby showing that these molecules bind to P-gp (although with lower affinities than for SUR) and are possibly transported by P-gp. They further conducted the competition experiments among these molecules as well as with typical P-gp substrates and found existence of a structural similarity between drug binding domains in the two proteins [27].

Recently, Hall et al. [28] have expressed that the cancer multidrug resistance (MDR) mediated by ATP-binding cassette (ABC) transporters presents a significant unresolved clinical challenge. They have proposed a strategy to resolve MDR by developing compounds that selectively kill cells overexpressing the efflux transporter P-glycoprotein (MDR1, P-gp, ABCB1). In this direction, they synthesized several novel molecules such as a lead compound NSC73306 (1, 1-isatin-4-(4′-methoxyphenyl)-3-thiosemicarbazone and its various analogues and tested against cancer cell lines. They found significant increase in MDR1-selectivity against most of the P-gp-expressing cell lines. In continuation of a quest to design and develop some new anticancer molecules against P-gp as a target, Palmeira et al. [29] have presented thioxanthones (1-Aminated thioxanthone) and their derivatives as dual inhibitors of P-gp and tumor cell growth. They have claimed that these molecules exhibited potential to act as both antitumor agents and P-glycoprotein (P-gp) inhibitors. However, Wei et al. [30] have investigated another drug, H1, a novel derivative of tetrandrine (Tet) which may reverse P-glycoprotein (Pgp)-mediated multidrug resistance (MDR) in KBv200, MCF-7/adr and their parental sensitive cell lines KB, MCF-7 by inhibiting the transport function and expression of Pgp. H1 inhibited Pgp expression and ATPase activity of Pgp in KBv200 cells in a dose-dependent manner, but had no effect on MDR1 expression.

5 Conclusions

Xenobiotics include all foreign substances such as synthetic chemicals, drugs, and antibiotics or any other artificial or man-made substances, which did not exist in nature. Most of these xenobiotics induce numerous toxic effects via acting at different targets. Many of these actively interact with different ATPases and efficiently modulate their structures and functions. ATPases in cellular and subcellular systems are involved in regulation of both the synthesis (F-ATPases present in mitochondria, chloroplasts, and bacterial plasma membranes) as well as degradation (V-ATPases present in eukaryotic vacuoles and lysosomes) of ATP molecules. The transmembrane ATPases (such as Na^+/K^+-ATPase) are known to import several metabolites necessary for cell metabolism and export toxins, wastes, and solutes that can hinder cellular processes. Out of several ATPases known so far, 190-kDa phosphoglycoprotein (P-gp), a multidrug resistance protein 1 (MRP1) (ABCC1) conferring resistance to a broad spectrum of anticancer drugs has been extensively studied. Keeping in view the key role played by ATPases in regulation of cellular homeostasis, these enzymes are now being extensively exploited as most viable targets to design and develop potential anticancer agents by analyzing their structure–function relationship and interactions with various drugs/xenobiotics using bioinformatics tools.

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Chapter 11
Emerging Role of Dysadherin in Metastasis

Kuntal Dey, Haim Garty, and Sajal Chakraborti

Abstract Dysadherin, a small regulator of Na^+/K^+-ATPase and a cell membrane glycoprotein, is associated with cancer metastasis. However, its role in metastasis is largely unknown. In this review we highlight the role of this recently identified protein in cancer progression. Dysadherin has been suggested to affect cancer progression by downregulating E-cadherin or by upregulating the chemokine production. Overexpression of dysadherin alters trans epithelial resistance (TER) indicating it's effect on paracellular permeability. Additional findings suggest that dysadherin also affects extracellular matrix. The expression of dysadherin can influence both the tumor cell as well as the cell matrix. Recent findings strongly suggest that dysadherin expression as an independent prognostic indicator of metastasis. Thus, dysadherin can be used as a molecular target for identification as well as prevention of cancer.

Keywords Dysadherin • Cancer • Na^+/K^+-ATPase • E-cadherin • CCL2 • RIC • FXYD5

1 Introduction

In the year 2001, the work of Ino et al. [1] showed that a monoclonal antibody NCC-3G10, produced upon immunization of BALB/c mice with human hepatoma Li-7 cells, recognize a cell membrane glycoprotein that is expressed in a wide variety of cancers cells. However, the expression was limited to normal cells such as stratified squamous epithelium, lymphocytes, and epithelial cells. It was also found that transfection of the cDNA of this protein inactivates E-cadherin function in post-transcriptional manner. Most importantly, this protein has an important role in

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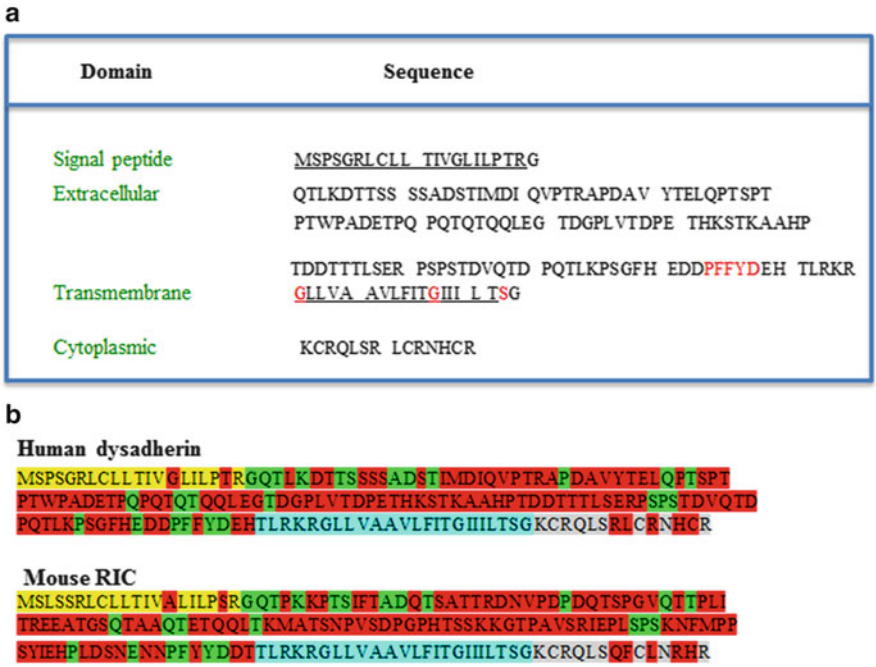

Fig. 11.1 (**a**) Amino acid sequence of human dysadherin showing the signal peptide, extracellular, transmembrane, and cytoplasmic domain. The *underlined* sequences are the two hydrophobic regions of the protein. (**b**) Comparison of the amino acid sequences of human dysadherin with mouse RIC. The same colors (except the *red* one) indicate the same region of dysadherin from the two species. The *red color* indicates the mismatch between the sequences

tumor progression and metastasis. They called this protein "Dysadherin" in order to symbolize its anti-adhesion property. Interestingly, global homology search showed that human dysadherin closely resembles mouse RIC (Related to Ion Channel) [1]. The signal peptide, the extracellular domain, the transmembrane domain, and the cytoplasmic region of dysadherin showed ~90 %, 21 %, 100 %, and 69 % similarity, respectively, with that of mouse RIC (Fig. 11.1a, b). RIC is a type of FXYD protein (FXYD5), a small protein that interacts and regulates the function of Na^+/K^+-ATPase [2]. It is now accepted that dysadherin and RIC (FXYD5) are identical.

2 Structural Features of Dysadherin

In general FXYD proteins are type I membrane proteins with an intracellular C terminus, an extracellular N terminus containing the signal peptide and a single transmembrane domain (Fig.11.1a). However, with the exception of dysadherin, the extracellular domain of other FXYD proteins is shorter than 40 amino acids, which includes a signal peptide that can be cleaved. The cDNA of dysadherin encodes 178

amino acids. For dysadherin, the extracellular domain is long containing ~140 amino acids. On the other hand, dysadherin has the shortest intracellular C-terminal segment with only 15 amino acids. The primary sequence of dysadherin predicts a number of O-glycosylation sites, but no N-glycosylation [3]. The work of Tsuiji et al. [4] suggests that the protein is heavily glycosylated. Work from different laboratories on different cell lines showed conflicting molecular mass of dysadherin [1, 2]. Lubarski et al. [2] observed the discrepancy of molecular weight between human dysadherin and mouse dysadherin and suggest that this difference may be due to species variation or may be a result of metastatic vs normal cells where dysadherin is differentially glycosylated. Interestingly it was also suggested that α- and β-subunit of Na^+/K^+-ATPase are not required for full processing of dysadherin and its trafficking to the plasma membrane. However, O-glycosylation of dysadherin is necessary for its stable expression [4].

Dysadherin was shown to be expressed in different types of cancers such as stomach, pancreatic, and breast tumors, whereas the expression was limited for relatively small number of normal cell types including lymphocytes and endothelial cells [1, 2]. Lubarski et al. [2] have demonstrated that dysadherin is expressed in epithelial tissues such as kidney, intestine, and lung. In kidney, the expression level appears to be highest in the cortex with reduced labeling in the medulla and papilla, whereas in intestines, dysadherin was found mainly in the duodenum. A more detailed characterization of the distribution of dysadherin in the nephron was documented by confocal fluorescence microscopy where dysadherin is localized in the basolateral membrane of the collecting tubule and in the intercalated cells of the collecting duct [2]. In NIH 3T3 fibroblasts, expression of a variety of oncogenes including E2a-Pbx1, v-Ras, Neu and v-Src shows concomitant upregulation of dysadherin. Transfection of human primary epithelial cells with c-Src or with E2F3 leads to the upregulation of dysadherin [5–7]. All these studies indicate that dysadherin is upregulated in cancer metastasis. However, a recent study of Gabrielli et al. [8] revealed identity of dysadherin in the male gonad and in spermatozoa. Its colocalization with E-cadherin and α4 isoform of Na^+/K^+-ATPase suggests a role for dysadherin as a modulator of sperm function.

3 Molecular Mechanism of Dysadherin Action

Dysadherin is highly expressed in several metastatic cancer cell lines compared with their respective normal cells [1, 9–11]. Overexpression of dysadherin in several cell lines resulted in a reduced cell–cell contact and enhancement of metastasis [9, 11]. In MCF7 and its derivative cell lines, overexpression of dysadherin has been observed in high-grade metastasis cells and not in the low grade one. Overall, these evidences strongly suggest that dysadherin plays an important role in metastasis. Till date two mechanisms have been proposed for dysadherin-mediated metastasis: E-cadherin dependent and E-cadherin independent. Figure 11.2 schematically represents how dysadherin could affects metastatic event.

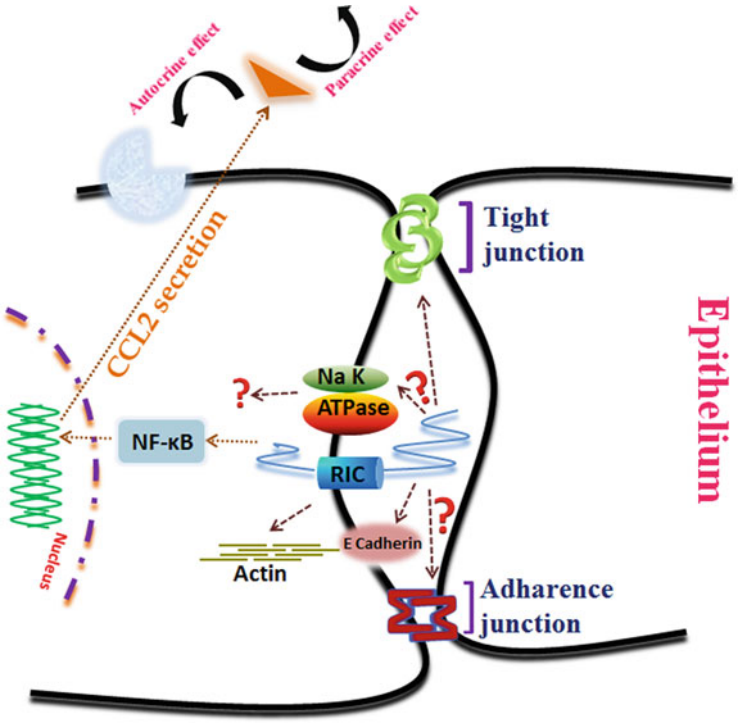

Fig. 11.2 Schematic representation of the probable pathways of the role of dysadherin in metastasis. Dysadherin can induce metastasis via E-Cadherin dependent as well as in independent pathways

3.1 Role of E-Cadherin in Dysadherin-Mediated Metastasis

During cancer invasion and metastasis, inactivation of E-cadherin has been suggested as an indispensable step. Dysadherin overexpression has been shown to inactivate E-cadherin posttranscriptionally and makes the cell metastatic [1]. Dysadherin also triggers accumulation of actin, suggesting some interplay between dysadherin and E-cadherin through actin. In vitro study also showed that in pancreatic cancer cells, modulation of dysadherin expression affected the cell morphology, actin organization as well as focal contact formation. Changes in cell motility have also been observed. Downregulation of dysadherin helps formation of paxillin containing focal adhesions and makes the cell more flattened and more adherent [9]. Thus, dysadherin may facilitate cell motility by recruiting actin filaments at the leading edge of the cell membrane. A recent report from Maeheta et al. [12] also showed that in differentiated-type carcinoma with submucosal invasion (DGCS), the expression of E-cadherin is inversely correlated with dysadherin expression. Although the underlying mechanism(s) regarding E-cadherin downregulation via dysadherin in DGCS are still unknown, yet dysadherin-positive and E-cadherin negative expression may be correlated with the more invasive DGCS. Moreover, combined

dysadherin and E-cadherin expression in glandular components may be valuable information for predicting aggressive tumor behavior and may help in the decision between endoscopic therapies and surgical resection.

Sato et al. [13] found an inverse relationship between dysadherin expression and E-cadherin occurrence in case of papillary carcinoma, follicular carcinoma, and undifferentiated carcinoma. A recent report of Colamaio et al. [14] correlated the miRNA level with dysadherin expression. They found that miRNA let-7a (let-7a belongs to the let-7 family of miRNAs) has an inverse correlation with dysadherin expression in thyroid follicular adenomas and carcinoma. Their results suggest a role of let-7a downregulation in the development of thyroid neoplasias of the follicular histotype, likely regulating dysadherin protein expression levels.

3.2 E-Cadherin Independency: Interaction of Dysadherin with Other Proteins

Dysadherin knockdown in breast cancer cell lines that expressed E-cadherin caused increased association of E-cadherin with the actin cytoskeleton. However, it was observed that in cells that had no functional E-cadherin (MDA-MB231), knockdown of dysadherin still suppress cell invasiveness, suggesting the existence of a novel mechanism of action. Global gene expression analysis in human breast cancer cells (MDA-MB-231) identified chemokine ligand 2 (CCL2) as the transcript most affected by dysadherin knockdown [15]. It was reported that CCL2 is expressed by a wide variety of cancer types, and numerous studies have showed that CCL2 generally facilitates tumor progression [16, 17], and have the role in angiogenesis [18]. CCL2 promoter contains sites for regulation by CAAT/enhancer-binding protein, NF-κB, c-ETS, and beta-catenin/TCF4. It was shown that dysadherin could play an important role in ER-negative breast cancer by promoting invasion and metastasis through a mechanism that involves enhanced signaling through the NF-kB pathway, leading to increased production of CCL2. CCL2 that is secreted by the tumor cell in response to dysadherin expression can promote tumor cell (pancreatic cancer) invasion in an autocrine manner and can also exert paracrine effects on endothelial cell recruitment that could enhance angiogenesis. It appeared that CCL2 mediate the effects of dysadherin also on the cytoskeletal rearrangement and hence stimulate cell motility, and contribute directly to the metastatic potential of human pancreatic cancer cells [5]. It is interesting to note that knockdown of dysadherin in ER-negative cell line (MDA-MB231) alter the expression of several hundred genes which means that there may be several other mechanisms exist for the action of dysadherin. Another interesting finding was a cDNA sequence, which is almost identical to that of dysadherin, was isolated from the library of human $CD34^+$ hematopoietic stem cells [19]. So there is good possibility of the regulation of stem cell dynamics by dysadherin.

It is well established that dysadherin is a tissue-specific regulators of the Na^+/K^+-ATPase. The work of Lubarski et al. [2] showed that dysadherin interacts with both the α and β subunits of the Na^+/K^+-ATPase and increases its V_{max}. FXYD5/

FXYD4 chimeras expressed in *Xenopus laevis* oocytes revealed that the transmembrane domain of dysadherin helps to increase the activity of the pump [20]. They further explored that the metastatic effect of dysadherin in mouse kidney collecting duct cell line, M1, may be due to an increase in paracellular permeability [20]. Expression of dysadherin in these cells revealed a large decrease in amiloride-insensitive transepithelial resistance as well as increased permeability to 4-kDa dextran. Impairment of cell–cell contact was also demonstrated by the alteration of tight and adherence junction markers zonula occludens-1 and β-catenin, respectively [21]. It was found that in M1, dysadherin inhibits transformation of adhered single cells from the initial radial shape to flattened, elongated shape in the first stage of monolayer formation accompanied by less ordered actin cables and fewer focal points. Structure–function analysis showed that the transmembrane domain of dysadherin, which also interacts with Na^+/K^+-ATPase and not its unique extracellular segment, mediates the inhibition of change in cell shape [21]. Expression of dysadherin in M1 cells resulted in a decrease in N-glycosylation of β1 Na^+/K^+-ATPase, while silencing it in H1299 cells had an opposite effect. This glycosylation pattern is important as because under normal condition glycosylation of β1 plays an important role in cell–cell contact formation [22]. These findings lend support to the possibility that dysadherin affects cell polarization through its transmembrane domain interaction with the Na^+/K^+-ATPase. However, interaction of dysadherin with other proteins cannot be excluded.

Another mechanism for dysadherin-mediated effect is given by Perk et al. [23]. They provide the first experimental report showing that dysadherin confers cancer stem cell (CSC) like properties to Hepatocellular carcinoma (HCC) cell lines. The following findings provided support for CSC in HCC cell lines. First, in vitro study showed that dysadherin enhanced the CSC properties of anti-apoptotic capacity, self-renewal, stem cell markers, and side population (SP) phenotype. Second, in vivo limiting dilution assay showed that dysadherin-positive cells were enriched for CSC. Third, in vitro knockdown of dysadherin also reduced stem cell-like properties. The SP phenotype has been known to facilitate the enrichment of CSCs and SP fractions and confers the capacity for both self-renewal and proliferation and largely responsible for in vivo malignancy. Park et al. [23] provided evidence in support of the SP population by showing that dysadherin transfected cells have a high expression level of ABC transporter. Although the exact mechanism is currently unknown, yet it has been suggested that the contribution of dysadherin to cancer stemness may be one of many mechanisms involved in dysadherin-mediated cancer progression [23]. Thus, dysadherin may represent a target molecule for the treatment of advanced cancer.

4 Pathology Associated with Dysadherin

Overexpression of dysadherin leads to several types of cancer as listed in Table 11.1. It is interesting to note that in several types of cancers like head and neck carcinoma or in lung cancer, dysadherin can primarily be used as a prognostic indicator.

Table 11.1 Expression of dysadherin in different cancers

Cancer type	Mechanism: E-cadherin dependency	Can dysadherin be used as an independent indicator?	Ref
Pancreatic ductal adenocarcinoma	No	Yes	[19]
Lung cancer	No	Yes	[24]
Breast cancer	Still not assessed	?	[1]
Gastric cancer	No	No	[25]
Tongue cancer	Yes	Yes	[26]
Thyroid cancer	Yes	?	[13]
Colorectal cancer	No	?	[27]
Head and neck squamous carcinoma	Yes	Yes	[28]

5 Conclusions

Since the field of research on dysadherin started just a decade ago, we still have a lot to learn about the role of dysadherin on cancer progression. First of all we need to know the other proteins that interact with dysadherin. The most likely candidate is Na^+/K^+-ATPase, since all FXYD family members function as tissue-specific modulators of this ion pump [2]. Dysadherin has been shown to interact with the α subunit of the Na^+/K^+-ATPase by its transmembrane domain [2]. High expression of dysadherin in the normal kidney cortex suggests that like other FXYD proteins, dysadherin could play a role in regulating ion flux in normal homeostasis [29]. It is now well established that besides its ion pumping activity, Na^+/K^+-ATPase also plays as role as a signal transducer [30, 31]. Moreover, the report from Barwe et al. [32] suggests that Na^+/K^+-ATPase may have a role in cancer metastasis and this effect is independent of the ion pumping activity of the pump. So all these pathways suggest a good possibility that dysadherin might contribute to cancer metastasis through mechanisms involving changes in ion flux through Na^+/K^+-ATPase.

Another good possibility is the relationship between dysadherin and E-cadherin expression. However, the detailed molecular mechanism by which dysadherin regulate E-cadherin expression is still not clearly known. It is also unknown about how dysadherin affect the CCL2 expression and hence modify the metastatic phenomena. If we look at the structure of dysadherin, the short cytoplasmic tail makes it relatively unusual to interact directly with other proteins (Fig. 11.2). However, the long extracellular domain of dysadherin, compared with other FXYD proteins, may facilitate interactions with other membrane proteins or with extracellular matrix components, and may affect several signaling pathways [9]. In MD-MB231 cells, the NF-κB pathway was affected with the expression of dysadherin. It is noteworthy that tumor invasion and metastasis are known to be influenced by numerous NF-κB-regulated gene products, including matrix metalloproteinases, interleukin-8 and various chemokines [9]. Lee et al. [33] have shown that dysadherin might play an important role in breast cancer by promoting invasion and metastasis through a mechanism that

involves enhanced AKT activation. Barwe et al. [32] reported that Na^+/K^+-ATPase is associated with the regulatory subunit of PI3-kinase that modulates the phosphorylation of AKT. The inhibition of Na^+/K^+-ATPase in highly motile MDCK cells suppresses cell motility. These findings support the possibility that dysadherin might contribute to AKT activation through mechanisms that involves the changes in Na^+/K^+-ATPase. The underlying molecular events require adequate attention.

Lubarski et al. [2] have demonstrated differences in the molecular weight as well as altered glycosylation patterns of dysadherin in the metastatic and normal tissue. This indicates that posttranslational modifications such as glycosylation and phosphorylation in modulating dysadherin localization and function are important and should be taken in to consideration. Tsuiji et al. [4] showed that dysadherin is a heavily O-glycosylated mucin like molecule and its cellular expression is regulated depending on its glycosylation status. Aberrant O-glycosylation inhibits stable expression of dysadherin and leads to upregulation of E-cadherin by an unknown mechanism, resulting in an increased cell–cell adhesion. This suggests the possibility that regulation of dysadherin expression may provide novel avenues in the field of oncology, while the carbohydrate-directed approach is expected to aid its development.

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Chapter 12
The Astrocytic Na^+, K^+-ATPase: Stimulation by Increased Extracellular K^+, β-Adrenergic Activation, Ouabain-Mediated Signaling, and Interaction with the Transporter NKCC1

Leif Hertz, Dan Song, and Liang Peng

Abstract The astrocytic Na^+,K^+-ATPase is important because increasing evidence indicates that increased extracellular K^+ in brain following neuronal excitation initially is accumulated into astrocytes. This is due to higher Na^+,K^+-ATPase activity in astrocytes than in neurons and because the extracellular K^+-sensitive site of the astrocytic Na^+,K^+-ATPase, in contrast to that of the neuronal enzyme, has low enough affinity for K^+ to be further activated by increased K^+ concentrations. However, K^+ must eventually be re-accumulated into neurons in order to prevent neuronal K^+ depletion. Accumulated astrocytic K^+ is released through Kir4.1 channels, but a presently unsolved problem is how renewed astrocytic uptake is prevented. Experiments in well-differentiated cultured astrocytes providing a solution of this problem are discussed. At the same time subunit composition of the astrocytic Na^+,K^+-ATPase and its influence on the enzyme's kinetic parameters is reviewed together with stimulation of the enzyme by noradrenaline and its functional importance. So are details of Na^+,K^+-ATPase signaling in response to submicromolar concentrations of ouabain and/or low mM K^+ concentrations without which the catalytic activity of the astrocytic enzyme is abolished. Two pathophysiological conditions are discussed, cerebral ischemia/reperfusion and hepatic encephalopathy. In the former ouabain signaling dependence on extracellular Ca^{2+} is crucial and provides therapeutic possibilities. In the latter the ability of NH_4^+ to mimic K^+ in both catalytic and signaling effects of the Na^+,K^+-ATPase is essential. In both conditions it is important that operation of the Na^+, K^+, Cl^- and water cotransporter NKCC1 is dependent upon ion gradients created by the Na^+,K^+-ATPase.

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Keywords Astrocyte • Brain ischemia • Brain potassium • Glutamate • Glycogenolysis • Na^+,K^+-ATPase subunits • Neuron • NKCC1 • Ouabain signaling • Potassium undershoot

1 Catalytic Activity of the Astrocytic Na^+, K^+-ATPase

1.1 The Astrocytic Na^+,K^+-ATPase, but not the Neuronal Enzyme, Is Stimulated by Above-Normal Extracellular K^+ Concentrations

Activation of the Na^+,K^+-ATPase requires simultaneous binding of Na^+ to an intracellular site and of K^+ to an extracellular site of the enzyme [1, 2]. In excitable cells, such as neurons, the activity of the Na^+,K^+-ATPase is generally regulated by the intracellular concentration of Na^+ ($[Na^+]_i$). The simultaneous increase in extracellular K^+ ($[K^+]_o$) during the excitation has normally no effect on neuronal Na^+,K^+-ATPase activity, because the affinity of this site in neurons is so high that it is saturated at normal $[K^+]_o$. This was convincingly shown by Grisar et al. [3], who determined kinetic properties of the Na^+,K^+-ATPase in mechanically isolated glial cells, neuronal perikarya, and synaptosomes from rabbit brain cortex as well as human cells and observed no increases in Na^+,K^+-ATPase activity in the neuronal preparations (Fig. 12.1). However, it has been shown by gel electrophoresis that the brain contains two distinct molecular forms of the Na^+,K^+-ATPase, which can be

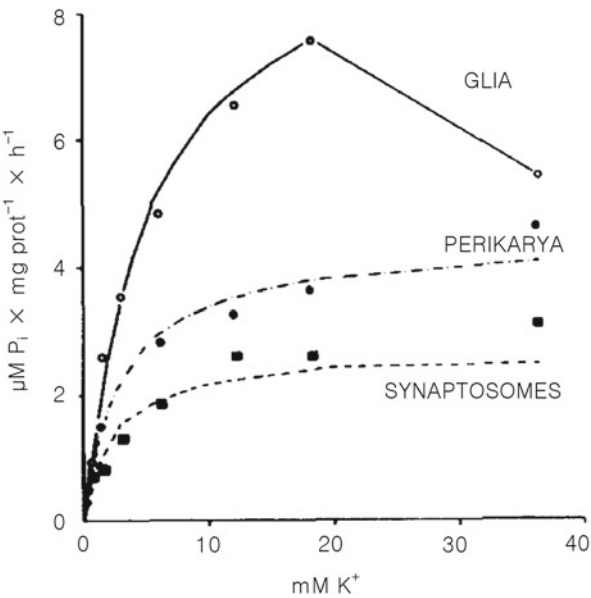

Fig. 12.1 Effect of different extracellular K^+ concentrations ($[K^+]_o$) on Na^+,K^+-ATPase activity in astrocytes and neuronal perikarya isolated from a single human brain. From where further methodological details are described. Reproduced with permission, where further methodological details are described

separated in their active form by gentle tissue fractionation procedures [4]. One is the only Na^+,K^+-ATPase of astrocytes, while the other is the only Na^+,K^+-ATPase of the axonal membrane. Figure 12.1 shows that in contrast to the neuronal enzyme isolated glial cells show a distinct increase in Na^+,K^+-ATPase activity in response to an increase in $[K^+]_o$, confirming previous results by Henn et al. [5]. This indicates that the K^+ affinity of the K^+-sensitive site is lower in astrocytes than in neurons. Subsequent kinetic analysis in homogenates of cultured mouse cerebral astrocytes and neurons [6] showed conventional Michaelis–Menten kinetics with a K_m value for K^+ of 1.9 mM in astrocytes and a K_m value of 0.43 mM in neurons. V_{max} in astrocytes was approximately twice of that in neurons. A similar affinity for Na^+,K^+-ATPase-mediated K^+ uptake was shown in rat cultures by Larsen et al. [7], consistent with the observation that the rate of active K^+ uptake is similar in rat and mouse cultures [8].

The reason for the difference in K^+ affinity between astrocytes and neurons is the different subunit composition. In freshly isolated cell fractions of mouse astrocytes and neurons, mRNA of the α_1 subunit has twice as high an expression in astrocytes as in neurons, whereas the α_2 subunit is almost restricted to astrocytes and the α_3 subunit to neurons (Fig. 12.2a) Nevertheless, the traces of α_3 in astrocytes and of α_2 in neurons are probably representative of the in vivo situation, since cross-contamination between fractions should also have led to neuronal β_2 expression. The β_1 subunit is more highly expressed in neurons than in astrocytes, but only astrocytes express both β_1 and β_2. The expression of the auxiliary protein FXYD7 is equal in the two cell types (Fig. 12.2b). Neuron-selective expression of mRNA for α_3 has also been shown by Cahoy et al. [9], and in cultures somewhat different from ours, Cameron et al. [10] reported that cortical astrocytes display α_2 and β_2 subunits and cerebellar granule neurons α_3 and β_1 subunits. The greater α_1 expression in astrocytes than in neurons shown in Fig. 12.1 is also consistent with immunochemical data by MacGrail et al. [11] and with conclusions based on low-affinity ouabain binding, reflecting content of α_1 protein, in our own cultured neurons and astrocytes [12].

Subunit composition is important for the kinetic properties of the $Na^+.K^+$-ATPase. In a study by Crambert et al. [13] nine different human Na^+,K^+-ATPase isozymes, composed of α and β isoforms, were expressed in Xenopus oocytes and analyzed for their transport and pharmacological properties. All human isozymes were functional but differed in their turnover rates depending on the α isoform. Variations in K^+ affinity and activation were a result of a cooperative interaction between α and β isoforms with α_2-β_2 complexes having the lowest apparent K^+ affinity. α Isoforms also influence the apparent internal Na^+ affinity in the order $\alpha_1 > \alpha_2 > \alpha_3$ [13, 14]. FXYD7 decreases the apparent K^+ affinity of α_1-β_1 and α_2-β_1, but not of α_3-β_1 isozymes [15]. These observations are consistent with the low affinity for K^+-induced stimulation of Na^+,K^+-ATPase activity in astrocytes and the ensuing ability of the astrocytic enzyme to be stimulated by above-normal $[K^+]_o$. As seen in Fig. 12.3, the low affinity of the α_2-β_2 complex was confirmed by Larsen et al. [7], who added the new information that depolarization increased the affinity of this complex. The α_2-β_2 complex is preferentially immunoprecipitated in mouse brain, whereas no α_1-β_2 or α_2-β_1 complexes were demonstrated [16]. However, under some conditions, e.g.,

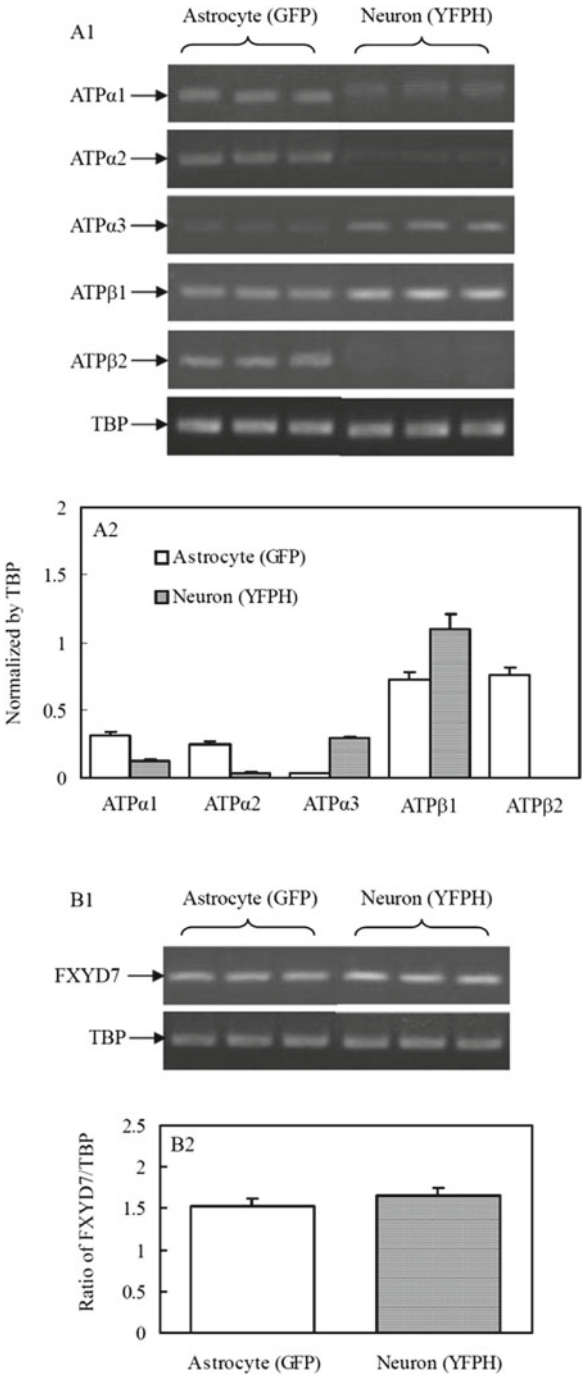

Fig. 12.2 Subunit composition of Na^+,K^+-ATPase from freshly isolated astrocytes and neurons obtained from mice where either the astrocyte-specific marker GFP or a neuronal marker, YFPH, had been linked to fluorescent compound, allowing isolation of an astrocytic respective a neuronal cell fraction by fluorescence-activated cell sorting (FACS). In each cell fraction mRNA expression was quantitated by reverse transcription polymerase chain reaction (RT-PCR). (**A1**): Products of PCR for α and β subunits and of the house-keeping gene TATA-binding protein (TBP) from three astrocytic and three neuronal samples; (**A2**): ratios between scanned expression of each subunit and TBP allowing quantitative determination of the expression of each subunit. Since different primers are used for each subunit the figure allows no quantitative comparison between expression of different subunits, but it provides reliable quantitation of astrocytic vs neuronal expression. (**B1**) and (**B2**): Similar results for FXYD7, the brain-specific FXYD. From Li et al. [17], reproduced with permission

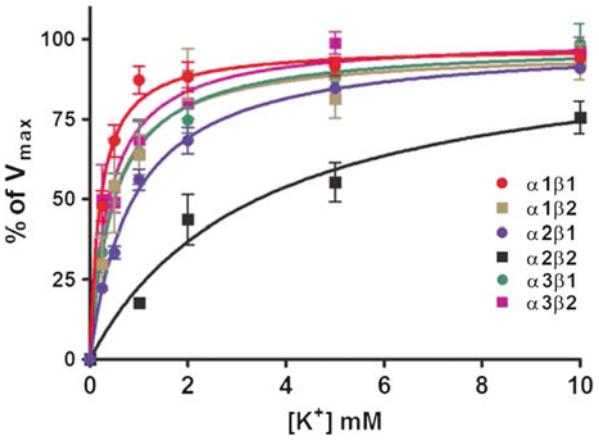

Fig. 12.3 Various subunit isoform compositions of the rat Na^+/K^+-ATPase were expressed in X. laevis oocytes. Na^+,K^+-ATPase activity as a percentage of V_{max} was determined as a function of $[K^+]_o$ and the curves fitted according to Michaelis–Menten kinetics. The graph shows the obtained activity at each $[K^+]_o$. From Larsen et al. [7], reproduced with permission

chronic treatment with the anti-bipolar drug carbamazepine the α_2 subunit is induced in neurons without concomitant induction of the β_2 subunit [17]. A submicromolar affinity for ouabain of the rat α_2 and α_3 subunits transfected into NIH 3 T3 cells is much lower than that of the α_1 receptor of ~50 μM [18]. Inhibition of K^+ uptake by different concentrations of ouabain is consistent with the values obtained by the binding studies ([19]; L. Hertz and W. Wal;z, unpublished experiments). The latter experiments also suggested that about three quarters of the K^+ uptake was mediated by the α_1 isoform.

Since the Na^+,K^+-ATPase and the gastric H^+,K^+-ATPase are the only P-type ATPases forming α-β complexes, it is likely that the obligatory β subunit plays a major role for K^+ transport [20, 21]. The β subunits facilitate correct membrane integration and packing of the catalytic α subunit, which is necessary for their resistance to degradation, acquisition of functional properties, routing to the cell surface, and determination of intrinsic transport properties [20]. In neurological diseases like familiar hemiplegic migraine type 2 (FHM2), the α_2 subunit shows mutations which are expressed in astrocytes; some of these mutations are found close to the interaction loci between α and β subunits and another mutation causes a reduced apparent K^+ affinity [21].

1.2 Both the Astrocytic and the Neuronal Na^+,K^+-ATPase Are Stimulated by Noradrenaline, but Different Subtype-Specific Receptors Are Involved

Both the astrocytic and the neuronal Na^+,K^+-ATPase are also stimulated by noradrenaline. In brain homogenates noradrenaline stimulation of Na^+, K^+-ATPase [22, 23] is inhibited by both α- and β-adrenergic antagonists [24]. Different noradrenergic receptor subtypes are involved in astrocytes and neurons with the β-adrenergic drug

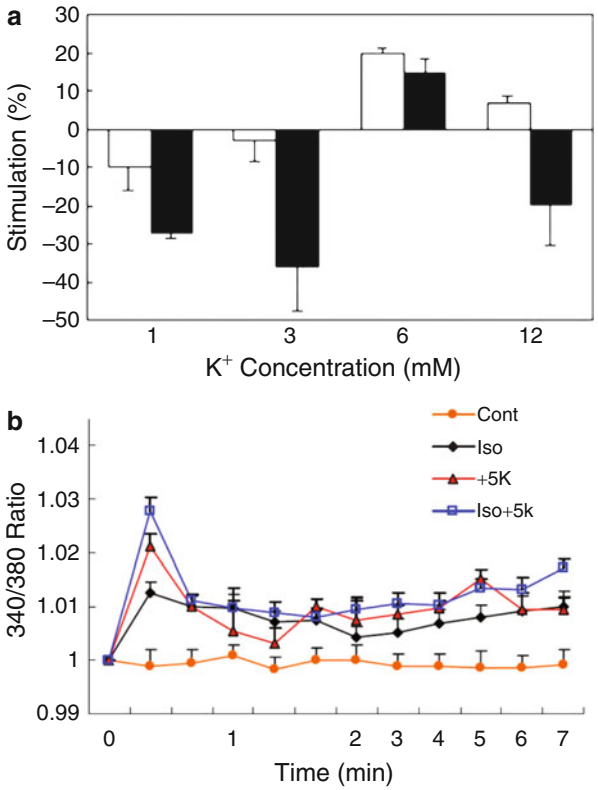

Fig. 12.4 Effects of noradrenaline or the β-adrenergic agonist isoproterenol and of $[K^+]_o$ on Na^+,K^+-ATPase activity or K^+ uptake in cultured astrocytes. (**a**) Stimulation or inhibition (negative stimulation) of Na^+,K^+-ATPase activity in homogenates of cultured astrocytes (*open columns*) or mouse cerebral cortical interneurons (*filled columns*) by 10 μM noradrenaline at different $[K^+]_o$. The activity in the same homogenates in the absence of noradrenaline is indicated as 0 %. (**b**) Increase in intracellular K^+ concentration in similar but intact cultures of astrocytes measured in arbitrary units by fluorescence of a K^+-sensitive drug under control conditions, after addition of 1 μM of the β-adrenergic drug isoproterenol, 5 mM KCl, or simultaneous addition of isoproterenol plus 5 mM KCl. (**a**) From Hajek et al. [6], reproduced with permission; (**b**) From Hertz et al. [58], reproduced with permission

isoproterenol stimulating astrocytic but not neuronal Na^+, K^+-ATPase [6]. The noradrenergic stimulation occurs only at close to normal $[K^+]_o$, so any additive effect by simultaneous exposure to elevated $[K^+]_o$ and noradrenaline is minimal (Fig. 12.4a) in either cell type. Rather, at aberrant $[K^+]_o$ noradrenaline has an inhibitory effect, especially in neurons. Similarly K^+-stimulated K^+ uptake into astrocytes is only marginally increased by 1 μM isoproterenol (Fig. 12.4b). The identical effects on Na^+, K^+-ATPase activity and K^+ uptake are important as the former is measured in a homogenate and the latter in intact cells. β_1-Adrenergic stimulation of the K^+ analogue rubidium has also been shown in pig hearts [25].

It is unknown why high K^+ and noradrenaline do not have a synergistic effect on astrocytic and neuronal Na^+,K^+-ATPases. However, in the proximal convoluted tubule of the kidney noradrenaline acting on α-adrenergic receptors is known to stimulate Na^+,K^+-ATPase activity via an increase in $[Ca^{2+}]_i$ and activation of the Ca^{2+}-dependent protein phosphatase 2B, calcineurin [26]. The α_1 isoform of Na^+,K^+-ATPase is the only catalytic Na^+,K^+-ATPase isoform expressed at this location and its dephosphorylation is increased at high $[Na^+]_i$, whereas protein kinase C (PKC) causes phosphorylation [27], which decreases Na^+,K^+-ATPase activity [28]. Ibarra et al. [27] concluded that the phosphorylation of a large pool of the Na^+,K^+-ATPase at a low $[Na^+]_i$ allows dephosphorylation (and thus activation) by α-adrenergic receptor activation. The pathway for α-adrenergic stimulation of pyramidal neurons from rat cerebral cortex includes PKC stimulation [29] and increase in $[Ca^{2+}]_i$ [30], and reduction in $[K^+]_o$ increases $[Na^+]_i$ in cerebral cortical neurons [31]. Similar effects as in the proximal convoluted tubule might therefore explain the noradrenergic stimulation of neuronal Na^+,K^+-ATPase activity at control levels of $[K^+]_o$ and the lack of effect or inhibition at least at decreased $[K^+]_o$. Since β_1-adrenergic stimulation of cultured astrocytes [32] leads to a G_s/G_i shift and subsequent PKC activation and increase in $[Ca^{2+}]_i$ (Fig. 12.5) and $[Na^+]_i$ is increased at low $[K^+]_o$ [31], the interaction between $[K^+]_o$ and noradrenaline on the astrocytic Na^+,K^+-ATPase can be explained in a similar manner at low K^+. Astrocytic $[Na^+]_i$ is not increased at high $[K^+]_o$ [31] and the astrocytic Na^+,K^+-ATPase is not inhibited by noradrenaline at 12 mM $[K^+]_o$ (Fig. 12.4a). It is reassuring that K^+/noradrenaline interactions in cultured astrocytes may be explained by effects determined in freshly obtained cells from the rat proximal tubule.

2 Signaling Activity of the Astrocytic Na^+,K^+-ATPase

The Na^+-K^+-ATPase is also a signaling molecule reacting to endogenous ouabain-like compounds, which are present in brain [33, 34] including astrocytes [35], as well as to minor increases in $[K^+]_o$. Activation of the tyrosine kinase Src in intact cells by ouabain acting on the Na^+,K^+-ATPase was first shown by Haas et al. [36], who found rapid activation of Src when nontoxic concentrations of ouabain were added to cultured neonatal cardiac myocytes. Activation of Src stimulated a pathway leading to phosphorylation of the epidermal growth factor receptor (EGFR) via Ras and eventually to phosphorylation of extracellular regulated kinases 1 and 2 ($ERK_{1/2}$). This pathway was confirmed by Zhang et al. [37], who also discovered an additional phospholipase C (PLC) and inositol trisphosphate (IP_3) receptor pathway, which lead to an increase in $[Ca^{2+}]_i$. These pathways also operate in cultured astrocytes [38], as shown by inhibition of $ERK_{1/2}$ phosphorylation induced by addition of 30 nM ouabain or 5 mM KCl by inhibitors of Src or EGF receptor phosphorylation (Fig. 12.6). An increase in $[Ca^{2+}]_i$ by ultralow concentrations of ouabain had previously been shown in such cells by Forshammer et al. [39]. Xestospongin, an inhibitor of the IP_3 receptor, inhibited a K^+-induced K^+ uptake (see Sect. 3). Thus,

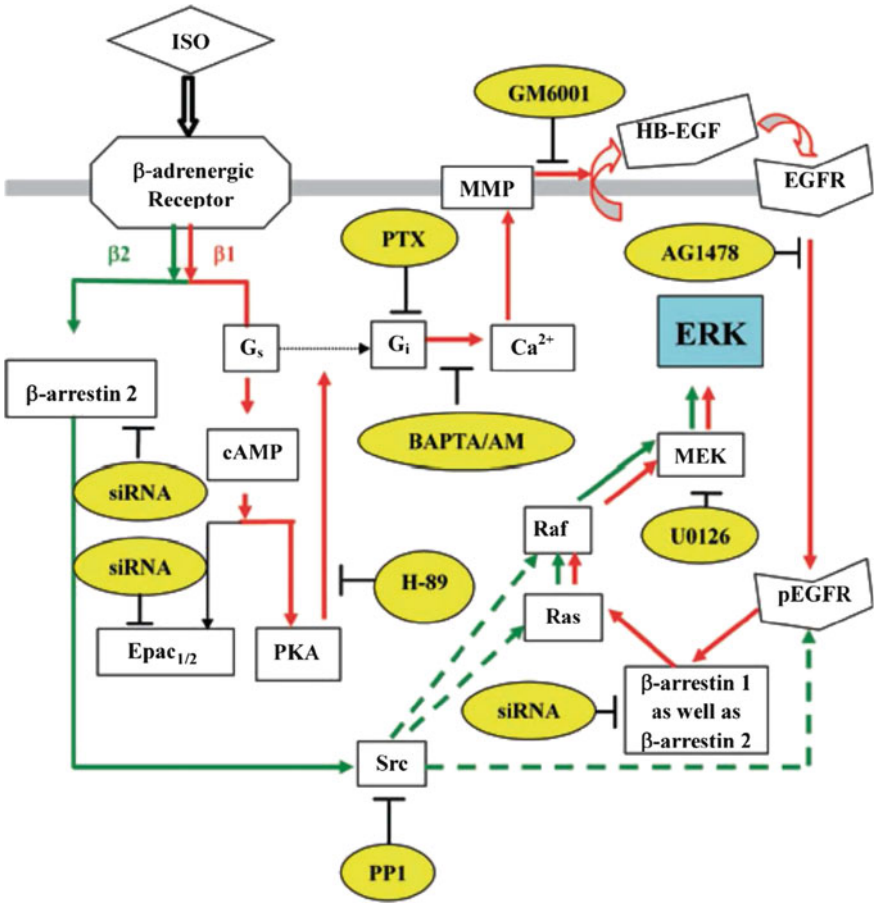

Fig. 12.5 Schematic illustration of stimulation of ERK phosphorylation by β-adrenergic receptors in astrocytes. Isoproterenol (ISO), binds to these receptors. At high concentrations (≥1 μM), the activation of the receptors induces a β_1-adrenergic (*red arrows*), PKA-dependent "G_s/G_i switch," which induces an enhancement of intracellular Ca^{2+} concentration by Ca^{2+} release from intracellular stores. The latter activates Zn-dependent metalloproteinases (MMPs) and leads to shedding of growth factor(s), such as heparin-binding epidermal growth factor (HB-EGF). The released HB-EGF stimulates autophosphorylation of the EGF receptor in the same and adjacent cells. The downstream target of the EGF receptor extracellular regulated kinases 1 and 2 $ERK_{1/2}$ (shown in *blue*) is phosphorylated via Ras/Raf/MEK pathway, contingent upon recruitment of β-arrestin 1. ERK phosphorylation by isoproterenol at a high concentration can be inhibited by H-89, an inhibitor of PKA, by PTX, an inhibitor of G_i protein, by BAPTA/AM, an intracellular Ca^{2+} chelator, by GM6001, an inhibitor of Zn-dependent metalloproteinase, by AG1478, an inhibitor of the EGF receptor, by siRNA against β-arrestin 1, and by U0126, an MEK inhibitor (all shown in *yellow*). In contrast, at a low isoproterenol concentration (≤100 nM) β_2-adrenergic (*green arrows*) activation of the receptors activates Src via the function of β-arrestin 2. Src stimulates ERK phosphorylation and phosphorylates the EGF receptor without involvement of the receptor-tyrosine kinase. $ERK_{1/2}$ phosphorylation is secondary to MEK activation, which probably is induced by direct activation of Raf by Src, whereas Src-mediated phosphorylation of the EGF receptor may not participate in the phosphorylation of $ERK_{1/2}$, which does not require recruitment of β-arrestin 1. The ERK phosphorylation by isoproterenol at low concentration can be inhibited by siRNA against β-arrestin 2, by PP1, a Src inhibitor, and by U0126, an MEK inhibitor. From Du et al. [32], reproduced with permission

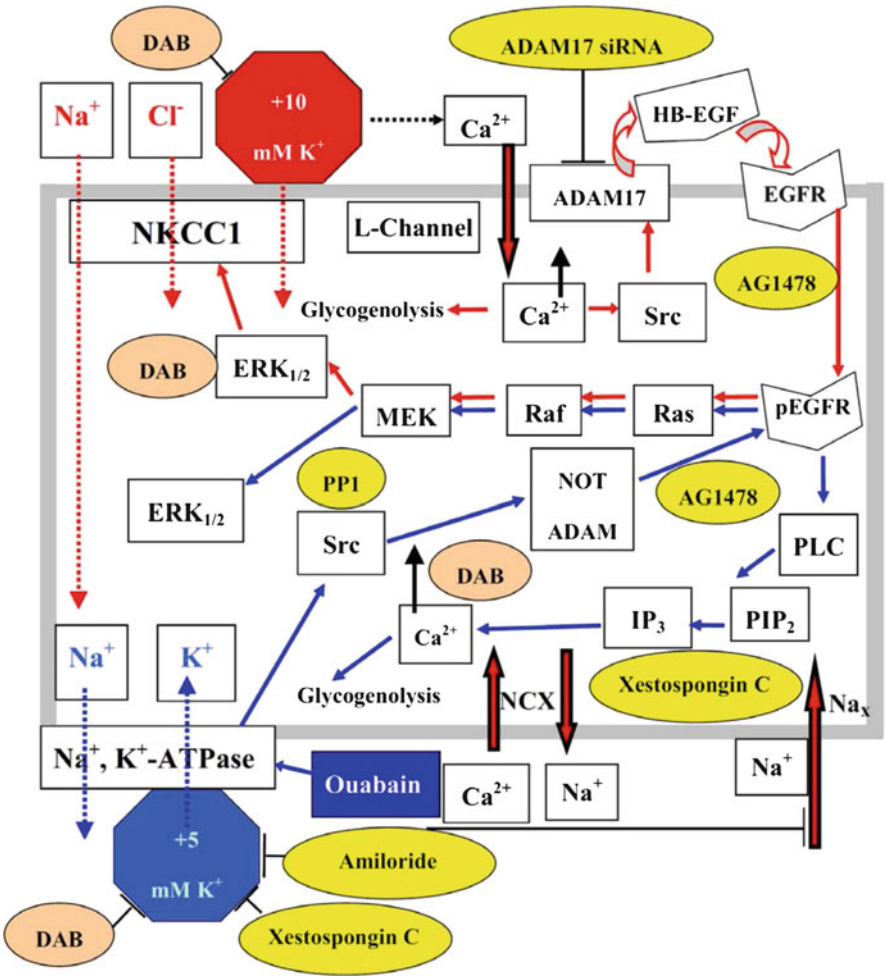

Fig. 12.6 Signaling pathways in cultured astrocytes for ouabain and $[K^+]_o$ increases ≤5 mM and ≥5 mM. The catalytic effects of the Na^+,K^+-ATPase and NKCC1 on ion fluxes are shown in the *left side* of the figure with Na^+,K^+-ATPase-mediated fluxes (+5 mM K^+) in *blue lettering at the bottom* and NKCC1 fluxes (+10 mM K^+) in *red lettering at the top*. All signaling pathways are shown in *black lettering*, with *blue arrows* for the signaling pathway of ouabain/Na^+,K^+-ATPase, activated by 30 nM ouabain or addition of 5 mM KCl (*bottom*) and *red arrows* for the signaling pathway leading to activation of NKCC1 (*top*). Transmembrane ion fluxes connected with signaling are shown by heavy *red arrows*. Increases in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) are shown by *black arrows*. Key points were verified by abolishment of ERK phosphorylation or prevention of the normal increase in intracellular K^+ content after addition of 5 mM KCl by the specific inhibitors or siRNA, shown in *brown* (the glycogenolysis inhibitor DAB) or *yellow ovals*. Note that the IP_3 receptor participates in signaling after addition of 5 mM K^+, but not after 10 mM K^+, making its inhibitor xestospongin C an important tool for distinction between activation of the two pathways. In contrast phosphorylation of the EGF receptor (EGFR) and of extracellular regulated kinases 1 and 2 ($ERK_{1/2}$), Src activation, increase in $[Ca^{2+}]_i$, and glycogenolysis occur in both pathways. Inhibition by amiloride of cellular increase in K^+ after addition of 5 mM KCl suggests inhibition of the Na^+ channel Na_x. Inhibition of Ca^{2+} entry via the Ca^{2+}/Na^+ exchanger NCX, needed in the ouabain signaling pathway, was not tested in our experiments (but see, Fig. 12.9).

in contrast to the inhibition of the Na^+,K^+-ATPase by usually applied ouabain concentrations, very low concentrations, which replicate the effect of endogenous ouabains, enhance K^+-mediated stimulation of the astrocytic Na^+,K^+-ATPase. Operation of a similar pathway in intact brain is shown by the demonstration that knock-out of the IP_3 receptor, which is an intermediate in the pathway leading to the increase in $[Ca^{2+}]_i$, increases $[K^+]_o$, and abolishes the normal increase in $[Ca^{2+}]_i$ in brain slices after high-frequency stimulation, and decreases the undershoot [40].

Even the slightest increase in $[K^+]_o$ also increases glycogenolysis in brain (Fig. 12.7), and the effect increases in parallel with further augmentation of $[K^+]_o$ [41]. The ouabain pathway opened by ouabain or 5 mM KCl also operates during K^+-induced stimulation of glycogenolysis by small increases in $[K^+]_o$. Figure 12.8 shows that interference with the ouabain pathway (by the IP_3 receptor antagonist xestospongin or a ouabain antagonist) inhibits stimulation of glycogenolysis by 5 mM KCl. In contrast nifedipine, an inhibitor of L-channel opening, does not impede glycogenolysis after addition of 5 mM K^+, although it inhibits the effect of addition of 10 mM KCl. The latter finding will be discussed later in connection with increased activity of the cotransporter of Na^+, K^+ and 2 Cl^- and water, NKCC1 [42, 43]. Here it suffices to mention that NKCC1 is a secondary active transporter, dependent on the ion gradients between extracellular and intracellular ion concentrations created by the Na^+,K^+-ATPase [44, 45] and that NKCC1 participates in astrocytic K^+ uptake when $[K^+]_o$ is increased by 10 mM or more [38]. It is also involved in the undershoot in $[K^+]_o$ following intense neuronal stimulation, shown by a reduction of the undershoot by the NKCC1 inhibitor furosemide [46]. Under pathological conditions, it is of major importance for development of brain edema after ischemia/reperfusion [47] and for a NKCC1-mediated regulatory volume increase after cell shrinkage [48]. These effects will be discussed in detail in Sect. 3.

The Na^+/Ca^{2+} exchanger NCX plays a major role in signaling by endogenous ouabains [49]. NCX is expressed in the plasma membrane, and most generally it extrudes one Ca^{2+} in exchange for 3 Na^+. However the transporter can also mediate Ca^{2+} entry, and the transport direction depends on Na^+ and Ca^{2+} gradients across the membrane and the membrane potential, which is influenced by $[K^+]_o$. Juxtaposition of plasma membrane and sarco(endo)plasmic reticulum membranes may permit NCX to regulate IP_3 and ryanodine receptor-mediated Ca^{2+} signaling [50]. This is the

Fig. 12.6 (continued) However in Ca^{2+}-free medium the K^+ uptake normally induced by addition of 5 mM KCl was abolished. The pathway activated by addition of ≥10 mM KCl and leading to activation of NKCC1 shown in *red* in the upper part of the figure had previously been determined for inhibition of ERK phosphorylation (using specific inhibitors) and found to include depolarization-mediated L-channel opening and metalloproteinase-induced release of an agonist of EGFR causing its phosphorylation (pEGFR). Additional inhibitor experiments shown in the figure indicated its dependence on glycogenolysis (inhibition by DAB) and the metalloproteinase ADAM 17, which is not involved in the pathway activated by addition of 5 mM KCl. Signaling connections between ERK phosphorylation and activation of NKCC1 or between ERK phosphorylation or increase in $[Ca^{2+}]_i$ and opening of Na_x have not been investigated. Modified from Xu et al. [38], reproduced with permission

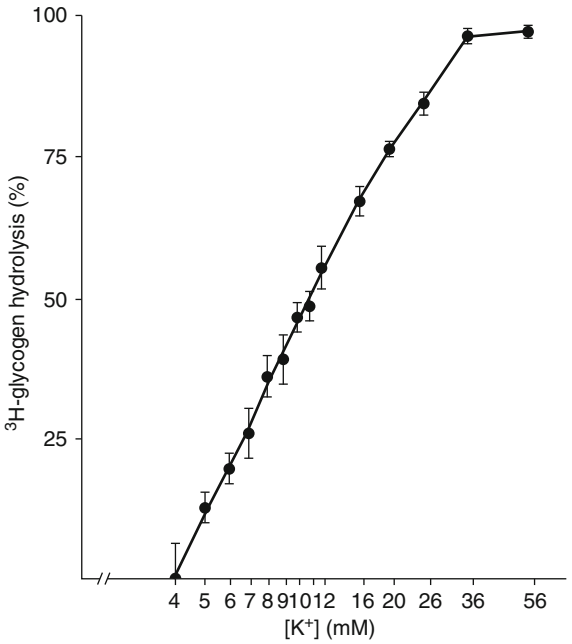

Fig. 12.7 Effect of increases in $[K^+]_o$ on mouse brain slices incubated in a physiological saline containing a total of 4 mM K^+. Note absence of glycogenolysis at this $[K^+]_o$ but increasing stimulation with increases in $[K^+]_o$. From Hof et al. [41], reproduced with permission, where further methodological details are described

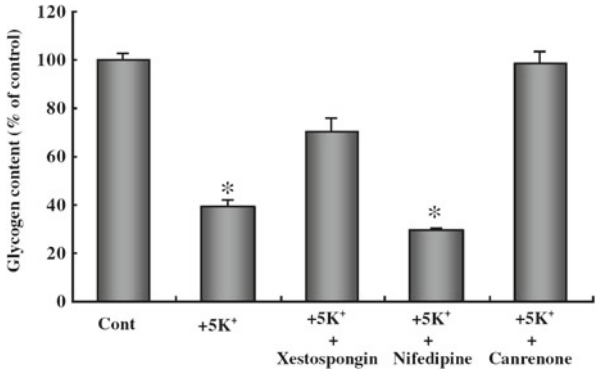

Fig. 12.8 Reduction of glycogenolysis, shown as decrease in glycogen content, caused by slightly elevated (+5 mM) $[K^+]_o$. The stimulation is inhibited by xestospongin and canrenone, an inhibitor of ouabain signaling, but not by nifedipine which inhibits L-channels for Ca^{2+} and stimulation of glycogenolysis by addition of ≥10 mM K^+ [52]. These results are consistent with the pathways shown in Fig. 12.6. From Xu et al. [52], reproduced with permission

case in arterial smooth muscle cells as shown in Fig. 12.9a, demonstrating that 100 nM ouabain causes an increase in $[Ca^{2+}]_i$, which is reduced by an NCX inhibitor and even more by removal of extracellular Ca^{2+}. Along similar lines, Wang et al. [40] found that a PAR-1 agonist that increases $[Ca^{2+}]_i$ in astrocytes, but apparently not in neurons [51], causes an elevation of intracellular K^+ in cultured astrocytes, which is

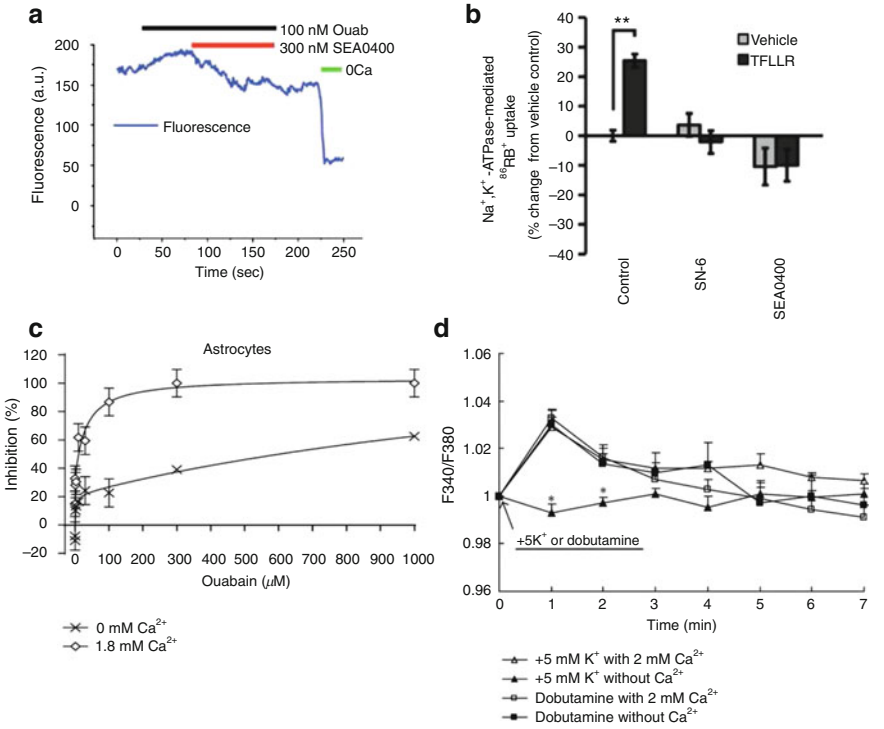

Fig. 12.9 Extracellular Ca^{2+} and its entry via NCX are required for ouabain-mediated increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and for K^+ uptake stimulated by addition of 5 mM KCl, but not for that stimulated by a β_1-adrenergic agonist. (**a**) In mouse mesenteric arteries $[Ca^{2+}]_i$ is increased by ouabain, but the increase is abolished by the NCX inhibitor SEA0400, and $[Ca^{2+}]_i$ further decreased in the absence of extracellular Ca^{2+}. (**b**) In rat astrocytes the PAR1-selective agonist Thr-Phe-Leu-Leu-Arg-NH2 (TFLLR) which increases $[Ca^{2+}]_i$ in astrocytes, but not in neurons, causes an increase in active uptake of the K^+ analogue ^{86}Rb, which is inhibited by two different inhibitors of NCX. (**c**) In the absence of extracellular Ca^{2+} the potency of ouabain on K+ uptake is drastically reduced, and (**d**) increase in intracellular K^+ by addition of 5 mM KCl, measured by fluorescence of a K^+-sensitive drug, is abolished during incubation in Ca^{2+}-free incubation medium, whereas that by 10 μM of the β_1-adrenergic agonist dobutamine is independent of Ca^{2+} depletion. (**a**) Modified from Blaustein et al. [50], reproduced with permission; (**b**) from Wang et al. [40], reproduced with permission; (**c**) from Song et al. [47], reproduced with permission; (**d**) from Song et al. [48], reproduced with permission

abolished by NCX inhibitors (Fig. 12.9b). The potency of ouabain as a K^+ uptake inhibitor in our cultured astrocytes is greatly reduced in the absence of extracellular Ca^{2+} and concentrations as high as 0.1 and 0.3 μM ouabain may have a stimulatory effect (Fig. 12.9c). In these cultures uptake of K^+, induced by a 5 mM increase in $[K^+]_o$ (and thus dependent on nanomolar ouabain signaling), is abolished during incubation in Ca^{2+} free medium [47], whereas that evoked by the β_1-adrenergic agonist dobutamine is maintained (Fig. 12.9d). Isoproterenol also stimulates astrocytic glycogenolysis, with no inhibitory effect by a β_2-adrenergic inhibitor, but pronounced although perhaps not complete inhibition by a β_1-adrenergic inhibitor [52].

Studies of increase in intracellular K^+ concentration in response to an increase in $[K^+]_o$ have given some additional information about the ouabain signaling pathway as will be discussed in Sect. 3. They include the dependence of the K^+ uptake upon glycogenolysis and Na^+ channel activity, as illustrated in Fig. 12.6.

3 Na^+, K^+-ATPase and Physiological Brain K^+ Homeostasis

3.1 Potassium Clearance

It is now well established that most clearance of increased $[K^+]_o$ following neuronal excitation is active [46, 53]. However, at highly elevated $[K^+]_o$ inwardly directed channel-mediated K^+ may also play a role [54], and Larsen et al. [7] also found a minor channel-mediated uptake after focal iontophoretic administration of K^+. A major reason why the astrocytic Na^+,K^+-ATPase is of interest is that it mediates the initial cellular re-uptake of K^+ [7, 54–57]. Neuronal activity increases the extracellular K^+ concentration $[K^+]_o$ both due to stimulation of neuronal glutamatergic receptors and resulting K^+ efflux [see 58] and due to action-potential-mediated cellular entry of Na^+ followed by exit of K^+ [55]. However, increases in $[K^+]_o$ can also occur after intense stimulation of cortical neurons expressing GABAergic receptors [59]. In hippocampus this K^+ release is dependent on bicarbonate-driven accumulation of Cl^- and subsequent stimulation of outward flux via the K^+, Cl^- cotransporter KCC2 [60]. During normal neuronal activity the increases in $[K^+]_o$ amount to $\leq$5 and often much less [61, 62]. Under these conditions cellular re-uptake is mediated exclusively by the Na^+,K^+-ATPase [38]. Released glutamate is predominantly [63, 64] and released GABA partly ([65], see however also [66]) taken up by astrocytes together with Na^+.

Since the astrocytic Na^+,K^+-ATPase in contrast to the neuronal Na^+,K^+-ATPase has sufficiently low affinity for K^+ to be stimulated by an increase in $[K^+]_o$ above its normal concentration (Sect. 1), it plays a major role in the initial clearance of $[K^+]_o$. However, it is obviously also stimulated by normal $[K^+]_o$, but stimulation of the astrocytic Na^+,K^+-ATPase in the absence of elevated $[K^+]_o$ may be prevented by its dependence on glycogenolysis. This dependence is indicated by the ability of the glycogenolysis inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) to prevent K^+-mediated uptake of K^+ (Fig. 12.10). It should also be kept in mind that even a slight increase in $[K^+]_o$ induces glycogenolysis in brain slices (Fig. 12.7). Such a complex regulation of the astrocytic Na^+,K^+-ATPase would allow neuronally released K^+ to (1) initially be taken up mainly into astrocytes [7, 54–57]; (2) afterwards be re-released via Kir4.1 K^+ channels [67], probably over a larger area due to connexin- and pannexin-mediated inter-astrocytic K^+ fluxes [68], preventing that $[K^+]_o$ is again increased; and (3) eventually be re-accumulated into neurons. The neuronal re-accumulation is a necessity in order to prevent depletion of neuronal K^+, since K^+ transport across the blood–brain barrier is very slow [69, 70]. This sequence does not explain how astrocytes re-accumulate lost K^+ in the absence of local increases in $[K^+]_o$, but this may be a situation that only occurs under deep anesthesia.

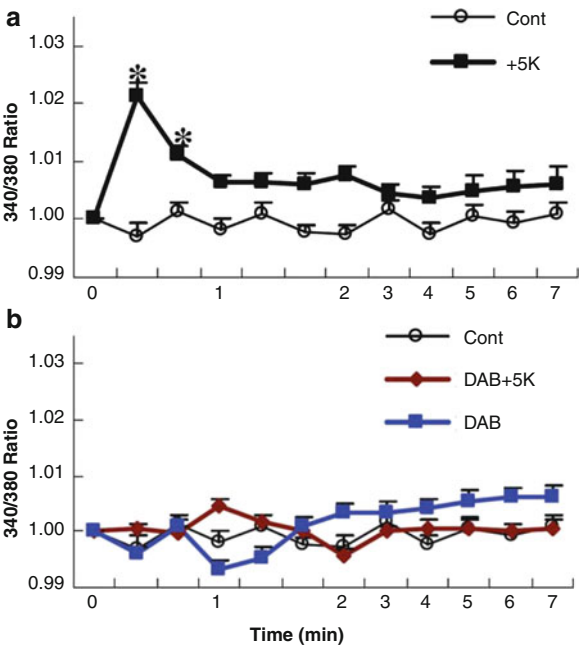

Fig. 12.10 Effect of the glycogenolysis inhibitor DAB (10 mM) on intracellular K^+ concentration, measured as in Fig. 12.4b, in intact cultures of astrocytes. (**a**) Effect of addition of 5 mM KCl alone. (**b**) Abolishment of K^+ effect by DAB. From Xu et al. [38], reproduced with permission

The reason why astrocytic Na^+,K^+-ATPase depends on glycogenolysis is that glycogenolysis is required for its signaling function, which in turn is needed for K^+-mediated stimulation of K^+ uptake. The signaling pathway shown in Fig. 12.6 was further examined by showing that DAB had no inhibitory effect on K^+ uptake into cultured astrocytes when extracellular Na^+ was increased. This is shown in Fig. 12.11a and must be due to stimulation of Na^+ uptake, since K^+ uptake could be inhibited by amiloride (Fig. 12.11b), an inhibitor of Na^+ channels, but at the concentration used probably not of NCX [71]. Consistency with the ouabain pathway shown in Fig. 12.6 is indicated by a similar ability of xestospongin, an inhibitor of the IP_3 receptor to inhibit K^+ uptake (Fig. 12.11c). Increase in $[Na^+]_i$ is needed because the Na^+,K^+-ATPase's intracellular site must be activated by Na^+ concomitantly with the K^+-mediated stimulation of the extracellular site. Since astrocytes are nonexcitable cells, the increased extracellular $[K^+]_o$ after neuronal excitation is not accompanied by an increased intracellular $[Na^+]_i$ in astrocytes. This complex regulation has up till now only been described in astrocyte cultures. However, during spreading depression large amounts of K^+ is accumulated by astrocytes in intact brain tissue, and inhibition of glycogenolysis enhances the speed with which the depression spreads over brain cortex, indicating impaired cellular uptake of K^+ [72]. An attempt to demonstrate that the rate of clearance of glutamate-induced increase in $[K^+]_o$ in brain slices is increased when glycogenolysis is inhibited [58] gave a negative result, in all probability because glutamate also causes a pronounced increase in $[Na^+]_i$. Similar studies should be repeated with electrical stimulation of

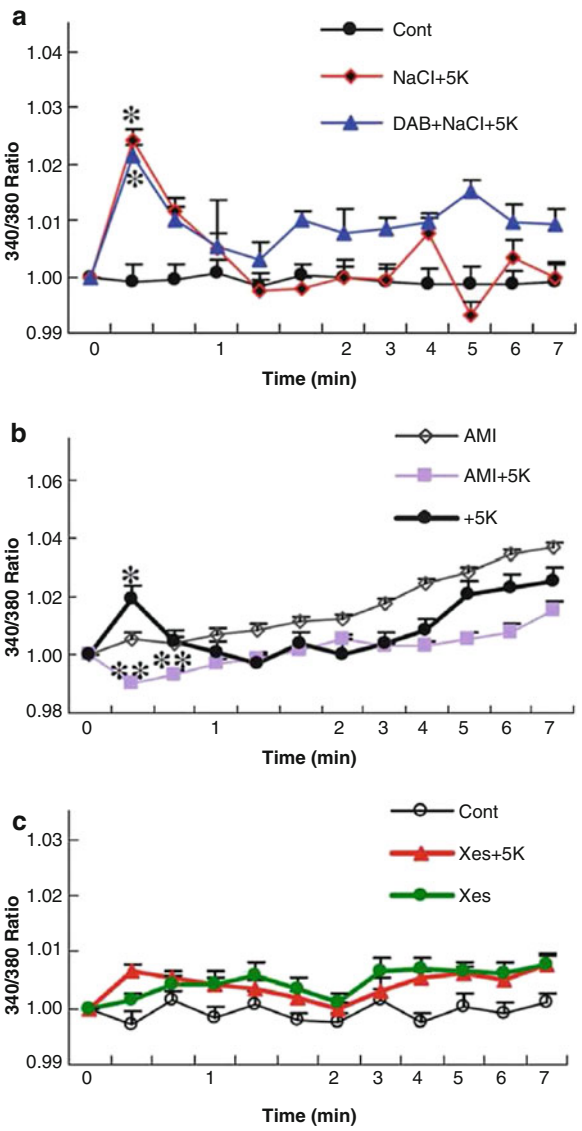

Fig. 12.11 (**a**) The effect of DAB on K^+-mediated increase in intracellular K^+ content is prevented by an increase in extracellular Na^+ concentration (+10 mM). On the other hand addition to a normal medium of 200 μM amiloride (**b**) an inhibitor of Na^+ channels or 500 μM xestospongine (**c**), an inhibitor of IP_3 receptors have a similar inhibitory effect as DAB. The findings in (**b**) and (**c**) are consistent with the pathway shown in Fig. 12.6. From Xu et al. [38]

brain tissue or of optic nerve, where K^+ release is secondary to action potential propagation. Another option might be to test DAB after iontophoretic application of K^+ to brain slices.

During spreading depression or seizures and in other situations with more highly elevated $[K^+]_o$ such as after brain ischemia [61, 73], where $[K^+]_o$ increases ≥10 mM occur, K^+ is in addition re-accumulated into astrocytes by NKCC1. These K^+ increases activate also the Na^+,K^+-ATPase, but not to any greater extent that K^+ increases ≤5 mM, which saturate the K^+-sensitive site of the Na^+, K^+-ATPase [38].

In the adult brain cortex NKCC1 is located both in glia cells, including astrocytes, where its activation by high $[K^+]_o$ can cause life-threatening edema (reviewed by Hertz et al. [19]), and at GABAergic terminals located on the axon initial segment of cortical neurons where Cl^- uptake via this transporter after intense stimulation is depolarizing and excitatory [74]. Cellular localization of NKCC1 is best determined by other than immunohistochemical techniques, since the immunological techniques can be deceptive [45, 75].

3.2 Post-stimulatory Undershoot in $[K+]_o$

It is now well established that Na^+,K^+-ATPase-mediated K^+ uptake plays the major role in cellular re-accumulation of increased $[K^+]_o$. However, the importance of NKCC1-mediated K^+ uptake stimulated by the β-adrenergic agonist isoproterenol for the establishment of the post-stimulatory undershoot in $[K^+]_o$ has only recently been suggested. In vivo evidence for such a mechanism includes that the undershoot is reduced by furosemide which inhibits NKCC1 [46] and its magnitude is increased by K^+ channel inhibition [53]. Since $[K^+]_o$ is not increased at this time, NKCC1 must be activated by a different stimulus. This is likely to be extracellular hypertonicity, known to occur after intense neuronal activity [76, 77] and possibly triggered by a 2:3 ratio between previous Na^+,K^+-ATPase-mediated cellular uptake of K^+ and release of Na^+ [78, 79], and causing cellular shrinkage. In cultured astrocytes bumetanide-inhibited NKCC1 activity is crucial for the subsequent regulatory volume increase (Fig. 12.12), and its rate is greatly enhanced by β_1-adrenergic stimulation [47], which increases the ion gradients driving NKCC1 [48]. The cellular accumulation of Na^+, K^+, Cl^- and water must lead to a corresponding decrease in

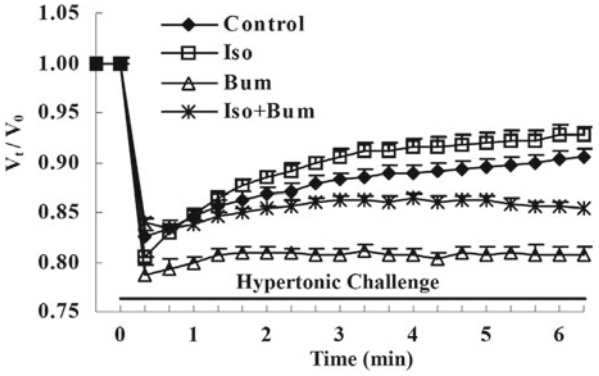

Fig. 12.12 After an initial decrease of the volume in isotonic medium (V_o) due to medium hypertonicity evoked by addition of 100 mM sucrose, a regulatory volume increase occurs. It is greatly accelerated by isoproterenol but this effect is inhibited by the NKCC1 inhibitor bumetanide. V_1: volume at any given time. From Song et al. [48], reproduced with permission

extracellular ions, except for Na^+, which is re-extruded by the Na^+,K^+-ATPase. Furosemide also inhibits another cotransporter KCC2, which is located in neurons, but KCC2 generally mediates outward transport [74], which would have the opposite effect on $[K^+]_o$, suggesting that this transporter is not involved. Extracellular hyperosmolarity also depresses population spikes and extracellular synaptic potentials [80], with neuronal gene expression changes blocked by the astrocyte-specific toxin fluoroacetate [81]. The transmitter-induced regulatory volume increase and concomitant reversal of extracellular hypertonicity may normalize neuronal activity and might play a role in inhibition of neuronal slow afterhyperpolarization, sAHP [58, 82]. As could be expected, the regulatory volume increase in cultured astrocytes is inhibited when glycogenolysis is prevented [47, 82]. Again, K^+ accumulated into astrocytes may subsequently be released via Kir4.1 channels, as suggested by the increase in the magnitude of the undershoot when these channels are inhibited [53].

4 Na^+, K^+-ATPase and Glutamate Uptake

Like many other amino acids glutamate is accumulated into astrocytes in association with Na^+ which provides the driving force and subsequently activates the intracellular Na^+-sensitive site of the Na^+,K^+-ATPase. Glutamate is accumulated into astrocytes by the transporters GLT-1 and GLAST [63, 64] and GLT exists in a macromolecular complex that includes the Na^+-K^+-ATPase, most of the enzymes involved in glycolysis, and mitochondria [83].

It was previously mentioned that the affinity for Na^+ is lower for α_2 than for α_1. It is even higher for α_3 but that is of little relevance for glutamate uptake since most glutamate uptake occurs into astrocytes [63, 64]. Illarionova et al. [84] used very young astrocyte cultures expressing GLAST to study the importance of α_1 and α_2 on glutamate uptake. Selective inhibition of α_2 resulted in a modest increase of $[Na^+]_i$ together with large decrease in uptake of aspartate, a glutamate analogue that is less metabolizable than glutamate itself. Moreover exposure to 200 μM glutamate caused a larger increase in $[Na^+]_i$ in α_1 than in α_2 overexpressing cells, and restoration of control levels of $[Na^+]_i$ took longer time in α_1 than in α_2 overexpressing cells.

5 Na^+,K^+-ATPase and Pathophysiological Brain K^+ Homeostasis

5.1 Brain Ischemia

During brain ischemia extracellular Ca^{2+} becomes greatly reduced (due to cellular uptake) whereas there is a large increase in $[K^+]_o$ [73]. This leads to NKCC1-mediated brain edema, which only becomes significant after re-oxygenation

Table 12.1 Brain water content in MCAO model with and without reperfusion

	No reperfusion		8 h reperfusion	
	Left hemisphere	Right hemisphere	Left hemisphere	Right hemisphere
Control	77.58 ± 0.20 (n = 5)	78.05 ± 0.29 (n = 5)	77.34 ± 0.18 (n = 3)	77.32 ± 0.14 (n = 3)
Ischemia 3 h	77.25 ± 0.16 (n = 5)	78.14 ± 0.25 (n = 5)	77.97 ± 0.17 (n = 8)	81.28 ± 0.34* (n = 8)

Water content was calculated as [(wet weight-dry weight)/wet weight] × 100 % in rats where a MCAO had been performed on the right side. In control rats no significant change occurred with or without reperfusion. In animals with MCAO in the right hemisphere, a small apparent increase in water content in this hemisphere after 3 h of ischemia was not statistically significant, whereas a larger increase after reperfusion marked with * was significant ($P < 0.05$). It was also significantly different ($P < 0.05$) from the small apparent increase without reperfusion. From Song et al. [47], reproduced with permission

(Table 12.1), reflecting its dependence on energy metabolism [47, 71]. There is abundant evidence that this edema occurs in astrocytes, but there must also be an effect on the blood–brain barrier bringing additional water into the brain (reviewed by Hertz et al. [85]). The specific NKCC1 inhibitor bumetanide [45] prevents the edema after ischemia/reperfusion (indicated by prevention of increase of water content in the tissue) and so do the same inhibitors (Table 12.2), which inhibit β_1-adrenergic signaling in astrocytes (Fig. 12.5). Moreover, the edema is not significantly counteracted by the Ca^{2+}-channel antagonist nimodipine [86], which prevents the Ca^{2+} uptake necessary for the development of NKCC1-mediated edema (Fig. 12.6). In the early experiments by the latter authors the edema developed already during the ischemic phase, possibly suggesting less complete arterial blockage than in the experiments shown in Tables 12.1 and 12.2. Both degrees of blockage may well be relevant for clinical stroke. The lack of effect by nimodipine points towards involvement of the other stimulus for NKCC1 activation, hypertonicity and cell shrinkage, and both of these were demonstrated by Matsuoka and Hossmann [86]. The inverse correlation between the magnitude of the increase in water space and the reduction of extracellular space demonstrated by these authors (Fig. 12.13) is consistent with swelling, and thus regulatory volume increase during the ischemic phase.

The prevention of water increase shown in Table 12.2 by β_1-adrenergic antagonists may seem peculiar because these inhibitors would normally not prevent the stimulation of the Na^+,K^+-ATPase driving NKCC1, which are mediated by the increased $[K^+]_o$. However, Fig. 12.9d showed that in the absence of extracellular Ca^{2+}, increase in intracellular K^+ mediated by an elevation of $[K^+]_o$ is abolished in Ca^{2+}-free media, whereas that mediated by the β_1-adrenergic agonist dobutamine is unaltered, and in brain ischemia extracellular Ca^{2+} is greatly reduced due to cellular uptake [73].

Complete similarity between the inhibitors blocking the pathway for β_1-adrenergic stimulation in cultured astrocytes (Fig. 12.5), those preventing regulatory volume increase in these cells [48], and inhibitors of brain edema in rats after ischemia and reperfusion (Table 12.2) supports the validity of the cultured astrocytes as models of astrocytes in situ. It is also of clinical significance. Goyagi et al. [87] have shown that administration of a β_1-adrenergic agonist 30 min after the onset of a 2-h-long isch-

Table 12.2 Brain water content in MCAO model after 3 h ischemia and 8 h reperfusion in the right hemisphere under control conditions (intra-cerebral saline only) and after injection of inhibitors of either the β_1- or the β_2-adrenergic pathway in astrocytes

	Left hemisphere	Right hemisphere
Saline	77.97 ± 0.17 (n = 8)	81.28 ± 0.34 (n = 8)*
H89	77.00 ± 0.42 (n = 3)	77.19 ± 0.09 (n = 3)
PTX	77.19 ± 0.11 (n = 4)	77.51 ± 0.26 (n = 4)
GM6001	77.08 ± 0.11 (n = 4)	77.15 ± 0.13 (n = 4)
AG1478	77.14 ± 0.11 (n = 3)	77.27 ± 0.04 (n = 3)
U0126	77.39 ± 0.10 (n = 4)	78.22 ± 0.67 (n = 4)
PP1	77.52 ± 0.26 (n = 5)	80.04 ± 0.33 (n = 5)*

In rats with MCAO in the right hemisphere drugs were added 15 min before the occlusion. Water content was calculated as [(wet weight-dry weight)/wet weight] × 100 %. In control animals (same value as in Table 12.1) an increase in the ipsilateral hemisphere was significant ($P < 0.05$), as marked with *. This was also the case after treatment with PP1, an inhibitor of Src, an intermediate in β_2-adrenergic signaling, but not after administration of any of the other inhibitors, which interrupt β_1-, but not β_2-adrenergic signaling as shown and discussed in Fig. 12.5. Most, but not all, inhibitors used to delineate the signaling pathways shown in that figure were tested in this Table. From Song et al. [47], reproduced with permission

emic period drastically reduces infarct size and improves neurological deficit score in rats after 7 days. Administration of subtype-specific β_1-adrenergic antagonists before experimental brain ischemia also provided neuroprotection against transient focal cerebral ischemia [88]. However, although the presence of β_1-adrenergic antagonists beginning 30 min before the onset of ischemia and continued for 24 h provided long-term improvement of histological outcome, they had no effect on neurological outcome and spatial memory retention 14 days later [89]. Iwata et al. [90] also found that administration of antagonists specifically of the β_1-adrenoceptor beginning 60 min after an 8-min bilateral carotid artery occlusion combined with hypotension reduced neuronal injury after forebrain ischemia, although motor activity was not improved. However, motor deficit index scores were significantly lower and neuronal survival better in rats treated with β_1-adrenoceptor antagonists beginning 30 min before 10 min of spinal cord ischemia and continued for 24 h [91]. Perhaps it is important that the β_1-adrenergic treatment, which also must have unwanted side effects on cognition and motor performance, is discontinued as soon as possible and not combined with other procedures that may enhance the side effects.

5.2 Hepatic Encephalopathy

It has been known for a long time that ammonia (NH_4^+) can substitute for K^+, but not for Na^+ in the stimulation of both the Na^+,K^+-ATPase and active transport of Na^+ and K^+ [92]. In cultured astrocytes exposure to 5 mM NH_4Cl activates NKCC after 24 h

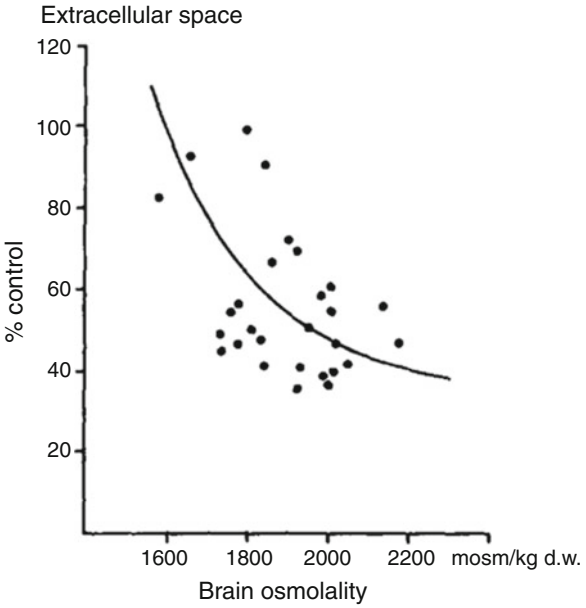

Fig. 12.13 An inverse correlation between the size of the extracellular space and tissue hypertonicity (hyperosmolality—mosm/kg dry wt), i.e., a correlation between the largest reduction in extracellular space and highest degree of hypertonicity, is consistent with the concept of a correlation between reduction in extracellular space and NKCC1-mediated ion uptake as part of a regulatory volume increase. This is especially the case since the water transport by NKCC1 does no fully compensate osmotically for its ion uptake [43]. The authors of the original paper are not responsible for this interpretation, but did regard such correlations as reflections of the interrelationship between ischemia and the development of brain edema. From Matsuoka and Hossmann [86], reproduced with permission

in a bumetanide-inhibited fashion [93]. A metabolic answer to NKCC1 activation, stimulation of oxygen consumption, is activated by even lower concentrations of ammonia than of K^+ [94, 95]. A third similarity between K^+ and NH_4^+ is that also NH_4^+ stimulates signaling by endogenous ouabains. In cultured astrocytes this is accompanied by an increased content of ouabain-like compounds [35]. Ouabain signaling activates production of reactive oxygen species (ROS) and nitrosactive agents which slowly sensitize NKCC1, explaining why cell swelling and brain edema normally take hours to develop after exposure to NH_4^+ ([96] and references therein). In cultured astrocytes, ammonia-induced cell swelling and ROS production (Fig. 12.14) can both be prevented by the main metabolite of spironolactone, canrenone, an aldosterone antagonist acting as a ouabain inhibitor [96, 97].

Since it is the α_2 isoform of the Na^+,K^+-ATPase which is stimulated by ultralow concentrations of ouabain, it is also this isoform that shows upregulated gene expression during exposure to elevated ammonia concentrations both in cultured astrocytes and in the brain in vivo [98].

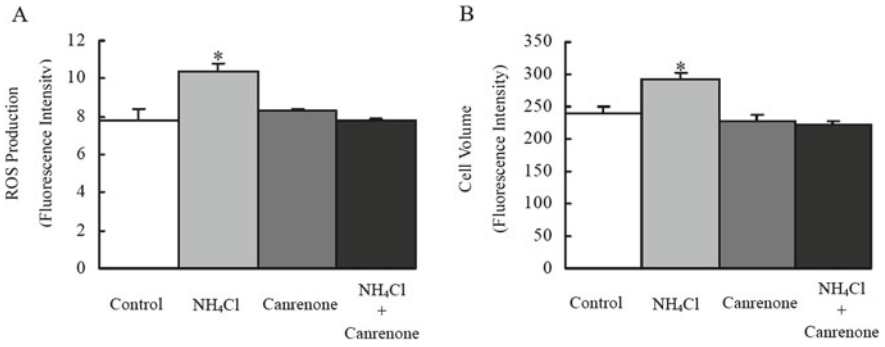

Fig. 12.14 Ammonia-induced ROS production and cell swelling can be inhibited by canrenone, an inhibitor of ouabain. (**a**) Cells were incubated with 0 or 3 mM NH_4Cl in the absence (control: no NH_4Cl, no canrenone) or presence of 100 μM canrenone for 2 h. ROS was determined as fluorescence intensity of oxidized carboxy-H_2DCFDA in individual cells in each of three cultures, averaged, and shown as mans ± SEM. (**b**) After incubation of the cells with 3 mM NH_4Cl for 12 h, cell volume was determined as fluorescence intensity of calcein, again in individual cells from three coverslips and averaged and expressed as in (**a**). From Dai et al. [96], reproduced with permission

In a recent paper Hadjihambi et al. [99] have suggested that the demonstration that an inhibitory effect of the NKCC1 inhibitor, bumetanide, potently suppresses ammonia-induced neurological dysfunction [100] points to a potential new target for treatment of hepatic encephalopathy. The authors express concern that the expression of NKCC1 also on astrocytes and on endothelial cells may produce off-target actions. In this context it should be noted that Kelly et al. [101] showed that bumetanide prevented several ammonia-induced abnormalities in cultured astrocytes and that both Jayakumar et al. [93] and Song et al. [97] found that bumetanide inhibited ammonia-induced swelling in such cells. Moreover, Jayakumar et al. [102] based on experiments in brain cortical slices treated with ammonia concluded "that targeting NKCC may represent a useful therapeutic strategy in humans with acute liver failure." Thus bumetanide treatment is not a new idea, and the effects on astrocytes and endothelial cells are therapeutic, probably for similar reasons as after ischemia and reperfusion.

Rangroo Thrane et al. [100] studied acute effects of very high plasma ammonia concentrations in intact, non-anesthetized mice and found evidence that the therapeutic effect of bumetanide was exerted on GABAergic neurons, where NKCC1 stimulation by NH_4^+ and an increased $[K^+]_o$ over-activate NKCC1. In turn this compromises inhibitory neurotransmission. This is similar to the effect described in Sect. 3.1 as a response by cortical neurons to a stimulation-induced increase in $[K^+]_o$, where Cl^- uptake via NKCC1 is depolarizing and excitatory [74]. The plasma ammonia concentrations obtained by Rangroo Thrane et al. [100] are at least one order of magnitude larger than those seen in hepatic encephalopathy [99]. This is especially important considering they were made in vivo in non-anesthetized animals, and hepatic disease leads to similar plasma concentration in rodents as in man [103].

Accordingly this study may be more directly relevant for the acute and deadly toxicity by very high concentrations of ammonia [104] a fact overlooked by Hadjihambi et al. [99]. Consistent with this concept virtually all animals died within 1 h and death was only postponed for ~10 min by bumetanide treatment [100]. This does not exclude that neuronal NKCC1 stimulation may contribute to the pathophysiology in hepatic encephalopathy provided the neuronal NKCC1 is also sensitized by oxidative and nitrosactive stress.

6 Conclusions

The present paper has attempted a comprehensive description of the mechanisms and roles of the astrocytic Na^+,K^+-ATPase. A considerable part of this is based upon experiments using mouse astrocytes in primary cultures and must ultimately be confirmed in intact brain tissue. However, several indications that they apply to astrocytes in situ are mentioned (similarity between K^+ effects on cells isolated from brain and on our cultured cells; effects of glycogenolysis in spreading depression; confirmation of β1-adrenergic pathway determined in cultured astrocytes using specific inhibitors by the ability of the same transmitters to prevent edema after ischemia and re-oxygenation). Moreover, initial uptake of excess K^+ in astrocytes is now well established and *must* be followed by return to neurons.

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Chapter 13
Uncoupling of P-Type ATPases

Yasser A. Mahmmoud

Abstract Cation-transporting P-type ATPases such as Na^+,K^+-ATPase, gastric H^+,K^+-ATPase, and sarcoplasmic reticulum Ca^{2+}-ATPase use energy from ATP hydrolysis to establish electrochemical gradients for ions across cellular membranes. These pumps also perform specialized functions. In particular, sarcoplasmic reticulum Ca^{2+}-ATPase is involved in nonshivering thermogenesis. We have identified the first chemical compound, capsaicin, which uncouples ATP hydrolysis from Ca^{2+} transport through Ca^{2+}-ATPase. Under physiological conditions, uncoupling of sarcoplasmic reticulum Ca^{2+}-ATPase is likely mediated by interaction with sarcolipin, a small protein highly expressed in skeletal muscle. In addition, we have characterized a drug that selectively abolishes K^+-dependent activity of the Na^+,K^+-ATPase, uncoupling Na^+- from Na^+,K^+ exchange. Here we provide basic information on the function and mechanism of P-type pumps. In addition, we review recent developments on the drug-mediated uncoupling of sarcoplasmic reticulum Ca^{2+}-ATPase and Na^+,K^+-ATPase.

Keywords P-type pumps • Sarcoplasmic reticulum Ca^{2+}-ATPase • Uncoupling • Membrane transport • Capsaicin • Capsazepine

1 Introduction

P-type ATPases (pumps) are integral membrane proteins that convert the free energy of ATP hydrolysis to ion gradients across cellular membranes [1, 2]. During turnover, P-type pumps discriminate between similar and abundant ions. To do so, they alternate between two major conformations, exposing high-affinity ion binding sites to either side of the membrane, to host the ion to be actively transported. P-type pumps are transiently phosphorylated from ATP during catalysis, so named

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"P-type." The major P-type ATPases found in man include plasma membrane Na^+,K^+-ATPase, sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), and gastric H^+,K^+-ATPase.

Na^+,K^+-ATPase is present in all animal cells. It exchanges intracellular Na^+ for extracellular K^+, at a stoichiometry of $3Na^+/2K^+$ per hydrolyzed ATP [3]. The enzyme consists of a catalytic α-subunit that undergoes ions- and ATP-dependent conformational transitions coupling ATP hydrolysis to the uphill transport of ions, and a glycosylated β-subunit that is important for function, folding, and plasma membrane delivery of the enzyme complex [4]. Small auxiliary proteins of the FXYD family interact with and regulate Na^+,K^+-ATPase activity in several tissues [5]. Under steady-state conditions, the Na^+,K^+-ATPase is challenged by several Na^+ influx events, associated with cellular processes such as nerve conduction, nutrient uptake, osmoregulation, and secretion. Remarkably, the Na^+,K^+-ATPase participates in a large number of physiological processes [6, 7], and emerging information indicates that the Na^+,K^+-ATPase also functions as a membrane receptor that converts extracellular signals to intracellular physiological responses [8, 9]. The receptor function of the pump seems to be uniquely controlled by interaction with cardiotonic steroids [10, 11], which are highly specific inhibitors of the NKA that increase cardiac inotropy (a member of this family is the plant-derived ouabain, the first known glycoside). Four different Na^+, K^+-ATPase α-subunit genes (α1–α4) and three β-subunit genes (β1–β3) have been described to date. α1 is expressed in almost all tissues, α2 is expressed in heart and skeletal muscle, α3 is found in the brain [12], whereas α4 is specifically expressed in testis [13]. Mutations in α3 have been found in several neurological disorders (see [14] for recent review).

Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps cytoplasmic Ca^{2+} into the sarcoplasmic reticulum (SR) lumen in exchange of luminal protons. Stimulation of the pumping activity of SERCA reverses the transient increases in cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$). In particular, SERCA plays a pivotal role in the rapid relaxation of heart and skeletal muscle cells. Cardiac SERCA is associated with the regulatory protein phospholamban (PLN), which regulates its pumping activity in response to adrenergic stimulation of the heart. In skeletal muscle, SERCA is associated with sarcolipin (SLN), a small protein believed to be involved in SERCA-mediated thermogenesis. Three SERCA genes are found in human; SERCA1 interacts with PLN and SLN in heart and skeletal fast-twitch muscle, respectively. SERCA2 is found in slow-twitch cardiac muscle, whereas SERCA3 is found in nonmuscular as well as cardiac tissue [15]. It is tempting to note that SERCA has no associated β-subunit, indicating a possible relationship between β-subunit in P-type pumps and active pumping of K^+ across the plasma membrane. SERCA mutations have been described in several diseases [16–18]. SERCA inhibitors are being tested as drugs for prostate cancer and cardiovascular disease [19–21].

H^+,K^+-ATPase is present in the parietal cells of the gastric mucosa. Stimulation of efferent vagus nerves to the stomach initiates a complex forward cascade that results in increased levels of Ca^{2+} and cAMP in the parietal cell, leading to stimulation of acid secretion by the H^+,K^+-ATPase and consequent acidification of the gastric lumen. The mammalian kidney contains a similar protein, the

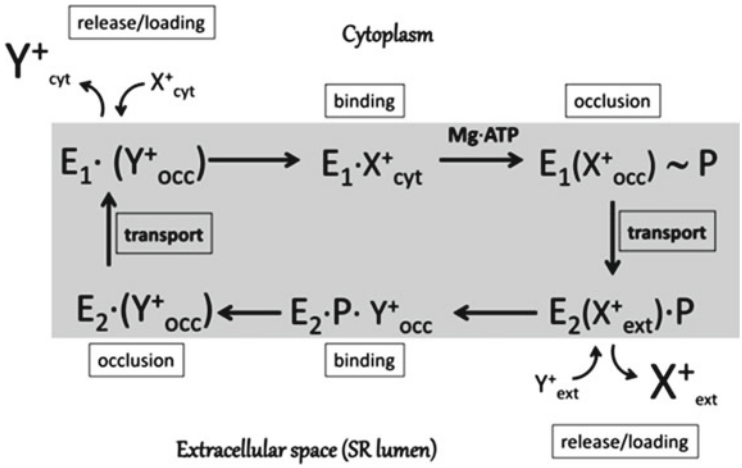

Fig. 13.1 A simplified Post-Albers mechanism for P-type pumps. Sequential steps of ion binding, occlusion, transport, and release are indicted in *boxes*. X represents a cytoplasmic ion; Na^+ (Na^+,K^+-ATPase), Ca^{2+} (Ca^{2+}-ATPase), or H^+ (H^+,K^+-ATPase). Y indicates an extracellular (or luminal) ion; K^+ (Na^+,K^+-ATPase and H^+,K^+-ATPase) or H^+ (Ca^{2+}-ATPase). ADP is omitted for clarity

nongastric H^+,K^+-ATPase, which is considered as a fourth member of the same group [22]. The H^+,K^+-ATPase is apparently not associated with regulatory proteins [23]. H^+,K^+-ATPase activity seems to be mainly regulated by membrane trafficking [24]; following stimulation of acid secretion in postprandial periods, inactive H^+,K^+-ATPase in intracellular vesicles is stimulated by translocation to the plasma membrane.

The Post-Albers scheme was proposed to describe the reaction mechanism of P-type pumps (Fig. 13.1). A number of cytoplasmic ions (X) bind to inward facing sites (i.e., located in the cytoplasm) in the E_1 form, providing the trigger for phosphoryl transfer in the presence of MgATP, forming an $E_1P(nX)$ intermediate with occluded ions. A conformational transition to the E_2P form releases the cytoplasmic ions to the extracellular or the luminal side of the membrane. Extracellular (or luminal) ions (Y) bind to the E_2P form, inducing dephosphorylation and formation of the $E_2(Y)$ intermediate with occluded ions. A transition to the E_1 form (aided by cytoplasmic ATP) releases Y to the cytoplasm (Fig. 13.1). Hence, ion binding configures the cytoplasmic domains to facilitate phosphorylation or dephosphorylation of the catalytic subunit and ATP facilitates release of the extracellular (luminal) ion to the cytoplasm.

P-type pumps share substantial structural similarities. This is expected as these proteins perform similar functions. However, structural dissimilarities between these pumps have been a matter of extensive investigations. Understanding the molecular bases of the dissimilarities would shed light on how these pumps discriminate between the different ions, a fundamental question in membrane transport

physiology. It is tempting to note that the different ion pumps establish widely different gradients across cellular membrane. Thus, Na^+,K^+-ATPase establishes an Na^+ gradient ($[Na^+]_{ext}/[Na^+]_{cyt}$) of ~15 folds across the plasma membrane. The Ca^{2+}-ATPase establishes a Ca^{2+} gradient ($[Ca^{2+}]_{SR}/[Ca^{2+}]_{cyt}$) of ~4 orders of magnitude across the sarcoplasmic reticulum membrane. H^+,K^+-ATPase establishes a H^+ gradient ($[H^+]_{lumen}/[H^+]_{cyt}$) of ~6 orders of magnitude across the parietal cell membrane. Information on how the structurally similar P-type pumps establish ion gradients with widely different magnitudes is beginning to emerge. In the case of the Na^+,K^+-ATPase, high extracellular Na^+ concentrations may impair active transport by forcing Na^+ to enter the extracellular sites in the E_2P form and reverse the enzymatic cycle. In marine organisms, extracellular Na^+ is as high as 400 mM. To ensure active transport under such conditions, four amino acids located at the external sites in squid pumps were found to be more positive compared to mammalian pumps [25]; hence, once Na^+ ions move in the forward direction, the reverse reaction is prohibited in squid pumps due to the excess positive charge at the site where reentrance may occur. In H^+,K^+-ATPase that establishes the largest gradient, forward pumping is secured by a different and unique mechanism. The N-terminus of the β-subunit has been shown to prevent the proton mediated reversal of the catalytic cycle by directly interacting with the phosphorylation domain [26, 27]. It is also notable that establishment of a large gradient by the H^+,K^+-ATPase would not be thermodynamically possible unless a pump stoichiometry change from $2H^+/2K^+$ to $1H^+/1K^+$ [28]. A recent electron crystallography structure of an E_2~$AlF(1Rb^+)$ intermediate grown under acidic conditions revealed a single occupied ion at site II [29], likely providing the structural basis for the hypothesized stoichiometry shift.

2 Uncoupling of P-Type Pumps

In all P-type pumps, hydrolysis of one ATP molecule has been thought to catalyze the transport of a fixed number of ions across the membrane. The rate of ion pumping (and consequently the rate of ATP hydrolysis) may increase by speeding up the conformational transitions, but the stoichiometry of transport during each cycle is expected to be constant. At least for the Na^+,K^+-ATPase, a change in pump stoichiometry in the presence of physiological substrate conditions is not expected under normal conditions but is likely to occur if the ion binding site(s) are modified. A modified binding site may fail to lodge the substrate ion despite its presence in the bulk medium. Such protein modification was made evident by the identification of spontaneous pump mutations having reduced interaction with Na^+ but normal interaction with K^+ [30]. Hence, uncoupling of Na^+,K^+-ATPase refers to the deviation from normal function described for unmodified enzyme ($3Na^+/2K^+/1ATP$). Pump function can be estimated by determining pump stoichiometry using radioactive isotopes (or other biophysical techniques), or preferably, by measuring the substrate-dependent stimulation of the enzyme's hydrolytic activity using different experimental approaches. Deviations from the normal $3Na^+/2K^+$ stoichiometry have

so far been observed under nonphysiological substrate conditions [31]. A decrease in the coupling ratio of the pump was reported to occur in the presence of cytoplasmic Na^+ concentrations as low as 200 μM (~25 folds less than normal cytoplasmic Na^+), where the pump is thought to operate with a partial number of ion binding sites [32]. In addition, incubation of red cells in media lacking Na^+ or K^+ is associated with ouabain-sensitive Na^+ efflux, referred to as uncoupled Na^+ efflux. It was reported to occur at a $3Na^+/1ATP$ stoichiometry [33] and is strongly dependent on extracellular Na^+ [34]. Whether or not protons are counter-transported during uncoupled Na^+ efflux is unclear. Investigations on K^+/K^+ exchange in red cells revealed that the Na^+,K^+-ATPase undergoes rapid phosphorylation/dephosphorylation cycles that substantially exceed the rate of ion exchange [35], implying that cation binding and substrate hydrolysis are linked together but are not tightly coupled. The Na^+,K^+-ATPase has also been shown to contribute to thermogenesis in animals [36]. Other studies speak against a role for liver Na^+,K^+-ATPase in thermogenesis [37]. To our knowledge, thermogenesis produced by purified Na^+,K^+-ATPase has not been unequivocally demonstrated by calorimetric studies. We consider it likely that uncoupling of the Na^+,K^+-ATPase is not directly linked to thermogenesis, but to events associated with the receptor (non-pumping) function of the Na^+,K^+-ATPase [9, 38–40]. Hence, the dynamic distribution of pumping and non-pumping pumps may give rise to fluctuations in ATP utilization and hence in the rate of heat production.

3 Heat Generation by Sarcoplasmic Reticulum Ca^{2+}-ATPase

In the case of SERCA, uncoupling refers to ATP hydrolysis not associated with Ca^{2+} transport to the SR lumen, giving rise to a [Ca^{2+} transport/ATP hydrolysis] ratio of less than one. In contrast to Na^+,K^+-ATPase, uncoupling of SERCA has been shown to be physiologically important. Ca^{2+} cycling across the SR membrane mobilizes considerable amounts of heat. Initially, Ca^{2+} cycling was thought to occur solely by two opposite mechanisms; passive Ca^{2+} efflux from SR through ryanodine receptor (RyR) and active Ca^{2+} influx to the SR through SERCA. Several studies have demonstrated a decrease in the coupling ratio of SERCA in the presence of Ca^{2+} gradient across the SR membrane [41, 42]. Indeed, heat emission from living organisms fully explains why different people have differential abilities to store fat. Energy released by oxidation of food is used to keep the basic metabolic rate, energize skeletal muscle activity, stored in tissues in the form of glycogen and fat, or released as heat. The heat produced is dissipated by subcutaneous vasodilatation which increases blood flow to the periphery, resulting in convective heat loss.

The importance of the sarcoplasmic reticulum proteins in regulating tissue thermal balance can be emphasized by looking into the heater organ of fish and the pathological condition known as malignant hyperthermia. The “heater organ” is a derivative of muscle that is relatively devoid of contractile elements. These specialized cells make up most of the superior rectus muscle in the orbit and generate heat

for the brain and eyes during cold-water dives, providing heat to maintain the function of the eye and adjacent brain at temperatures as high as 14 °C over the water temperature. Like typical muscle cells, heater cells possess abundant acetylcholine receptors and have an extensive network of sarcoplasmic reticulum and T-tubules. Mitochondria are also extremely abundant in heater cells, comprising over 60 % of total cell volume [43, 44]. Interestingly, RyR channels expressed in this organ are similar to that in the mammalian slow-twitch muscle (which is thought to be more important for thermogenesis than fast-twitch muscle). Thermogenesis in heater cells is initiated by depolarization, which causes calcium release by the sarcoplasmic reticulum. ATP is then consumed by Ca^{2+}-ATPase, which returns calcium to the sarcoplasmic reticulum. Reduction in the ATP pool drives fuel oxidation. Thus, depolarization-induced calcium entry into the cytoplasm is directly linked to Ca^{2+} cycling and subsequent thermogenesis [45].

In mammals, the potential of Ca^{2+} cycling is demonstrated by malignant hyperthermia, wherein in genetically predisposed individuals or animals (a mutation in the skeletal muscle ryanodine receptor [46]), certain environmental factors such as some anesthetics, or stress, can make the sarcoplasmic reticulum leaky, with an ensuing hyperthermia. Furthermore, it is possible that some leakage occurs in the normal resting muscle contributing to obligatory thermogenesis. Based on observations made in isolated sarcoplasmic vesicles, it was estimated that the calcium recycling across the sarcoplasmic membrane could account for 30–70 % (depending on the calcium pool size in the muscle) of the resting muscle energy expenditure. As mentioned, muscle is a major site of nonshivering thermogenesis in birds, and it has been found that SERCA1 and RyR channels increase in muscle of ducklings during cold adaptation. The above-mentioned observations support to the hypothesis that sarcoplasmic reticulum calcium leak; coupled to rapid recapture by SERCA could subserve a thermogenic role.

4 Identification of SERCA Uncoupling Drugs

ATP hydrolysis by SERCA has been proposed to be the major source of heat in skeletal muscle [47, 48]. SR preparations have been shown to undergo slippage, where the pump fails to actively transport occluded Ca^{2+}, leading to a decrease in the coupling ratio. Slippage increases significantly in the presence of high Ca^{2+} concentration in the SR lumen [49]. SERCA is thought to be quiescent in the resting skeletal muscle (perhaps with basal activity associated with Ca^{2+} dependent signal transduction events). We have identified the first chemical factor that produces SERCA uncoupling [50]. Capsaicin is an active component of chili peppers, a plant that belongs to the genus *Capsicum*. It produces a burning effect resulting from stimulation of the transient receptor potential vanilloid type (TPRV1), highly expressed in sensory neurons. The burning effect is due to a capsaicin-mediated Ca^{2+} influx through TPRV1. We have documented that capsaicin produces severalfold stimulation of the hydrolytic activity of SERCA. The stimulation by

capsaicin requires Ca^{2+} gradient across the SR membranes, as detergent-opened membrane fragments are stimulated by capsaicin to a much lower level. A series of kinetic and biochemical analyses were performed to unravel the mechanism whereby capsaicin modulates SERCA [50]. Capsaicin did not affect Ca^{2+} accumulation into SR vesicles nor did it affect equilibrium Ca^{2+} binding, showing that the plant-derived vanilloid did not affect the high-affinity Ca^{2+} (E_1) form. Capsaicin increased the rate of phosphoryl transfer ($E_1 \rightarrow E_1P$) but decreased the rate of ADP-dependent dephosphorylation. The drug increased the rate of E_2P hydrolysis and this could explain the significant stimulation of steady-state ATP hydrolysis. The observed effect of capsaicin on SERCA, i.e., accelerating phosphoryl transfer and hydrolysis without affecting Ca^{2+} binding, is consistent with the conclusion that capsaicin interacts with the cytoplasmic domains of the pump, but not the transmembrane domains. This conclusion is supported by proteolytic cleavage experiments. Digestion with proteinase K of SERCA stabilized in the E_1 form produces a cleavage between Thr^{242} and Glu^{243} (producing an N-terminal p28N and a C-terminal p83C [51]). An extra cleavage occurs between Leu^{119} and Lys^{120} in the presence of ligands stabilizing the E_2 form (producing an N-terminal p14N and a C-terminal p95C [52]). Capsaicin was found to increase the amount of the p28N fragment compared to control samples, showing that capsaicin exposes the link between the A domain and M3 [50]. Hence, we concluded that capsaicin controls the position of the A domain, explaining how capsaicin affects phosphoryl transfer and phosphoenzyme hydrolysis, both events are controlled by A domain rotation [53]. The proteolytic cleavage data strongly indicate that capsaicin interacts directly with SERCA, that is, the increased SERCA activity is not due to increased Ca^{2+} influx through other passive Ca^{2+} routes. The discovery that capsaicin induced direct effects on SERCA indicates that capsaicin (or related drugs) may directly induce uncoupled ATP hydrolysis in the resting muscle cell, in other words, increased passive Ca^{2+} transport through channels in the SR (or in the plasma membrane) is not a prerequisite for the increase in the hydrolytic activity of SERCA. The effect of ingested capsaicin on heat generation may thus be accounted for (at least in part) by its direct effect on SERCA. The cross-reaction of capsaicin with at least three Ca^{2+}-handling proteins together with its low affinity effect on SERCA precludes its use as an uncoupling pharmacon. However, the discovery of this plant-derived drug would suggest the existence of animal-derived uncoupling agents.

Focus was then directed to animal-derived molecules that can function similar to capsaicin. Searching among different structurally related molecules sheds light into several endogenous lipid metabolites of the multifunctional endocannabinoid family of drugs, which function as effectors of the cannabinoid receptor (CB) in the central nervous system [54], and vanilloid receptors in sensory neurons [55]. We have identified *N*-arachidonoyl dopamine (NADA) as a relatively potent stimulator of SERCA uncoupling ($K_D = 0.36 \pm 0.12$ μM). The effect of NADA is highly specific, as the structurally related molecules *N*-palmitoyl dopamine and *N*-oleoyl domapine produce no effect on SERCA. Interestingly, NADA was found to modulate SERCA through a mechanism distinct from that of capsaicin [56]. NADA interacts potently with SERCA in the presence of sub-μM Ca^{2+} concentrations,

i.e., comparable to that in the resting muscle cell. Higher Ca^{2+} concentrations increase the K_D for NADA stimulation (i.e., decreases the affinity of SERCA for NADA). NADA also requires a Ca^{2+} gradient to produce SERCA uncoupling; however, in the absence of Ca^{2+} gradient, NADA instead inhibits SERCA activity (the biochemical basis of the inhibition of SERCA by NADA in open membrane fragments is outside the scope of this report). In contrast to capsaicin, NADA was found to stabilize an E_1 form of SERCA, as evidenced from the NADA induced decrease in Ca^{2+} interaction with the luminal sites. NADA decreased the rate of SERCA dephosphorylation which is unexpected for a drug that increases the hydrolytic activity of SERCA. Proteinase K cleavage showed that NADA increased the protection of phosphorylated SERCA at neutral pH [56], in opposition to what was obtained after capsaicin treatment [50]. We have proposed that NADA modifies SERCA by modifying the communication between the A and P domains. This would likely impair the $E_1P \rightarrow E_2P$ transition and hence coupled Ca^{2+} transport across the membrane [50]. Investigations on cultured skeletal muscle cells revealed that 100 nM NADA was enough to produce a significant decrease in cytoplasmic ATP levels in the absence, but not in the presence, of 5 mM glucose [56]. This result is expected as glucose would rapidly restore ATP levels through increased oxidative phosphorylation. The decrease in cellular ATP levels was fully reversed by pretreatment with Tg, indicating that the effect is mediated through direct interaction of the drug with SERCA.

Figure 13.2 shows the effect of another member of the endocannabinoid family, arachidonoyl ethanolamine (AEA, anandamide), on SERCA activity in the presence of different Ca^{2+} concentrations. Strikingly, the apparent affinity for AEA is strongly dependent on the free Ca^{2+} concentration present in the extravesicular medium, i.e., cytoplasmic Ca^{2+}. Hence, the uncoupling produced by these molecules requires low cytoplasmic Ca^{2+}. Uncoupling relies on Ca^{2+} binding, phosphate transfer from ATP, but failure to transport Ca^{2+} to the SR lumen and hence Ca^{2+} return to the cytoplasm. Low cytoplasmic Ca^{2+} concentrations would favor this Ca^{2+} slippage from the binding sites. It was concluded that the long chain polyunsaturated fatty acid chain is responsible for the uncoupling effect of these drugs, with the different head group structure of the uncoupling molecule responsible for the fine kinetic effects on SERCA. It would be of particular importance to develop molecules that interact with SERCA but not with the other targets (CB1 or TRPV1 receptors [54, 55]). This strategy will develop SERCA uncoupling drugs without indirect stimulation of passive Ca^{2+} fluxes across the SR membrane through the other routes.

SLN is now well recognized that sarcolipin, as a SERCA uncoupling protein, increases thermogenesis by skeletal muscle cells [57, 58]. This demonstrates that SERCA uncoupling may be produced by several independent factors. The importance of the SERCA-SLN interaction has recently emphasized by new crystal structures showing that SLN co-crystallizes with an E_1-like form of SERCA grown in the presence of Mg^{2+} and in the absence of Ca^{2+} [59, 60]. SLN was found to stabilize the Ca^{2+} empty E_1-Mg^{2+} state, lying between M2, M6, and M9, and seems to retard the movement of M2 and hence transition to the $E_2(2Ca^{2+})$ form. Biochemical investigations are necessary to understand the dynamics of the M2 movement mediated by SLN and how it leads to phosphoryl transfer not coupled with Ca^{2+} transport.

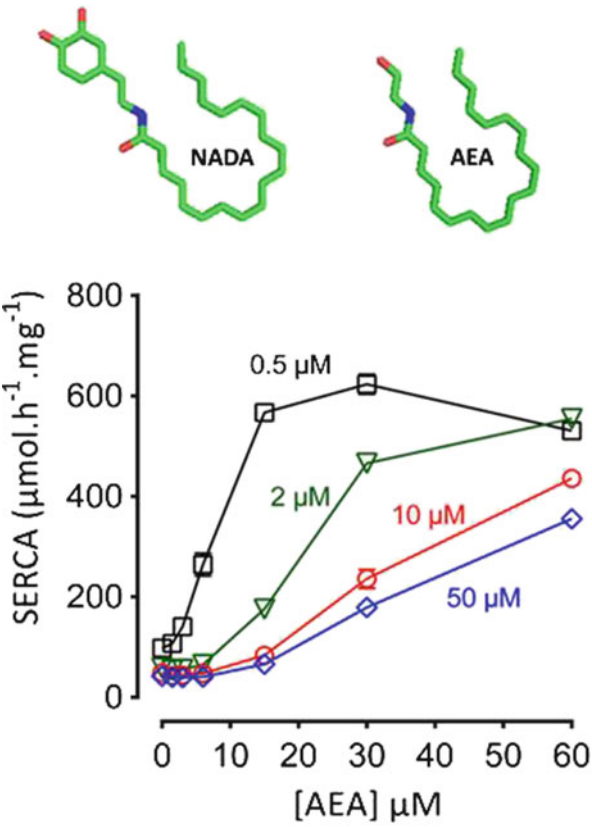

Fig. 13.2 SERCA uncoupling molecules. *Top*. Structure of *N*-arachidonoyl dopamine (NADA) and arachidonoyl ethanolamine (AEA). *Bottom*. Effect of arachidonoyl ethanolamine (AEA) on SERCA activity, measured at 37 °C as described in [56]. The ATPase reaction contained 30 mM MOPS buffer pH 7.2, 0.5 μg SR vesicles, 130 mM KCl, 3 mM MgATP and the indicated AEA concentrations. The reactions were performed at different Ca^{2+} concentrations (calculated using WinmaxC), as shown

5 Drug-Mediated Uncoupling of the Sodium Pump

Capsazepine (CPZ) is a synthetic vanilloid antagonist that was developed to abrogate the stimulation of TRPV1 by capsaicin [61, 62]. CPZ was found to produce no effect on the hydrolytic activity of the Na^+,K^+-ATPase in the absence of K^+. However, CPZ inhibits the pump in the presence of K^+, showing that the drug selectively abolishes the contribution of K^+ to pump activation. In order to understand how this is achieved, we have performed a large set of biochemical and biophysical experiments. CPZ was found to increase the apparent affinity for K^+ with no effect on Na^+ affinity [63]. The drug strongly decreased the steady-state phosphoenzyme level measured in the presence of micromolar ATP concentrations at 0 °C. However, CPZ was found to stabilize this remaining small amount of phosphoenzyme. The phosphoenzyme intermediate stabilized by CPZ is increased by ADP, showing that CPZ stabilizes an ADP-insensitive, unique pump conformer. Strikingly, CPZ has no effect on active $^{22}Na^+$ influx into liposomes containing inside-out pumps when the ATP concentration was 10 μM, but inhibits the flux at 300 μM ATP. These results indicate that CPZ separates an Na^+ cycle from an Na^+/K^+ cycle in the pump; the Na^+/

K^+ cycle seems inoperative in the presence of low ATP concentrations but only operates at saturating ATP. CPZ only attacks the K^+ cycle, leaving the Na^+ cycle intact. This conclusion challenges the concept of coupled $3Na^+/2K^+$ transport and suggests that one Na^+ has its own entrance and exit pathway. A similar proposal was indeed concluded in early studies on reconstituted Na^+,K^+-ATPase; detailed kinetic analyses indicated the presence of two separate sodium pump cycles, one releasing a single Na^+ per ATP molecule hydrolyzed [64, 65].

Additional biochemical and biophysical studies have been performed to understand the CPZ modification of the Na^+,K^+-ATPase. Patch clamp experiments showed that CPZ decreased pump-mediated current in the presence of extracellular K^+. In the absence of K^+, however, CPZ increased pump current, indicating increased Na^+ efflux [66], in full agreement with the in vitro measurement on the kidney enzyme [63]. This conclusion was further supported by employing Asante NaTRIUM green II (ASG II), an Na^+ sensitive dye. Pump-mediated change in intracellular Na^+ was estimated by measuring the fluorescence of ASG II loaded into cultured human embryonic kidney cells. In the presence of extracellular K^+, CPZ increased ASG II fluorescence, indicating an increase in intracellular Na^+ as a consequence of inhibition of the coupled Na^+/K^+ transport. However, in the absence of K^+ (Na^+/Na^+ exchange conditions) the fluorescence instead decreased, showing an increase in Na^+ efflux from the cells [66]. Thus, CPZ abolishes the K^+ transporting steps (and hence the Na^+ transport associated with it) but stimulates Na^+ release.

The above studies were further confirmed using shark Na^+,K^+-ATPase reconstituted in lipid vesicles, where the effect of CPZ on the electrogenicity of the pump can be estimated. Liposomes containing inside-out reconstituted pumps (orienting their cytoplasmic domains outside the liposomal lumen) can be stimulated by MgATP to catalyze Na^+ influx ($3Na^+/2Na^+$ stoichiometry) into liposomes (cellular efflux). This brings a net positive charge across the liposomal lumen, producing an inside positive membrane potential that can be registered as a function of time using the potential sensitive dye oxonol VI. When reconstituted pump was incubated in media containing Na^+ and Mg^{2+} and then stimulated by 75 μM ATP, oxonol VI fluorescence increased, likely reflecting the exchange of two intravesicular Na^+ with three extravesicular Na^+ and hence establishment of a membrane potential across the liposomal membrane, positive inside. The fluorescence reached maximum (plateau) when the inside positive potential impairs active Na^+ release from the $E_2P(Na^+)$ conformer. Addition of CPZ increased the fluorescence to new plateau with a magnitude that was twice the plateau obtained in the absence of CPZ [66]. Thus, CPZ seems to decrease the sensitivity of the extracellular sites for Na^+. When the intravesicular medium was made to contain 30 mM K^+, the same result was obtained except for the higher rate of oxonol fluorescence associated with Na^+/K^+ exchange, reflecting the difference in affinity between extracellular K^+ and extracellular Na^+. The increase in fluorescence seen after CPZ treatment in the presence of K^+ is likely an effect on Na^+/Na^+ exchange, as K^+ is rapidly washed out of the liposomes. The most striking observation was the effect of CPZ on oxonol VI fluorescence in the presence of ion gradient across the liposomal membrane. In the presence of a K^+ gradient, CPZ produced an ATP-independent increase in

fluorescence. This effect is K^+ specific; a similar Na^+ gradient produced no effect on fluorescence. Thus, the increase in fluorescence is not associated with active transport but is likely related to electrogenic K^+ binding facilitated by the chemical gradient across the membrane. It does not seem likely that K^+ is transported to inside the vesicles. Extravesicular (cytoplasmic) K^+ likely binds to a site in the protein and produces a shift in the orientation of the negatively charged oxonol dye in the membrane and hence an increase in fluorescence.

Studies on purified enzyme showed that the insensitivity of Na^+-ATPase to CPZ depends on the medium pH. Thus, CPZ was found to inhibit Na^+-ATPase at basic pH values, showing that the interaction of CPZ with the pump is switched by protons. In other words, CPZ blocks the K^+ sites at acidic and neutral pH and apparently blocks all three sites at basic pH (as observed from the complete loss of Na^+-ATPase activity induced by CPZ). Wang and Horisberger observed an inward proton current through the pump in the absence of external Na^+ and K^+; the current is stimulated by a decrease in extracellular pH or at more negative membrane potential [67]. Proton transport has been linked to the unique Na^+ site as evidenced from site-directed mutagenesis combined with electrical measurements [68]. Recently, patch clamp studies revealed that proton leak occurs independently from Na^+/K^+ exchange, but was found to be facilitated by impairment of forward K^+ binding/occlusion [69]. Similar conclusions were attained using pumps expressed in oocytes [70]; however, the authors stated that "binding of two Na^+ or two protons to sites I and II inhibits proton transport." These results point to a high degree of interaction between the Na^+ unique site and the shared sites.

Thus, we concluded that one of the Na^+ sites (likely site III) functions independent of the two other sites [63, 66]. The pump may catalyze uncoupled Na^+ transport wherein a single Na^+ ion is able to bind from the intracellular side and be released to the external medium. For this to occur, it is necessary to assume that two of the sites are either constitutively occupied with two K^+ ions or blocked [66], and that the site through which uncoupled transport occurs may have entrance and exit pathways distinct from the other sites. That transport across the Na^+ specific site occurs through a distinct pathway independent from the shared sites has been proposed earlier [71]. In order to investigate these proposals, we sought to use ion congeners that bind the different sites on exclusive basis. Studies by the group of Artigas have introduced what we have been looking for. Based on early studies on Na^+ channels [72, 73], they tested the responsiveness of the Na^+,K^+-ATPase to several alkali metal and organic cations. They concluded that guanidinium (Gua^+) functions as an Na^+ congener at the Na^+ unique site but not the shared sites, as it produces voltage-dependent inhibition of an outward current in the presence of external K^+ and produces inward current by permeating the pump at negative potentials [74]. On the other hand, acetamidinium ($Acet^+$) and formamidinium ($Form^+$) were reported to function as K^+ congeners, interacting exclusively with the shared sites [75].

Trypsin cleavage has been used to study conformational transitions of the α-subunit of the renal Na^+,K^+-ATPase [76, 77]. Incubation with trypsin in the presence of Na^+ produces two cleavages at the N-terminal third of the α-subunit (site T_2 at the N-terminal tail and site T_3 in the A domain) whereas in the presence

of K^+ a single cleavage at the middle of the α-subunit occurs (site T_1 in the N domain [76, 77]). We were the first to study the effect of Gua^+ on pump conformation [66]. Gua^+ produced cleavage preferentially at T_3, i.e., a similar cleavage pattern as expected for the $E_1(Na^+)$ form. Importantly, a short peptide was exposed to cleavage by trypsin in the presence of Gua^+ (site T_4). This fragment is derived from the C-terminus of the α-subunit following cleavage between R1005 and P1006 [66], showing that the distal part of M10, expected to be buried in the membrane, becomes exposed to trypsin attack in the Gua^+ bound form. Figure 13.3 shows the location of the T_4 site in the crystal structure of the C-terminal M8M10 region of the α-subunit in the $E_1{\cdot}AlF_4^-{\cdot}ADP{\cdot}3Na^+$ form (PDB accession nr 3WGU [78]) and the $E_2{\cdot}MgF_4^{2-}{\cdot}2K^+$ form (PDB accession nr 2ZXE [79]). The architecture of the M8M10 region is almost identical in both structures, except for a tiny disposition of the C-terminal tail (~2.5 Å) and an even smaller shift in the M8M9 loop. Factors that expose the C-terminal tail include Gua^+, high pH (known to shift the conformational equilibrium of the pump toward the E_1 form), and CPZ [66]. Notably, Gua^+ does not produce ATP hydrolysis either alone or in combination with Na^+ and K^+ despite being occluded in the pump.

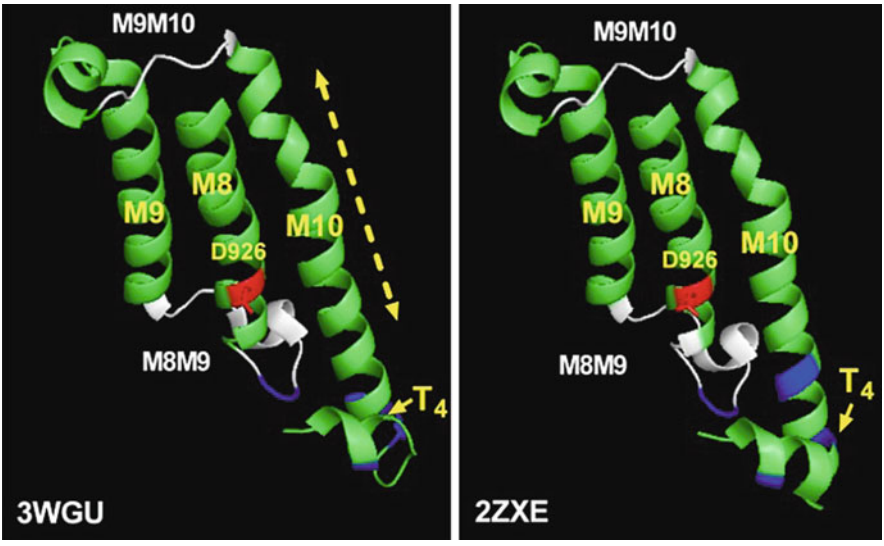

Fig. 13.3 Structure of the C-terminal part of the Na^+,K^+-ATPase α-subunit. The figure shows the M8M10 part in the two available conformations. The location of the T_4 site is indicated by *arrow*. D926, located in M8, is shown in *red*. The intracellular M8M9 loop and the extracellular M9M10 loop are shown in *white*. Positive amino acids in the M8M9 loop and in the C-terminal tail are shown in *blue*. The *dashed yellow line* indicates a hypothetical situation where movements of M10 perpendicular to the membrane—likely regulated by changes in membrane potential—would change the position of the C-terminal tail. Movement of M10 would likely change the position of D926 in M8, thereby regulating Na^+ interaction with the pump. D926 is an important, Na^+ affinity-determining residue. Taken from [66]

6 Conclusions

Novel information on the uncoupling of SERCA and Na^+,K^+-ATPase has been uncovered. We have identified the first SERCA uncoupling agent and characterized its interaction with SERCA. In addition, we provided information on how uncoupling would be achieved in mammals through interaction of Ca^{2+} transport proteins with multifunctional lipid metabolites of the endocannabinoid family. These studies will hopefully persuade future studies on how the lipid metabolites are synthesized and released to their precise sites of action (e.g., [80]).

We have demonstrated uncoupling of the sodium pump by chemical modification. The uncoupled pumps release a single Na^+ with the shared site likely filled with strongly bound K^+. Previous studies on H^+,K^+-ATPase have indicated that a mutation adding a positive charge in M6 (neutralizing a negative charge on Glu820), mimicking K^+ binding, fully activate H^+,K^+-ATPase activity and compensate for the absence of K^+ [81]. Whether uncoupling of the Na^+,K^+-ATPase may have physiological significance remains to be determined. A major unresolved issue is how the Na^+ specific site communicates with the shared sites [66]. Gua^+ and H^+ were found to leak through what is meant to be site III in the pump. In contrast, Na^+ was found not to permeate [75]. The pump likely contains a sort of valve that prevents the reverse flow of Na^+ but allows other ions to permeate. Our results [66] indicate that the highly polar M10 may be involved in the regulation of ion interaction with site III, thereby regulating ion interaction with the shared sites.

In the crystal structure of the E_1 form [78], D926 coordinates Na^+ in the unique site, explaining the strong effect of the mutation of Na^+ affinity without affecting K^+ interaction. Interestingly, D926 is the only ion-coordinating residue present in M8; all the other coordinating residues are located in M4, M5, and M6. Based on our results as well as literature data, it seems likely that αM10 functions as a membrane potential responsive element. In studies on Ca^{2+}-ATPase, the αM7-M10 domain has been thought of as a membrane anchor [82, 83]. Yet, αM10 contains a relatively large number of polar amino acids and is thus expected to be loosely associated with the membrane. This expectation is supported by thermal denaturation studies reporting the exposure of the M9M10 loop to the extracellular medium upon heating [84–86]. In consistence, we showed previously that low temperatures expose cytoplasmic stalk segments of the regulatory protein FXYD10, implying movement of the domains toward the cytoplasm [87]. A large number of studies have indicated the importance of the C-terminus of the pump in ion selectivity and voltage dependence [74, 88–91], suggesting its involvement in the regulation of an ion permeation pathway. That αM10—carrying the C-terminal tail—moves perpendicular to the membrane would explain how the ion permeation pathway is regulated. The distal part of αM10 contains several positively charged amino acids, favoring the aforementioned idea. In this regard, the K1003E mutation, located in the membrane upstream the C-terminal tail, was shown to shift the voltage dependence of charge translocation (Na^+/Na^+ exchange conditions) by −12 mV compared to wild-type enzyme [92]. Finally, the exposure of the C-terminal tail to trypsin

cleavage in response to the different stimuli [66] indicates that αM10 moves during conformational changes (dashed arrow in Fig. 13.3). In principle, modifying the position of αM10 (and the associated C-terminal tail) would regulate two functionally important events; (1) indirect effect on the position of M8 carrying D926 and (2) direct interaction with the ion binding core through the C-terminal tail, thereby modulating the inclination of αM5 (expected to modulate ion selectivity at the shared sites). Crystal structures—based on protein crystals grown in the absence of membrane potential—would not be able to judge the effect of membrane potential on pump structure. Biophysical and biochemical studies will be required to confirm whether or not αM10 moves in response to changes in membrane potential.

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Part II
Modulatory and Regulatory Aspects

Chapter 14
Phospholemman: A Brief Overview

Sajal Chakraborti, Kuntal Dey, Md Nur Alam, Amritlal Mandal, Jaganmay Sarkar, and Tapati Chakraborti

Abstract Na^+/K^+-ATPase (NKA) plays the key role in maintaining Na^+ and K^+ gradients in cells, which is essential for regulation of cell volume and membrane potential. PLM (aka FXYD1) interacts with NKA and Na^+/Ca^{2+} exchanger (NCX) and modulates their activities in tissue specific and physiological state specific manner. Protein kinase A (PKA) and protein kinase C (PKC) targets different pools of PLM associated with NKA and NCX, thereby regulating their activities. Additionally, some signal transducers such as phosphatases, phosphodiesterases and nitric oxide play important roles in modulating functions of PLM, especially under phosphorylated conditions, toward the activities of NKA and NCX. Understanding the above phenomenon is of significance in developing novel therapeutics for recovery of patients suffering from a variety of pathophysiological conditions, especially cardiovascular and neural diseases.

Keywords Phospholemman • FXYD1 • Na^+/K^+-ATPase • Na^+/Ca^{2+} exchanger • Ageing • Exercise

1 Introduction

Na^+/K^+-ATPase (NKA) consists of two subunits, α and β. The enzyme plays an important role in maintaining Na^+ and K^+ gradients in cells, which is essential for preservation of cell volume and membrane potential. Phospholemman (PLM or FXYD1) is one of the members of the NKA regulatory proteins, FXYDs. FXYD denotes for the conserved extracellular Phe.ala-X-Tyr-Asp motif. PLM was first

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identified in 1985 as a 15 kDa sarcolemmal (SL) protein [1]. In humans, PLM gene is localized to chromosome 19q13.1 [2]. PLM is expressed predominantly in tissues that are electrically excitable or are specialized in transport [3, 4], while in other tissues, PLM mainly functions as a regulator of cell volume via NKA [5, 6].

PLM interacts specifically with the NKA and modulates its functional properties in a tissue specific and physiological state specific manner [7–10]. The functions of PLM are mediated by its cytoplasmic domain, which associate with different regions of the α-subunit of NKA and regulate the enzyme activity by mechanisms such as phosphorylation, glutathionylation and palmitoylation [11].

Understanding the physiological functions of PLM in different tissues, for example, in heart are evident from studies with PLM knockout mice [12]. NKA enzymatic activity is higher in heart of PLM knockout mice, which has been shown to reverse the NKA inhibition caused by PLM. Higher NKA activity in the PLM knockout heart results in lower$[Na^+]_i$ and that leads to a decrease in cardiac contractility [13]. However, basal $[Na^+]_i$ was similar between wild-type and PLM knockout cardiac cells, for example, myocytes [14, 15], indicating that PLM does not affect the basal tone.

2 Localization of PLM

All of the FXYD proteins, except PLM, were primarily found in the basolateral plasma membrane. PLM was found to colocalize and sequester in the endoplasmic reticulum (ER) by its last three carboxy terminal amino acids (RRR) motif. The C-terminal cytoplasmic tail of human, dog, rabbit and rat PLM contains three serines (at residues 62, 63 and 68) and one threonine (at residue 69), which is replaced by serine in mouse PLM [7]. Cellular stimulation activates second messengers leading to the phosphorylation of SSST: 62, 63, 64, 69 amino acid residues of PLM. The increased translocation of phosphorylated PLM to the plasma membrane may reflect its ability to override retention signals in the ER. Alternatively, phosphorylation of PLM may allow it to interact with other factors and traffic to the plasma membrane. Upon entry to the plasma membrane, the phosphorylated PLM associate with other proteins to perform its physiological function [16]. Understanding the regulation of PLM phosphorylation could provide insights into the function of PLM and its putative protein partners. The relationship between PLM and the Na^+ pump is dynamic and that PLM can dissociate from the pump once it comes at the cell surface [17].

3 Structural Aspects of PLM-NKA

PLM is synthesized as a 92 amino acid peptide with a 20 amino acid N-terminus signal peptide, which is cleaved off during processing. The modified protein contains 72 amino acid residues of which the first 17 amino acids reside in the extracellular domain (H1). The transmembrane (TM) region contains 20 amino acids

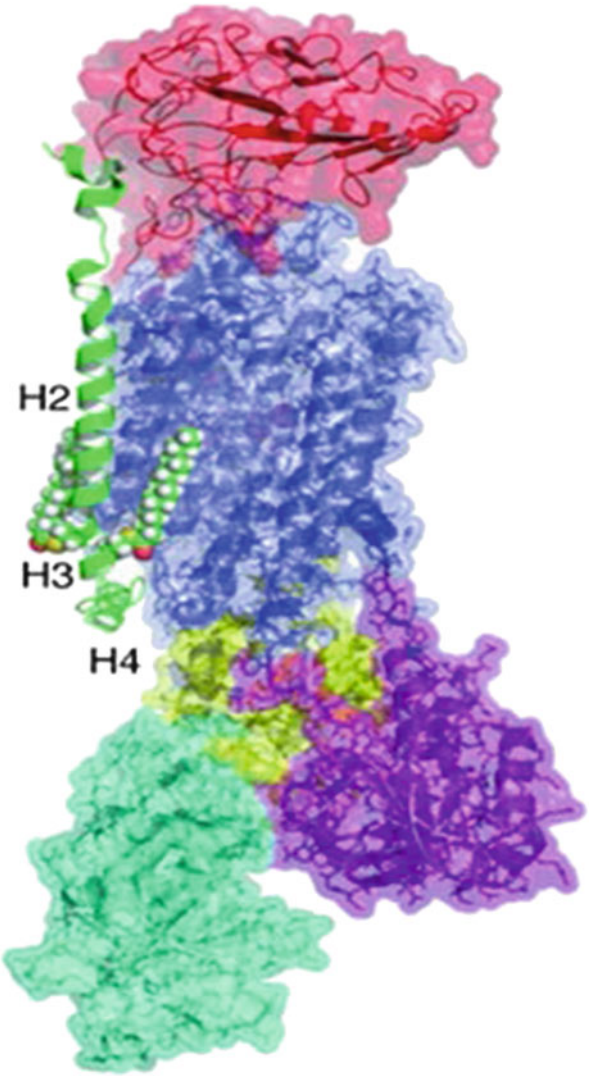

Fig. 14.1 Position of PLM relative to the α-subunit of NKA is shown in *green*, N domain in *cyan*, P domain in *yellow*, and A domain in *purple*. The β-subunit is *red*. Sodium (*purple sphere*) is shown in its proposed binding sites. Helices H2, H3 and H4 of the PLM are labelled. Taken from Ref. [74] with permission

(H2) followed by a short chain (H3) and a stretch of amino acids (H4), which are located at the C-terminus in the cytoplasm [4, 18]. Two domains of PLM, the membrane domain (H2) and the C-terminal domains (including H4) are involved in binding with NKA (Fig. 14.1).

4 PLM and NKA Activity

PLM is a major substrate for both protein kinase A (PKA) and protein kinase C (PKC). PLM can be phosphorylated by PKA at Ser68 and by PKC at both Ser63 and Ser68 [19], which paralleled positive inotropic response of the heart [20, 21].

PLM is unique among FXYD proteins in that it has consensus sequence for phosphorylation by PKA (RRXS), PKC (RXXSXR)n and never in mitosis aspergillus (NIMA) kinase (FRXS/T). PLM is also a substrate for myotonic dystrophy protein kinase [4]. Elevation of cAMP that occurs in β adrenergic receptor activation upon isoproterenol treatment to cells activates cAMP-dependent kinase (PKA), which in turn phosphorylates Ser68 on PLM [21]. This not only overcomes PLM inhibition of the Na^+/K^+ pump, but also stimulates the NKA activity by increasing V_{max}, thereby augmenting sensitivity of the pump to intracellular Na^+ [22]. The stimulatory effect of isoproterenol on the pump has not been observed in PLM knockout mice [24]. Figure 14.2 depicts schematically the relationship between PLM and NKAα subunit and subsequent alterations induced by PLM upon phosphorylation.

PKA and PKC targets different pools of PLM associated with different NKA isozymes. PKA phosphorylates the pool of PLM which is associated with NKAα1β1 isozyme and increase their apparent Na^+ affinity [23]. On the other hand, PKC phosphorylate PLM which is associated α2β1 isozymes, whereas activation of α2β1, but

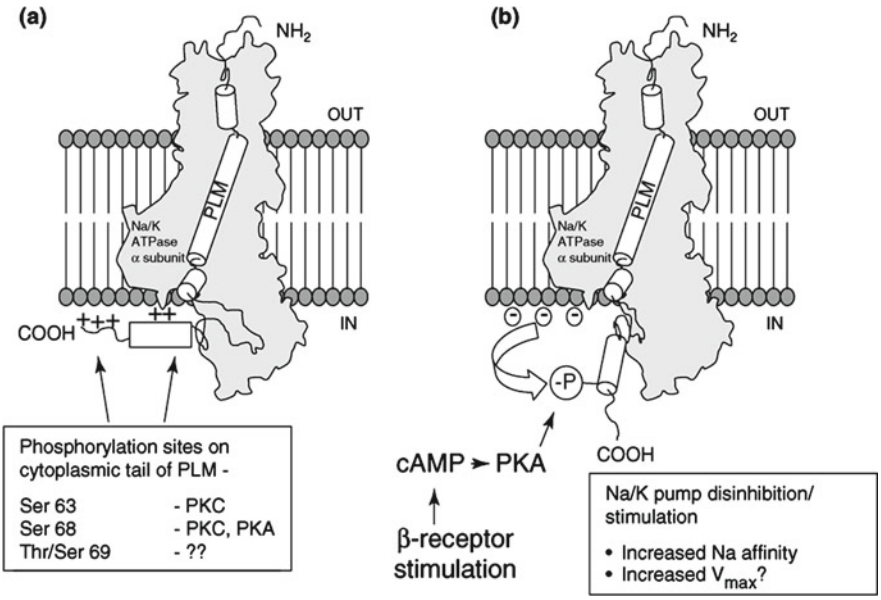

Fig. 14.2 Diagram showing the suggested relationship between phospholemman (PLM) and the NKAα-subunit and alterations induced by PLM phosphorylation. PLM is a single transmembrane spanning protein that inserts into the sarcolemma with its carboxy terminus on the cytoplasmic site of the membrane. The cytoplasmic tail of PLM contains three phosphorylation sites at Ser63, Ser68 and Thr69 (Ser69 in mouse) (**a**), the helical structure of PLM normally orients with the cytoplasmic region of the α-subunit close to, or inserted into, the membrane. The basic nature of this region (+) means that it associates with the negatively charged phospholipids of the membrane. However, when PLM is phosphorylated at any or all of the phosphorylation sites indicated in (**a**); the orientation of the cytoplasmic tail may shift altering its interaction with the NKAα-subunit and disinhibiting/stimulating ion transport (**b**). Taken from Ref. [75] with permission

not α1β1, isozyme produces functional effects in cells [23]. The α2β1 isozyme contributes to only about 11 % of total NKA [24], albeit that plays a prominent role in cardiac contractility compared to that of the α1β1 isozyme [25] due to its preferential localization in T tubules and their colocalization with NCX [24, 26].

Thus, PLM phosphorylation by PKA and PKC play differential roles in regulating different NKA isozymes. PKA and PKC produce additive effects in phosphorylation on PLM on NKA activity, which are observed in cells such as cardiac myocytes [22] and pulmonary artery smooth muscle cells [23], leading to an increase in Na^+ extrusion, which limits Na^+ overload during sympathetic stimulation in both the left and right ventricles.

5 PLM and NCX Activity

Na^+/Ca^{2+} exchanger (NCX) is one of the major Ca^{2+} efflux mechanisms, thereby restoring $[Ca^{2+}]_i$ to resting levels and maintains steady state Ca^{2+} balance [28]. Steady state $[Ca^{2+}]_i$ in each excitation-contraction cycle mainly depends on the proper functioning of NCX [28]. NCX function has interdependency on the activities of NKA [27].

NCX1, the most important member of NCX family, is a 938 amino acid (939 amino acids in rat) comprising the first five TM segments, a large intracellular loop and a –COOH terminal domain consisting of the last four TM segments [28, 29]. The $–NH_2$ terminus of NCX1 is extracellular and the –COOH terminus is intracellular. The α repeats in TM segments 2,3 and 7 of NCX1 are important in Ca^{2+} transport, while the large intracellular loop contains the regulatory domains of the exchanger [30, 31]. PLM interacts and associates with residues 218–358 of the intracellular loop of NCX1 [32]. Molecular model of phospholemman and NCX is schematically represented in Fig. 14.3.

Phosphorylation of PLM by PKA and PKC at Ser 68 causes inhibition of NCX activity and mutation in this single amino acid completely eliminates the capability of PLM to inhibit NCX activity [33]. In HEK293 cells, upon co-expression of both NCX and PLM, the stimulatory effects of the PKC activator, PMA on NCX activity was inhibited by increased PLM phosphorylation [34]. Co-expression of PLM and NCX inhibited both forward and reverse mode of NCX and decreased Na^+-dependent Ca^{2+} uptake. Inhibition of NCX by PLM seems specific because ablation of dual PKA and PKC phosphorylation at Ser68 in PLM by Ala replacement led to the loss of function and nullifies its inhibitory effect on NCX activity. In cardiac myocytes, over expression of PLM causes an increase in $[Ca^{2+}]_i$ and subsequently produces contraction [33]. Thus, the effects of PLM on cardiac excitation-contraction coupling not only occur due to changes in Na^+ ion gradient resulting from PLM inhibition on NKA activity [29], but also by interaction of PLM with NCX [27, 35, 36].

Mutation in Ser 63 (Ser63A) results in modest inhibition of NCX when compared with wild type PLM, which suggests that phosphorylation at Ser63, also con-

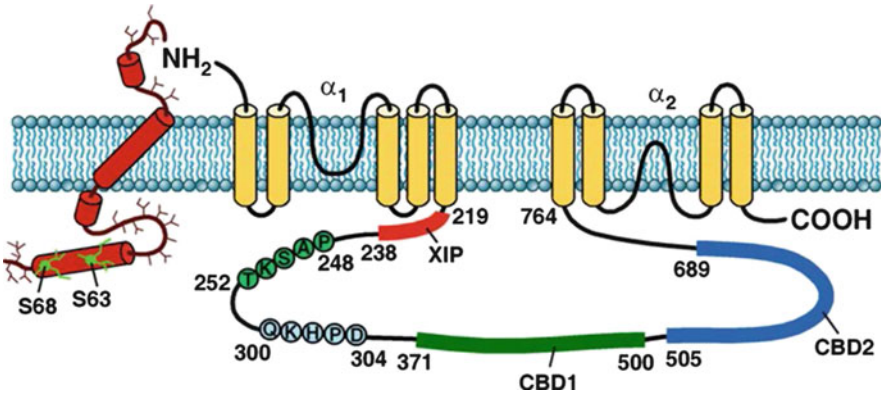

Fig. 14.3 Molecular model of phospholemman and Na^+/Ca^{2+} exchanger. *Left*: nuclear magnetic resonance studies of highly purified phospholemman in micelles reveal four helices of the protein with a single transmembrane (TM) domain. The FXYD motif is in the extracellular domain, and the physiologically important phosphorylation sites Ser63 and Ser68 are in the cytoplasm. *Right*: the mature Na^+/Ca^{2+} exchanger is modelled to consist of nine TM segments with two reentrant loops (between TM2 and TM3 and between TM7 and TM8) as of the conserved α repeat motifs that are important in ion transport activity. The N-terminus is extracellular and the C-terminus is intracellular. Between TM5 and TM6 is a large intracellular loop (residues 218–764) which contains the regulatory domains of the exchanger. Specifically, the proximal linker domain (residues 218–358) which interacts with PLM, the exchange activity peptide (XIP) region (residues 219–238), the two calcium binding domains 1 (residues 371–500) and 2 (residues 505–689) connected in tandem by a short linker (residues 501–504), and the interaction site for endogenous XIP (residues 562–679) all reside within the intracellular loop. The two specific segments (residues 248–252 [PASKT] and residues 300–304 [QKHPD]) in the proximal linker domain responsible for inhibition of Na^+/Ca^{2+} exchanger by phospholemman as shown. Taken from Ref. [76] with permission

tributes to the inhibitory effect of PLM on NCX [37]. However, Ser68 phosphorylation plays a major role in the inhibition of NCX by PLM. Changes in PLM conformation by mutating Ser68 (Ser68A) alters its interaction with NCX by not allowing it to interact with PKC, conceivably due to steric hindrance [34].

PLM exist in at least two subpopulations based on its accessibility: one only to PKA and the other only to PKC indicating that only one population of NKA is available to PKA, and another proportion to PKC. It has been suggested that these subpopulations represent two isoforms (α1β1 and α2β1) of NKA [23, 36].

6 Physiological Significance

In intact heart, β-adrenergic stimulation mediated depolarization increases Na^+ influx into the myocytes. In addition, L-type Ca^{2+} currents also increase upon β-adrenergic stimulation, resulting in an enhancement of Ca^{2+} entry. To maintain

steady state Ca^{2+} balance, Ca^{2+} efflux by forward mode of NCX and subsequent entry of Na^+ occurs into the cell. Therefore, an increase in NKA activity by PLM phosphorylation during β-adrenergic stimulation prevents intracellular Na^+ overloads [34]. Importantly, unchecked stimulation of NKA activity could decrease $[Na^+]_i$, thereby augmenting the NCX activity, which causes further $[Ca^{2+}]_i$ depletion. Therefore, mechanisms in which PLM upon phosphorylation at Ser68 enhances NKA activity [5, 8], but inhibits NCX activity, have physiological significance, for example, ischemia-reperfusion induced contraction to blood vessels leading to hypertension [34, 38].

7 PLM-Thr69 Phosphorylation

In cardiac myocytes, a new phosphorylation site has been identified in PLM-Thr69 residue of PLM, which is phosphorylated by PKCα/ε [39]. A notable property of Thr69 phosphorylation in cardiac myocytes is its transient nature especially in comparison to Ser63 and Ser68 phosphorylation [16]. In kidney cells, Thr69 phosphorylation plays an important role for transport of PLM from ER to the cell membrane [16]. However, in cardiac myocytes, PLM mainly resides in the sarcolemma and T-tubules [35, 37], and the transient nature of agonist induced Thr69 phosphorylation could not induce PLM trafficking from the intracellular store, while sustained PKC activation is necessary to induce PLM trafficking in kidney cells [16]. The physiological relevance of Thr69 phosphorylation of PLM remains unclear.

8 Role of Phosphatase

The phosphorylation status of Ser68 of PLM is known to be regulated by protein phosphatase inhibitor (PPI), inhibitor-1 (I-1), PKA and PKC [40], whereas the phosphorylation status of Ser63 is mainly governed by PP2A and PKC (Fig. 14.4). So, in addition to their phosphorylation, the phosphorylation status of the two pools of PLM is determined by colocalization with a phosphatase [36]. Armouche et al. [40] have reported that hyperphosphorylation of PLM in the failing human heart may be due to the down regulation of the inhibitor-1 and also protein phosphatase-1 (PP-1) activity [41].

PLM phosphorylation and cardiac NKA activity are negatively regulated by protein phosphatase-1 (PP-1). PP-1 has been implicated in modulating cardiac β agonist response and is a negative regulator of cardiac contractility [42–44]. Ser68 is a downstream target of I-1, which allows a cross talk mechanism in which PKA regulates the function of NKA by regulating I-1 [40].

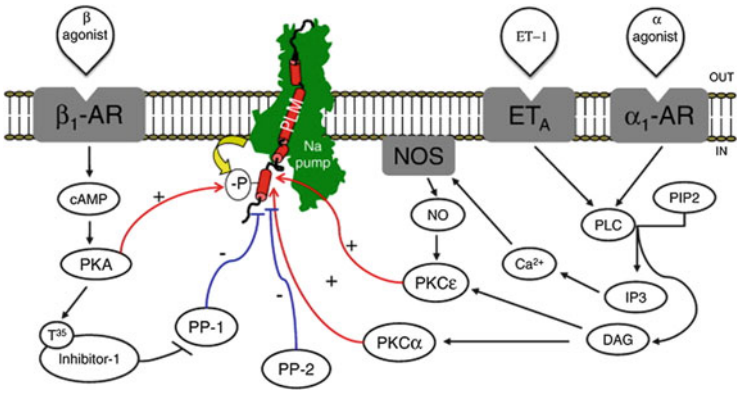

Fig. 14.4 Signalling pathways regulating Na^+ pump activity via phosphorylation of PLM. PLM phosphorylation state is regulated by the kinase activity of PKA and PKC and phosphatise activity of PP-1 and PP2A, PKA and PKC phosphorylate phospholemman and thus stimulate Na^+ pump (denoted by +), whereas PP-1 and PP2A remove phosphates from phospholemman and thus inhibit the pump (denoted by -). *ET-1* endothelin-1; ET_A endothelin receptor; *NOS* nitric oxide synthase; *AR* adrenergic receptor. Taken from Ref. [77] with permission

9 Phosphodiesterase and PLM

Sildenafil, a selective inhibitor of phosphodiesterase enzyme type 5 (PDE5) prevents the hydrolysis of cGMP and plays an important role in ameliorating ischemia-reperfusion injury [45, 46]. Sildenafil induces PKG mediated phosphorylation of PLM during ischemic reperfusion injury and stimulates the NKA activity. This would limit Na^+ and Ca^{2+} overload [49].

Two pools of cGMP exist in a cell: particulate guanyl cyclise (P-GC) is present in cell membrane, while soluble guanylate cyclase (S-GC) is a cytosolic enzyme and both types of GCs upon activation produce cGMP [22]. P-GC can trigger discrete signal transduction compared with the soluble enzyme (S-GC) in elevating cGMP locally instead of globally in a cell [47, 48]. Sildenafil mediated cardioprotection may involve stabilization of cGMP produced in the particulate fraction in contrast to that derived from S-GC, since sildenafil could not inclusively maintain the elevated level of cGMP [46]. Figure 14.5 outlines the cardioprotective function of sildenafil. Sildenafil at reperfusion activates PKG leading to the phosphorylation of PLM Thr69, but not Ser63 or Ser68, in a PKC-dependent manner. The phosphorylation of PLM at position Thr69 leads to the activation of NKA, which provides a mechanism for sildenafil-mediated cardioprotection against reperfusion injury mainly by attenuating cellular Na^+ overload [46].

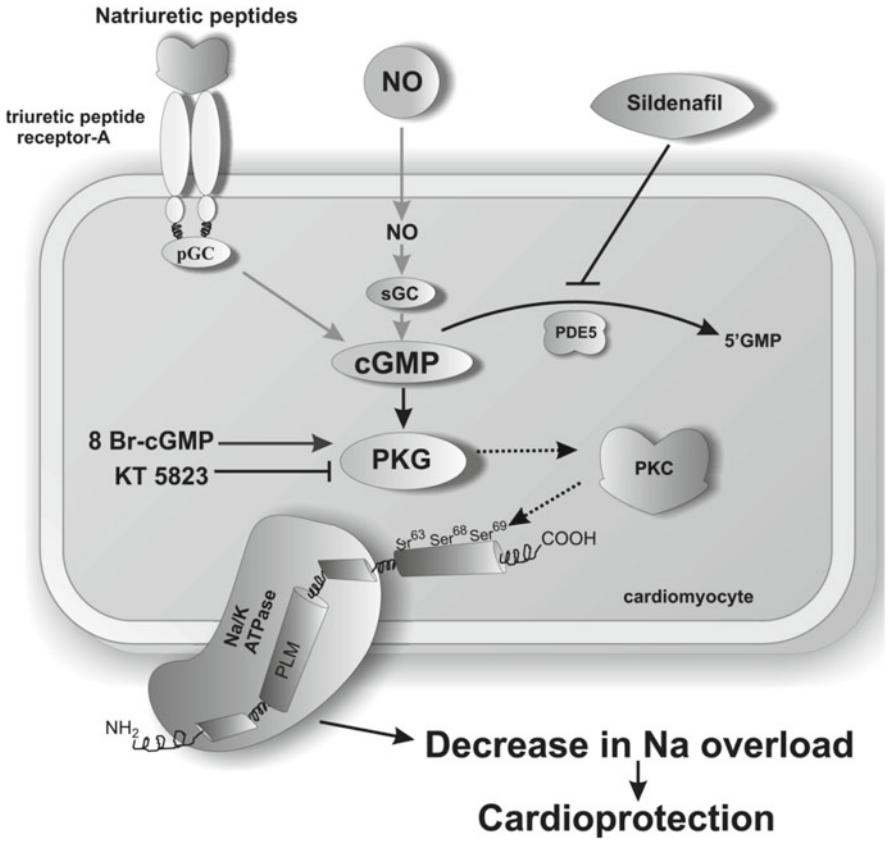

Fig. 14.5 Schematic diagram to illustrate the potential cardioprotective signalling mechanism, for sildenafil at reperfusion. *NO* nitric oxide; *sGC* soluble guanylate cyclase; *PDE5* phosphodiesterase enzyme type 5; *8-Br- cGMP* 8-bromo-cGMP. Taken from Ref. [46] with permission

10 Nitric Oxide and PLM

Endogenous NO maintains Na^+ and Ca^{2+} homeostasis, for example, in isolated myocytes. Exogenously added NO increases apparent Na^+ affinity of NKA activity in rat myocytes in a dose-dependent manner [48]. NO is known to stimulate NKA activity via PKCε induced phosphorylation of PLM at Ser63 and Ser68 [48].

NO has been reported to both inhibit and stimulate NKA activity. At low doses, NO stimulates NKA activity by increasing its apparent Na^+ affinity [49]. Conceivably, the differences in the NO donors used and the concentration of NO generated may explain the apparent ambiguity of NO. The NO donor, *S*-nitroso-*N*-*acetylpenicillamine* (SNAP) produces biphasic contractile effects in cardiac tissue, with a positive inotropy at low NO concentrations and negative inotropy at high concentrations [50]. Inhibition of endogenous NO has been shown to cause positive

inotropy. It is possible that the inhibitory effect of excess production of NO could be due to the formation of the potent oxidant, peroxynitrite ($NO + O_2^{\bullet -} \rightarrow ONOO^{-\bullet}$), which inhibits NKA activity [31, 32]. The signalling pathway through which NO exert its effects could occur via PKCε-mediated phosphorylation of Ser63 and Ser68 residues of PLM [51].

11 Palmitoylation and Glutathionylation

11.1 Glutathionylation

PLM in cells is susceptible to glutathionylation. Mutagenesis identified the specific reactive cysteine in the cytoplasmic terminal. Na^+ pump β1 subunit glutathionylation induced by oxidative signals results in the pump inhibition [52]. Glutathionylation of the β1 subunit increases in myocardium of PLM−/− mice [36]. By facilitating deglutathionylation of the β1 subunit, PLM reverses oxidative inhibition of the Na^+ pump and plays a dynamic role in its regulation [36].

The transmembrane domain of the β subunit is detached from the transmembrane domain of the α-subunit and their interaction is stabilized by single hydrogen bond [58]. Movements of α/β subunits relative to each other during the change to the E1 conformation [53, 26] may disrupt the interaction and shift Cys46 into a domain accessible to the aqueous environment of the cytosol where glutathionylation can occur.

There may be pathological implications of PLM-dependent redox regulation of membrane transport. An enhancement in the level of neurohormones during oxidative stress [54, 55] and subsequent dysregulation of cytosolic Na^+ and Ca^{2+} handling have been suggested to contribute to the pathophysiology of heart failure [56]. Decreased PLM expression may accentuate several abnormalities associated with Na^+ and Ca^{2+} dysregulation, for example, ischemia-reperfusion [35] and that may also be modified by glutathionylation of PLM and NKAβ1 subunits [36].

11.2 Palmitoylation

Protein S-palmitoylation is currently considered as an important and common post translational modification in a variety of cells [57]. S-palmitoylation is the reversible covalent post translational attachment of palmitic acid to the thiol groups of cysteine via an acyl thioester linkage [58]. Protein-S-palmitoylation is catalyzed by palmitoyl acyl transferases and reversed by protein thioester, and that occurs dynamically and reversibly in a manner analogous to protein phosphorylation [58]. In silico algorithms and analysis suggest that human PLM can be palmitoylated at Cys-40 and Cys-42 residues, which resides in the intracellular region of PLM [59].

Phe28 of PLM and Glu960 of the α-subunit of NKA are critical for their interaction [60]. Palmitoylation of PLM at Cys42, but not Cys40, could impinge on PLM

Phe28 and α subunit of Glu960, thereby alter their interaction. Importantly, PLM protects the Na^+ pump from oxidative inhibition through oxidation of an intracellular cysteine [61], which could be of significantly greater functional consequence for the pump if a pool of non-oxidized PLM exists to exchange with the recently oxidized Na^+ pump associated PLM [36].

11.3 Relationship Between Palmitoylation and Glutathionylation

Glutathionylation of PLM at Cys42 enhances Na^+ pump activity by relieving the inhibition of the Na^+ pump, which occurs due to oxidative modification [61] and the mechanism seems complex. Palmitoylation and glutathionylation, therefore, could compete for the same cysteine. The ability of the Cys42 to receive either glutathione or palmitate will depend on whether it has already been modified with the other. PLM may act as a pump activator or inhibitor depending on its phosphorylation and redox state [64]. Oxidant stress itself leads to PLM phosphorylation [62], whereas an inhibition of the pump by palmitoylation has also been demonstrated.

12 PLM and Brain CSF

PLM is highly expressed in selective structures of the CNS [51]. It is most abundant in cerebellum, where it was detected, for example, in Purkinje neurons and in axons traversing the granule cell layer. PLM is particularly enriched in choroid plexus, the organ that secretes CSF in the ventricles, where it colocalizes with NKA in the apical membrane [63]. In cerebellum and choroid plexus, PLM physically associates with the NKA and regulates brain ventricle size upon playing a role as a "volume sensor". Either an increase or decrease in CSF volume can be pathological throughout life [63].

The NKAα subunit regulates brain development with the involvement of PLM. NKA acts as a key regulator of brain ventricle formation by impacting three processes: neuroepithelium formation, neuroepithelial permeability and CSF production. Involvement of NKA in water movements has been shown to occur either with aquaporins in plasma membrane or through the paracellular pathway. Aquaporins are strongly expressed in "choroid plexus" and its knock-out mice have abnormal CSF production [63, 51].

13 PLM in Ageing and Exercise

Both insulin and exercise induce phosphorylation of PLM in muscle [38]. Insulin and exercise are known to promote PLM phosphorylation via PKA and PKC [19].

Endurance exercise training has been shown to reverse some of the ageing related deficits. The level of expression of PLM was not modified by advancing age, but was significantly increased by exercise training in aged rats [64]. Exercise training alters expression of PLM by transiently increasing the phosphorylation of PLM Ser63 and Ser68 without modifying Thr69, while ageing modulates the level of PLM associated α1 isoform and exercise training modulates the levels of PLM associated α1 and α2 isoform. The increase in PLM associated α1 in comparison to that of α2 with age indicates that PLM plays a more important role in regulating α1 than α2 during ageing [65].

PLM is less likely to be involved in long-term skeletal muscle adaptations. Endurance training induced changes in Na^+ and K^+ handling during skeletal muscle contraction due mainly to the adaptive increase in NKA and PKCα/βII have been demonstrated to play a role as an upstream kinase that phosphorylates PLM is response to acute exercise [66]. PLM contains a potential phosphorylation site for CaMKII. It, therefore, remains to be determined whether CaMKII is upstream or downstream to the PKCα/βII for PLM phosphorylation during acute exercise [18].

14 Summary

PLM is the only among FXYD member that regulates the activities of both NKA and NCX, which suggests that PLM, NCX and NKA form a macromolecular complex. There are significant differences, however, between the mechanisms by which PLM regulates the activities of these two important ion transporters. Phosphorylation of PLM relieves inhibition of NKA, whereas it is the phosphorylated form of PLM that actively inhibits NCX [35]. NKA is known to play a role in cell volume regulation [67]. Inhibition of NKA by PLM, therefore, may have profound consequences during cardiac ischemia when disordered cell volume regulation and electrical instability are present [68]. Alpha2β1 isozyme of NKA has been demonstrated to play a more important role in cardiac contractility in comparison to the α1β1 isozyme due to its preferential localization in T-tubules, where it colocalizes with NCX [22].

PLM is highly expressed in heart and brain (cerebellum and choroid plexus) [63], but in kidney it is limited to the juxtaglomerular apparatus [69]. An increase in PLM expression was observed in response to cardiac infarction and nerve injury [66, 69].

Juel et al. [70] have demonstrated that PLM Ser68 phosphorylation was higher after exercise compared with rest in type II (slow twitch) fibres, whereas the type 1(fast twitch) fibres showed no discernible change. PKA and PKC mediated PLM Ser68 and PLM Ser63 phosphorylations, in addition to some other kinases, could non-specifically phosphorylate other residues after exercise in type II fibres resulting in an increase in NKA activity [71].

15 Conclusions

Some signals that influence the trafficking of NKA through the ER have been identified to some extent. For example, a stretch of negatively charged amino acids act as a signal for transport from the ER to the cell membrane [16]. However, the proteins that are involved for the translocation of NKA to the cell membrane are currently unknown and require investigation.

PLM upon phosphorylation at Ser63, Ser68 and Thr69 enhances NKA activity, but inhibits Na^+/Ca^{2+} exchange activity in some types of cells, for example, cardiac myocytes. The consequences of NKA activation on the one hand and NCX inhibition on the other on cellular Ca^{2+} homeostasis and contractility seems complex. Notably, phosphorylations of Ser68 and Ser69 have been well studied in contrast to the Thr69 phosphorylation [34]. Thus, mechanism(s) that regulates Thr69 phosphorylation in modulating NKA activity requires further research.

Bossuyt et al. [72] have demonstrated hyperphosphorylation in a rabbit model of volume overload induced dilated heart failure. Armouche et al. [40] have reported hyperpolarization of PLM in the failing human hearts attributable to the down regulation of inhibitor-1 (I-1) and increase in protein phosphatase-1 (PP-1) activity. So, considering only up- or the downregulation of PLM phosphorylation do not seem to explain properly progression of a disease, for example, heart failure. Future studies are required to determine whether any correlation exists between I-1 and PP-1 with cell volume regulation during PKA and PKC mediated hyperphosphorylation of Ser63, Ser68 and Thr69 of PLM.

PLM has unique distribution in axons of cerebellum. Considering the three NKAα isoforms and three β isoforms that are expressed in different brain cell types in different combinations [51, 63, 73], the potential subunit diversity of the Na^+ pump and their physiological significance in axons of brain cells requires adequate attention.

Endurance exercise training has been demonstrated to reverse some of ageing related dysregulation of NKA activity [65]. The level of PLM expression is not modified by advancing age, but has been shown to significantly increase by exercise training in aged animals. An increase in the level of PLM may modulate NKA activity upon phosphorylation. Thus, systematic studies on long-term vs acute effects of exercise in trained and untrained subjects may provide important insights on the regulation of NKA activity regulated by exercise.

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Chapter 15
Regulation of the Cardiac Na^+/K^+-ATPase by Phospholemman

Hansraj Dhayan, Rajender Kumar, and Andreas Kukol

Abstract Phospholemman (PLM) is a regulatory subunit of the cardiac Na^+/K^+-ATPase (NKA), but exists also as an independent tetramer. The membrane-spanning protein consists of 72-amino acid residues and is the first member of the FXYD motif-containing family of tissue-specific NKA regulatory subunits (FXYD1). A comparative model of the human PLM/NKA complex shows the interactions between NKA and the extracellular FXYD motif as well as the transmembrane helix-helix interactions. A variety of intracellular posttranslational modifications point to a highly dynamic picture of interactions between NKA and the intracellular part of PLM. Posttranslational modifications of PLM include NKA-activating phosphorylation, inhibiting palmitoylation and activating glutathionylation. PLM gene expression has the potential for posttranscriptional regulation by the formation of potassium-ion-stabilised G-quadruplex structures in pre-splicing mRNA. The overall physiological role of cardiac PLM is to protect the heart under conditions of increased heart rate and oxidative stress avoiding calcium overload of the cytoplasm and arrhythmias. The PLM tetramer possibly exists as a storage pool in order for the heart to react quickly to changing conditions.

Keywords fxyd1 • Cardiac protein • Transmembrane protein • Protein-protein interactions • Posttranslational modification • Oligomerisation

1 Introduction

The Na^+/K^+-ATPase (NKA) is a P-type ATPase originally discovered by Skou [1] and responsible for the export of three sodium ions to the outside of the cell and the import of two potassium ions. This ion transport against the concentration gradient

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is driven by the hydrolysis of ATP. The ion gradient established by NKA is essential to many membrane transport processes including the generation of action potentials in nerve and muscle cells. NKA is composed of a minimum of two subunits, namely the catalytically active α subunit with a molecular mass of approximately 100 kDa and the β subunit with approximately 33 kDa (in humans), which is required for intracellular transport of NKA to the plasma membrane [2]. At least four isoforms of α subunits and three β isoforms are known, while in cardiac muscle $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are mainly expressed. With ouabain-based photoaffinity labels a third γ subunit (also known as FXYD2) was discovered in the porcine kidney [3] and later it was reported that NKA is generally associated with a third subunit. Whether this association is permanent or transient remains a matter of investigation. The identity of the third subunit varies in different tissues, but the FXYD (phenylalanine-X-tyrosine-aspartate) sequence motif is common to the third subunit in all tissues [4]. In cardiac muscle tissue the third subunit is termed phospholemman [5] or FXYD1, which is also used as the name of the gene encoding for the protein phospholemman (PLM).

While the structure and function of NKA have been reviewed in Chap. 1 of this volume, this chapter focuses on the structural and biochemical aspects of the NKA regulation by PLM and its physiological consequences. First, the protein structure of PLM is discussed with reference to various sites of posttranslational modification, followed by the structure of the human FXYD1 gene and its potential for regulation at the level of transcription and mRNA processing, and finally the physiological consequences of PLM-NKA interaction are reviewed. Many excellent reviews about PLM and its physiological role in particular have appeared in the literature [6–9] and should be consulted by the interested reader in addition to this chapter.

2 Phospholemman Sequence and Structure

The protein phospholemman (PLM) was originally characterised as a major plasma membrane substrate of protein kinase A (PKA) and C (PKC) that consisted of 72-amino acid residues in the mature protein and a cleavable 20-residue N-terminal signal sequence [10]. PLM is expressed in the heart, skeletal muscle, smooth muscle and liver, while it is absent from the brain and the kidney. Later, as outlined above, PLM was classified as the first member of the FXYD class of proteins [4]. The phosphorylation sites have been identified in later studies as Ser63 (PKC) and Ser68 (PKA and PKC) and Thr69 (PKC) [11]. Palmitoylation occurs at Cys40 and Cys42 [12] and glutathionylation at Cys42 [13]. Due to the increasing number of genome sequencing projects we can, at the time of writing, identify 25 sequences of FXYD1 from UniProt [14]. Except the *Ophiophagus hannah* (king cobra) FXYD1, all other 24 organisms FXYD1 sequence show >50 % sequence identity. The multiple sequence alignment of FXYD1 protein sequences without signal sequence is shown in Fig. 15.1 colour coded by conservation with a blue-red gradient. It can be seen that there are very few variations to the FXYD motif. Other regions of high conservation are the transmembrane domain as well as the intracellular C-terminal domain between Cys40 and Arg66 with some exceptions. Note that PLM follows

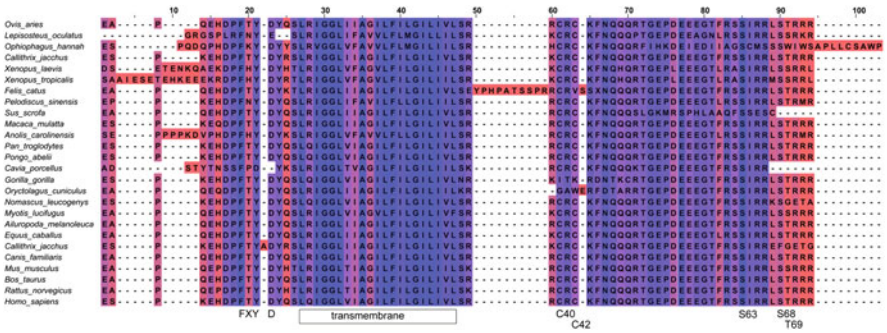

Fig. 15.1 Multiple sequence alignment of FXYD1 orthologues. The alignment is coloured according to alignment quality (=conservation) with a *red* (low conservation) to *blue* (high conservation) gradient. Important functional sites of the human sequence discussed in the text are indicated. The figure was prepared with JalView [54]

the positive-inside rule for transmembrane proteins [15] showing a higher number of positively charged residues on the intracellular part of the protein as well as aromatic residues Tyr11 and Tyr13 at the membrane water interface.

2.1 The Phospholemman Monomer

The 3D structure of the PLM monomer was determined by solution-state NMR spectroscopy (PDB-ID: 2JO1) in SDS micelles at pH 5 [16]. It reveals, in addition to a long helix from Gln14 to Phe44 encompassing the transmembrane domain, short helical segments in the N-terminal part and a longer helical segment from Phe60 to Thr69 in the C-terminal part. The angle of the helical axes between the two main helical segments is approximately 90° giving rise to the characteristic L-shape of the PLM monomer. The C-terminal part including the helix is very basic containing altogether six Arg residues between Phe60 and Arg72; thus it is positively charged at physiological pH. This explained the association with the negatively charged micelle surface in the NMR study. Teriete et al. suggested that negative charges introduced by phosphorylation could lead to a reorientation of the C-terminal segment facilitated by the flexible linker region between Asp45 and Thr59. Interestingly there is a high level of conservation in the linker region among FXYD1 orthologues (Fig. 15.1).

2.2 The Phospholemman Tetramer

Gel electrophoresis of synthetic PLM transmembrane peptides with perfluorooctanoate, a detergent that keeps transmembrane protein complexes intact, has shown that PLM forms tetramers [17]. In the same study it was shown by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) that the

lipid-membrane-embedded tetramers are α-helical and have a predominantly transmembrane orientation similar to the monomer structure obtained in SDS micelles. A solid-state magic angle spinning ^{17}O-NMR study of the lipid-membrane-embedded PLM transmembrane domain indicated that the tetramer is not completely symmetric, but may have a C_2 (dimer of dimers) or C_1 rotational symmetry along the bilayer normal [18]. An atomic structural model of the tetrameric PLM transmembrane domain was obtained by a combination of orientational constraints derived from site-specific infrared dichroism (reviewed in [19]) and a systematic conformational search based on molecular dynamics simulation of a transmembrane helical bundle protein [20]. The PLM transmembrane helical bundle reveals an average helix tilt angle of 7.3° in line with the previous solution state NMR-study of the monomer. The tetramer is closely packed and does not show any indication of an open pore in the centre of the tetramer. However, the helix-helix packing shows the unusual feature that some small residues, such as Gly19 and Gly20, point to the outside of the helical bundle (Fig. 15.2a), which supported the hypothesis that a PLM tetramer may occur in vivo as a storage form that readily interacts with NKA [21]. A two-stage model of PLM-NKA interaction was proposed that involves a slow interaction of the PLM tetramer with NKA leading to the abstraction of one PLM monomer (Fig. 15.2b). In the second stage the remaining PLM trimer interacts fast with other NKA molecules. The existence of PLM tetramers in human embryonic kidney cells has been confirmed by fluorescence resonance energy transfer (FRET) and it was shown that the tetramer is stabilised by phosphorylation [22]. More recently the existence of PLM homo-oligomers was reported in cardiac muscle [23]. In this carefully conducted study a significant proportion of PLM (>50 %) was identified that was not associated with NKA but formed a separate pool of multimeric PLM. The stoichiometry of multimeric PLM was not clearly identified, but using formaldehyde cross-linking without denaturation prior to electrophoresis resulted in a band consistent with a tetramer that was detected by Western-blotting probed with a PLM-phospho-Ser68-specific antibody. The phosphorylation pattern of NKA-associated and multimeric PLM is different, with NKA-associated PLM being unphosphorylated or phosphorylated at Ser68, while multimeric PLM shows Ser63 phosphorylation in addition to phosphorylation at other sites. It was suggested that phosphorylation does not change the distribution between multimeric and NKA-associated PLM, but the protein phosphatase PP2A associated with NKA leads to a dephosphorylation of PLM-Ser63 [23]. The PLM multimer in cardiac muscle cells located in the same membrane compartment as NKA does not seem to interact with other proteins; thus it was suggested that it acts as storage pool for PLM confirming the postulation made in earlier work [21]. There is a potential parallel to phospholamban (PLB) that regulates the sarco/endoplasmic reticulum Ca-ATPase (SERCA). Although not related by sequence similarity, PLB is structurally similar to PLM with 52-amino acid residues and one transmembrane domain. It associates with and regulates SERCA via phosphorylation and exists as a pentamer in its non-associated form (reviewed in [24]). Since PLB shows measureable ion channel activity, the function of the PLB pentamer is still under discussion [25, 26], while based on the structure of the PLM tetramer transmembrane domain, no potential for ion conduction was found [21], thus the hypothesis of the PLM tetramer as a storage pool for regulation of NKA seems the most likely one.

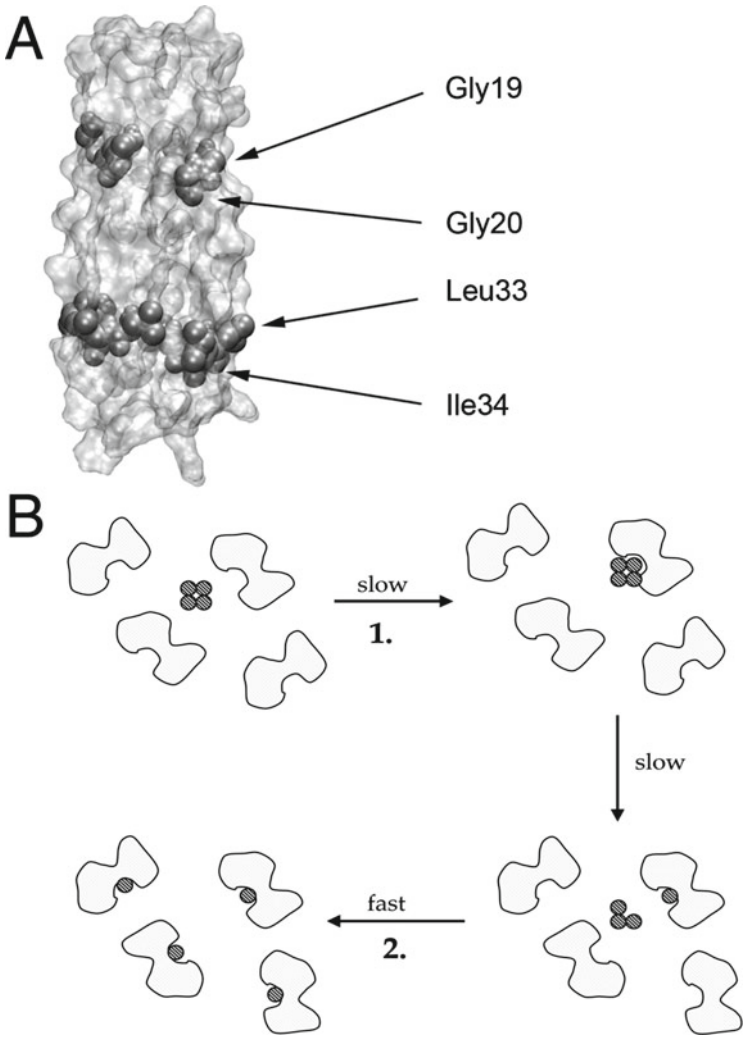

Fig. 15.2 (**a**) The structure of the PLM transmembrane domain tetramer showing the protein solvent-accessible surface. Residues in dark shade have been used to derive orientational constraints with site-specific infrared dichroism. (**b**) A putative model of the PLM tetramer–NKA interaction utilising the tetramer as a storage form of PLM

2.3 The Structure of the Phospholemman-NKA Complex

The atomic structure of the cardiac human phospholemman-NKA complex is not known, but a number of potassium-bound homologue structures are available in the Protein Data Bank, namely the pig renal NKA with parts of FXYD2 at 3.5 Å resolution (PDB-ID: 3B8E) [27], a shark NKA model in the E2 state with FXYD10 at 2.4 Å resolution (PDB-ID: 2ZXE) [28] and a sodium bound form from pig kidney

at 4.2 Å resolution (PDB-ID: 4HQJ) [29]. In particular the high-resolution shark NKA model shows a large portion of the FXYD10 subunit including the FXYD motif. The sequence identity between the shark and the human NKA subunits is 88 % for the α-subunit, 65 % for the β-subunit and 35 % between FXYD10 and PLM, while a 30 % sequence identity is considered as the lower limit for comparative modelling [30]. For this review we have prepared a comparative model of the human NKA/PLM complex using the shark 2ZXE and the human PLM monomer structure (2JO1, 100 % sequence identity) as templates with the explicit inclusion of three potassium ions, one Mg^{2+} and one phosphate analogue MgF_4^{2-} ion found in the 2ZXE template. The structure of this comparative model shown in Fig. 15.3 is discussed in the following text. The PLM backbone structure is coloured according to sequence conservation showing a tendency towards lower conservation of residues facing away from NKA. The arrangement of subunits is shown in Fig. 15.4a, revealing the details of the interactions of the FXYD1 motif with NKA (Fig. 15.4b), namely Phe9 is interacting with residues Val183, Gln69 Val72, Ala73 and Phe186 of the β-subunit, and Tyr11 is interacting with Tyr68, Asp70 (β-subunit), Lys984 and Pro985 (α-subunit), while Asp10 is not involved in close interactions with its side chain pointing towards the solvent.

The palmitoylation sites Cys40 and Cys42 are located at the C-terminal end of the long helix and face to opposite sites of the helix. Cys40 points towards the outside (Fig. 15.4c), while Cys42 (hidden in Fig. 15.4c) points towards the α-subunit with the closest contact being the guanidinium group of Arg353 at a distance of 5.8 Å from the cysteine sulphur atom. The positive Arg328 could stabilise the thiolate anion of Cys42 at physiological pH, which may explain the susceptibility of Cys42 to glutathionylation (see Sect. 4.3). The thiolate anion is much more reactive towards forming disulphide bonds than the protonated form. Some information about the structural consequences of phosphorylation comes from fluorescence resonance energy transfer (FRET) experiments between cyan fluorescent protein-labelled NKA and yellow fluorescent protein-labelled PLM. Upon phosphorylation of Ser63 and Ser68, the amount of FRET decreased indicating that the intermolecular distance between PLM and NKA was increased [31]. It is possible that phosphorylation increases the flexibility of the PLM C-terminus and releases it from NKA binding sites. Another possibility is that the phosphorylated PLM C-terminus shifts to other phospho-specific NKA-binding sites as indicated by experiments with PLM knockout mice. Upon addition of a synthetic Ser68 phosphopeptide (PLM residues 54–72), NKA activation was observed [32]. A FRET study that investigated alanine mutations on the α1-subunit of NKA at Phe956, Glu960, Leu964 and Phe967 identified the interaction between Glu960 and Phe28 of PLM to be among the critical interaction sites between PLM and NKA. The comparative model of NKA/PLM developed for this review identifies a close interaction between Glu960 and Phe28 as shown in Fig. 15.5, although this information was not included in the modelling protocol. The structure of the short C-terminal helix containing the phosphorylation sites is entirely based on the solution-state NMR structure of the PLM monomer; thus the orientation and any interactions shown are not indicative of the native PLM-NKA complex. In the absence of structural detail, even available through comparative modelling, molecular dynamics simulations of the PLM-NKA

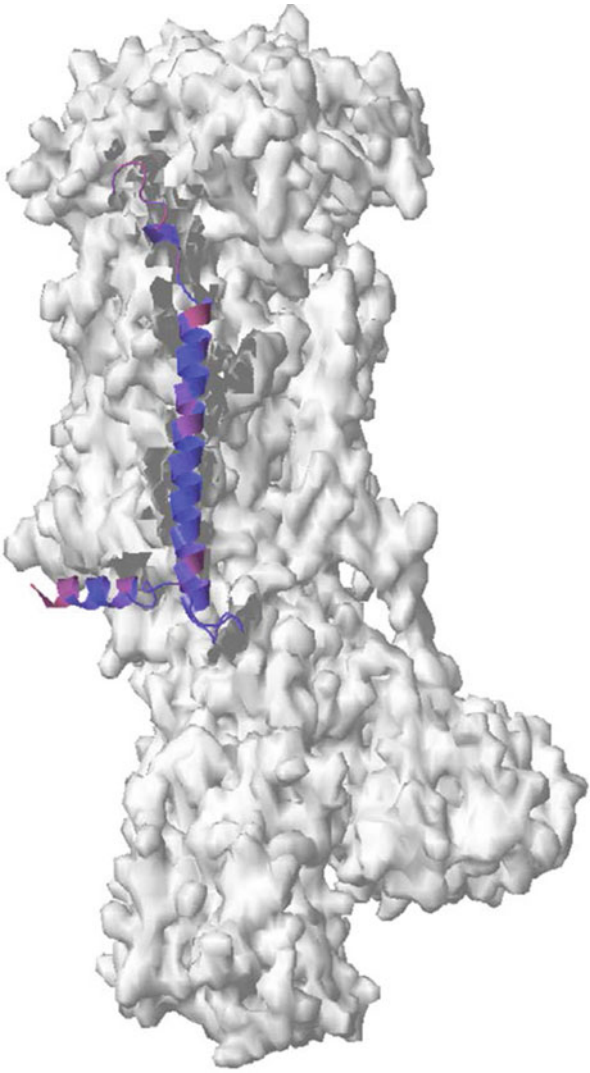

Fig. 15.3 A protein surface model of NKA with PLM shown as ribbons colour coded by conservation calculated from the alignment shown in Fig. 15.1. Protein structure displays were made with Rasmol [55]

complex in realistic lipid bilayers [33] could be carried out to assess the molecular details of this interaction.

3 The Phospholemman/FXYD1 Gene

The human FXYD1 gene is located on the q arm of chromosome 19 from bases 35,138,789–35,143,055 (assembly GRCh38) amounting to 4286 bases in the full transcript. There are in total eight exons including one 5′ and one 3′ untranslated exon. As outlined above the PLM protein is a target of significant posttranslational

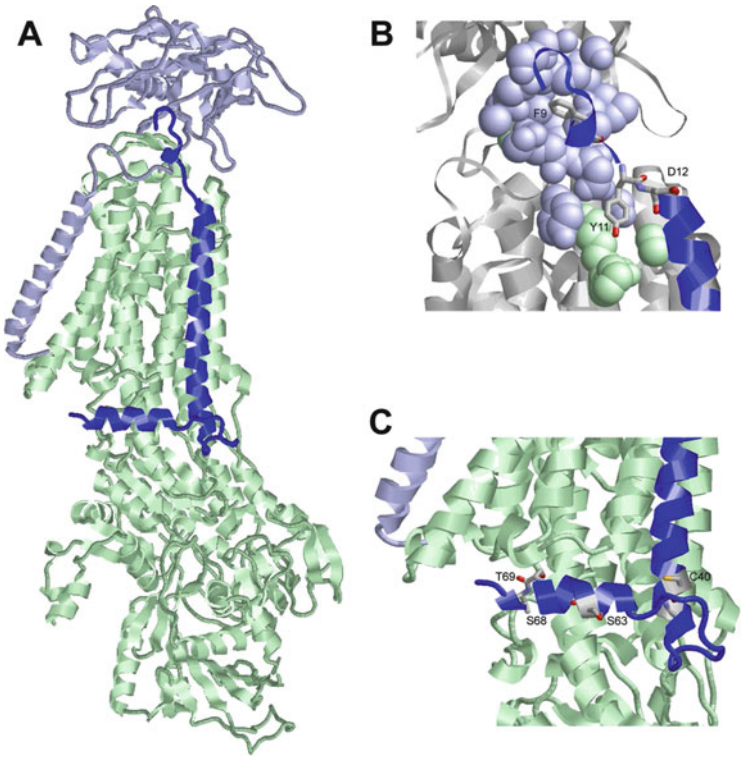

Fig. 15.4 (**a**) A backbone ribbon display of the NKA/PLM complex based on comparative modelling with Modeller 9v6 [56] as explained in the text. (**b**) Details of the interaction of PLM (*blue*) with NKA α1 (*green tint*) and β1 (*blue tint*) subunits. The amino acid residues from NKA interacting with PLM are shown in spacefill representation, while PLM residues are shown as sticks. (**c**) Structural details of the C-terminal phosphorylation and palmitoylation/glutathionylation sites

modification. Another way to regulate proteins is via the regulation of gene expression. As part of the ENCODE project transcription factors associated with genes were identified with ChIP-Seq (chromatin immuno-precipitation sequencing) experiments [34]. Using the ENCODE ChIP-Seq Significance Tool [35] a number of potential transcription factors were found around the transcription start site of FXYD1 (shown in Table 15.1). Among general transcription factors, such as TAF required for the DNA transcription activity, there are a number of transcription factors involved in cell differentiation, such as BHLHE40 and EGR1. P300 is the only cardiac-related transcription factor implicated in the enlargement of cardiac myocytes. This may reflect the variety of cell types analysed in the ENCODE project with underrepresentation of cardiac myocytes due to the difficulties of obtaining functional cardiac myocytes in cell culture. It can, however, be concluded that the FXYD1 gene is under the control of transcription factors.

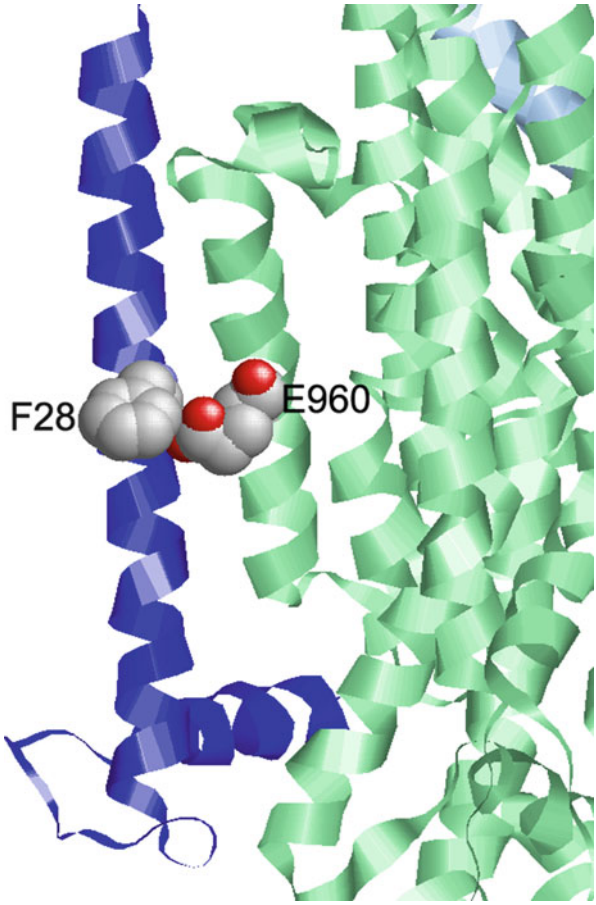

Fig. 15.5 Detail of the interaction between PLM-Phe28 and NKA-α1-Glu960 shown to be important in FRET experiments

The initial pre-mRNA transcript is subjected to the process of splicing and 11 transcripts are reported in Ensembl Human Release 77 [36]. Nine of those are supported by at least one expressed sequence tag (transcript support levels 1–3). Most transcripts differ in the position of the transcription start site and the length and or existence of the last non-protein-coding exon. Transcript FXYD1-008 encodes for a 115-residue protein that contains a 27-residue insert after the transmembrane domain (after LIVLS in Fig. 15.1). The 115-residue protein appears to have a 12-residue shortened signal peptide, but a 5′ truncation in the transcript evidence prevented the complete assignment of the coding sequence; thus the transcript may indeed contain the full 20-residue signal peptide. Two of the well-supported transcripts, FXYD1-004 and FXYD1-010, have a retained intron but do not contain any protein-coding region as annotated by the HAVANA team [37].

A computational analysis of the *Homo sapiens* FXYD1 pre-mRNA alongside 16 orthologues revealed that the FXYD1 pre-mRNA contains sequences capable of

Table 15.1 Fxyd1 transcription factors detected in the ENCODE project based on ChIP-Seq (minimum false discovery rate = 0.05). A region from 2500 bases upstream to 500 bases downstream of the transcription start site was included

Transcription factor[a]	Function[b]
BHLHE40	Circadian control and cell differentiation.
CEBPB	Transcriptional activator involved in immune and inflammatory responses.
CMYC	A proto-oncogene involved in cell division (negative regulation), apoptosis.
EGR1	Transcription activator of genes required for mitogenesis and differentiation.
FOXA1	Embryonic development, establishment of tissue-specific gene expression,
GABP	modulates transcriptional activity of nuclear hormone receptors, cell-cycle
HNF4A	regulation, regulation of apoptosis, glucose homeostasis.
HNF4G	Transcription activator, necessary for the expression of the adenovirus E4 gene.
MAX	Required for the transcription of alpha 1-antitrypsin, apolipoprotein CIII,
MBD4	transthyretin genes and HNF1-alpha, may be essential for development of the
MXI1	liver, kidney and intestine.
P300	Acts in complex with CMYC as repressor and in complex with MAD as activator.
POL2	DNA *N*-glycosylase involved in DNA repair.
RXRA	Competes with CMYC for binding to MAX, thus antagonises CMYC function.
SP1	Histone acetyltransferase P300, regulates transcription via chromatin remodelling,
TAF	promotes cardiac myocyte enlargement, participates in circadian rhythms.
USF1	Subunit of RNA polymerase II, that synthesises mRNA precursors.
ZBTB7A	Retinoic acid receptor, regulates gene expression in complex with other nuclear receptors, regulates various biological processes.
	Regulates expression of a large number of genes involved in cell growth, apoptosis, differentiation and immune response.
	General transcription factor required for transcription of genes.
	Upstream stimulatory factor one, binds to the promoter of a variety of genes.
	Transcription repressor, possibly involved in the development of B-cells among other roles.

[a]Factors in *bold* are supported by the strongest level of evidence

[b]Function obtained from UniprotKB [14]

folding into higher order intramolecular RNA structures called G-quadruplexes and that this feature is conserved in evolution [38]. G-quadruplexes are formed by square planar arrangements of four guanine bases (G-tetrads) stabilised by hydrogen bonds. At least two G-tetrads stack together stabilised by sandwiched potassium ions [39]. In FXYD1 and orthologues it was found that stretches of three Gs were conserved indicating that three G-tetrads could form a stable G-quadruplex structure. Using synthetic oligonucleotides with sequences taken from human and bovine FXYD1 pre-mRNA, the formation of G-quadruplexes was shown in vitro using fluorescence spectroscopy and native polyacrylamide gel electrophoresis [38]. It is interesting to note in the context of the sodium-potassium exchanging NKA that the required potassium ion concentration for stable G-quadruplexes to form is in the region of 100 mM, which is similar to the intracellular potassium ion concentration of 120 mM. Considering the evolutionary conservation of G-quadruplex-forming sequences it was suggested that G-quadruplex formation of FXYD1 pre-mRNA may control the splicing (either through inhibition or favouring alternative spliced

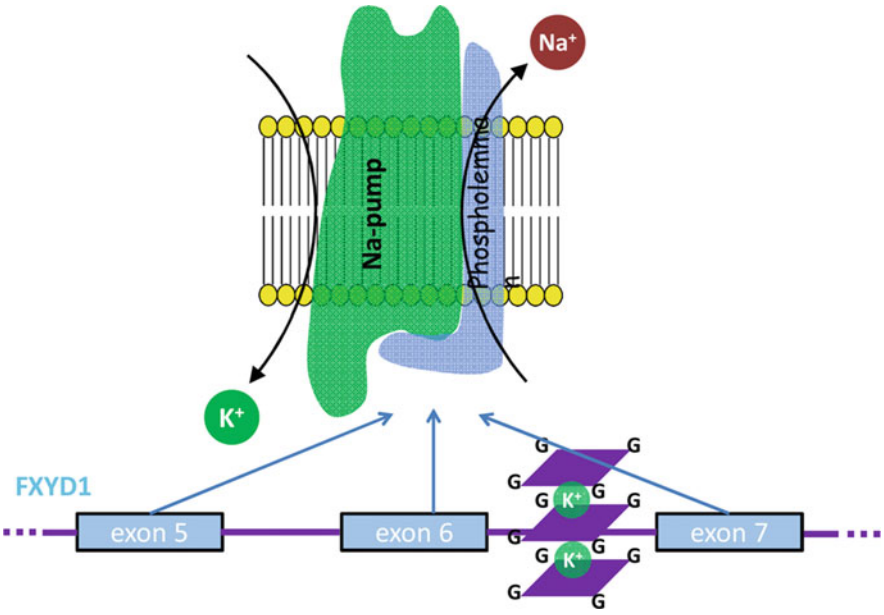

Fig. 15.6 Cartoon of G-quadruplex formation in FXYD1 pre-mRNA between exon 6 and exon 7 that may affect expression of the PLM protein product

products) and thus the expression of the phospholemman protein product [38] (see Fig. 15.6). However, these in vitro results await further experimental confirmation in particular in cardiomyocytes.

4 Physiology of NKA Regulation by Phospholemman

While the presence of PLM is not essential for survival as PLM knockout experiments have shown [40], PLM may have a protective role for the heart under conditions such as increased heart rate and oxidative stress. PLM knockout mice showed slightly depressed cardiac contractile function as well as a mild cardiac hypertrophy. Under the conditions of increased stimulation frequency and β-adrenoreceptor activation PLM knockout mice showed a larger increase in intracellular sodium concentration, a larger calcium load of the sarcoplasmic reticulum and a larger calcium transient leading to more arrhythmias compared to wild-type mice [40]. Therefore it was postulated by Pavlovic et al. that the physiological role of PLM "may be to limit the rise in intracellular Na^+ during sympathetic stimulation and thereby preventing Ca^{2+} overload and triggered arrhythmias in the heart" [8].

4.1 Phospholemman Phosphorylation and Dephosphorylation

PLM is phosphorylated by protein kinase A (PKA) at Ser68 [41] and at Ser63, Ser68 and Thr69 by protein kinase C [11]. The biochemical consequence of PKA phosphorylation is an increase in sodium affinity [42], while PKC phosphorylation increases v_{max} [43]. Overall PLM phosphorylation stimulates NKA, while unphosphorylated PLM inhibits NKA activity [5, 32] explaining the physiological role of PLM mentioned above.

The regulation of PLM by phosphatases has been investigated to a much lesser extent than kinases, but recently it was shown that the Ser/Thr phosphatase 1 (PP-1) acts on Ser68 [44], but not on Thr69 under physiological conditions as the reported EC_{50} of 2.7 μM for Thr69 was too high. Ser63 is dephosphorylated by Ser/Thr protein phosphatase 2A (PP-2A) [44], which was shown to be associated with the NKA complex [45]. As outlined above the PLM Ser63 dephosphorylation occurs most likely, when PLM is associated with NKA, but not in the PLM tetramer complex [23]. Taken together PLM phosphorylation exerts a protective effect on the heart muscle [46]. In the absence of PLM an increased heart rate and β-adrenergic stimulation would lead to a higher intracellular sodium concentration and a larger calcium content of the sarcoplasmic reticulum leading to more arrhythmias. NKA activation via phosphorylation of PLM prevents this.

4.2 Phospholemman Palmitoylation

As outlined in Sect. 2, PLM is palmitoylated at Cys40 and Cys42 [12]. The effect of palmitoylation is an increase of the half-life of PLM and a decrease of NKA activity. Furthermore, PLM phosphorylation at Ser68 increased its palmitoylation [12], which is surprising as phosphorylation and palmitoylation events are causing opposite effects. At this point the physiological significance of PLM palmitoylation has not been established. The enzymes responsible for palmitoylation of proteins are palmitoyl-S-transferase enzymes (known as DHHC proteins) and depalmitoylation is catalysed by thioesterase enzymes. It was shown in human fibroblast-derived cardiomyocytes that overexpression of DHCC5 decreased NKA pump currents by 55 %, while siRNA knockdown of DHCC5 increased NKA pump currents by 38 % [47]. Thus, DHCC5 contributes to palmitoylation of PLM leading to a subsequent decrease in NKA activity. In the same study it was postulated that the damage caused by reoxygenation of cardiac tissue after an ischemic event may be related to PLM palmitoylation. Upon reperfusion massive endocytosis of cardiac cell membrane including the NKA/PLM complex occurs that limits the recovery after an ischemic event. Several observations linked PLM palmitoylation to this event, namely internalised PLM was palmitoylated to a higher level than cell-surface-bound PLM and the extent of massive endocytosis was reduced in hearts lacking PLM or DHHC5. Thus reperfusion damage may be reduced by inhibition of palmitoylation [47]. The normal physiological function of PLM palmitoylation

may be to regulate NKA under conditions of metabolic stress or to contribute to NKA/PLM turnover.

4.3 Phospholemman Glutathionylation

The sensitivity of NKA to oxidative stress and to the cellular sulfhydryl redox status is already known since a long time [48, 49] and linked to glutathionylation of the α [50] and β1 subunit [51], which both have inhibitory effects. PLM has been shown to reverse glutathionylation of the β1 subunit by becoming itself glutathionylated at Cys42, thus exerting an activating effect on NKA [13]. However, the physiological role of PLM glutathionylation is complicated, as it is linked with phosphorylation by PKA and palmitoylation that also occurs at Cys42. Palmitoylation of PLM, which has an inhibitory effect, is promoted by PKA phosphorylation at Ser68 [12], and to further complicate matters, oxidative stress can activate PKA [52]. Possibly electrophysiological modelling of cardiomyocyte activity taking explicit account of PLM could explain some of the complexities. Models of heart failure that highlight the importance of sodium currents and NKA activity have been recently presented [53], albeit modulation of NKA by PLM was not included.

5 Conclusions

PLM has emerged as an important modulator of NKA in particular under conditions of stress, such as increased heart rate or metabolic stress. A number of posttranslational modifications of PLM have been characterised and their physiological consequences have been described. Further research is needed to understand the interplay between different posttranslational modifications and how they relate to overall cardiac physiology. Additionally, PLM regulation may occur at the level of gene expression, involving transcription, processing of mRNA and regulation of the stability of mRNA and the rate of transcription. Gene regulation with regards to PLM is largely unexplored, but the exciting possibility of potassium ion-stabilised higher-order G-quadruplex structures of PLM pre-mRNA warrants further investigations. Progress has been made in the area of structural biology with the determination of high-resolution human homologue NKA/PLM structures, the structure of a PLM monomer and a tetramer complex. These structures can be used for comparative modelling of the human NKA/PLM complex as it was attempted for this review. However, experimentally determined protein structures and comparative models provide only a static picture, while the NKA-PLM interactions modulated by post-translational modifications are surely dynamic. Most experimental methods investigating this dynamic interaction will be confounded by the heterogeneity of PLM posttranslational modifications in cardiomyocytes; thus single-molecule-based experimental methods together with complementary in silico simulation methods may be required to resolve this heterogeneity.

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Chapter 16
Regulation of Brain Na-K ATPase Activity by Noradrenaline with Particular Reference to Normal and Altered Rapid Eye Movement Sleep

Megha Amar, Abhishek Singh, and Birendra N. Mallick

Abstract Rapid eye movement sleep (REMS) is naturally expressed at least in all the mammals, including humans, studied so far. It is regulated by interplay among complex neuronal circuitry in the brain involving various neurotransmitters. Although the precise function and role of REMS is yet to be deciphered, loss of REMS increases brain excitability; however, the mechanism of action was unknown. As Na-K ATPase is the key molecule that maintains ionic homeostasis across neuronal membrane and modulates the excitability status of neurons, we proposed that REMS deprivation (REMSD) could affect the neuronal Na-K ATPase activity. On the other hand, evidences suggest that REMSD would elevate noradrenaline (NA) level in the brain and it has been proposed that REMS maintains brain NA at an optimum level. Therefore, while attempting to understand and explain the mechanism of action we hypothesized that REMSD-induced elevated NA could modulate Na-K ATPase activity in the brain and thus modulates the neuronal and brain excitability. In this chapter first we discuss the mechanism of increase in NA level in the brain after REMSD. Then we discuss the effect of such elevated NA on neuronal and glial Na-K ATPase activity. We observed that REMSD-induced increase in NA affected neuronal and glial Na-K ATPase activities in opposite manner, while it increased neuronal Na-K ATPase, and it decreased the same in glia. An intricate regulation of Na-K ATPase activity in neurons and glia is likely to be responsible for maintenance of ionic homeostasis in the brain during normal situation, which when disturbed including upon REMS loss patho-physiological changes and symptoms are expressed.

Keywords Adrenoceptors • Brain excitability • Calcium ion • Glia • Ionic homeostasis • Neuron

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1 Introduction

Basic rest and activity cycle (BRAC) is among the fundamental properties of a living system. In addition to the physical movement, the conscious and cognitive levels of the animals (particularly those higher in evolution) also differ during these states. Normally higher levels of consciousness and cognition are associated with waking state of an individual as opposed to that of during sleeping state when the levels remain subdued. As of our present-day understanding these states are manifested in an appreciable manner in animals higher in evolution having evolved brain. Notwithstanding, although cognition and consciousness may exist in other lower living species (organisms) without having brain or having a rudimentary brain, we do not understand them as yet due to lack of more fundamental, objective and stringent criteria to define and identify them. As the brain is made up of specialized excitable tissues the neurons, factors modulating the neuronal behaviour would affect the cognitive and conscious levels of an individual and vice versa.

One of the main characteristic properties of neurons as compared to other living cells in the body is its dynamic excitability, which is a reflection of the instantaneous changes in the potential difference across the neuronal membrane, the transmembrane potential. The transmembrane potential difference is a fundamental characteristic of all living cells, including neurons, and is caused due to differential ionic concentrations across the semipermeable biological membrane of a living cell. As the ionic concentrations across the membrane of a neuron are in a dynamic equilibrium, transmembrane potential needs to be continuously and effectively maintained at an optimum level for the brain to function effectively.

The Na-K ATPase plays a major role in maintaining such ionic balance across living cells including neurons [1–3]. Therefore, alteration in the Na-K ATPase activity is likely to affect the neuronal excitability and associated brain-controlled behaviour including cognitive and conscious levels and vice versa. As differences in the levels of consciousness and cognition are among the fundamental criteria to differentiate between sleep from other conscious states, we hypothesized that the brain Na-K ATPase activity is likely to be affected upon sleep disturbances resulting in associated behavioural changes, and conversely changes in the said enzyme activity would affect sleep. Further, while understanding the mechanism of action, we observed that noradrenaline (NA) is responsible for inducing the sleep-loss-associated changes in the Na-K ATPase activity, which we will explain subsequently.

2 Sleep-Wakefulness-REMS

Sleep and wakefulness are expressed in animals higher in evolution and are considered to be modified form of BRAC. These states have been objectively defined and identified by the presence of electrophysiological signals recorded simultaneously from the brain, the antigravity muscles and due to the eye movements. These electrophysiological signals not only helped classifying different levels of consciousness and mental states, they also discovered a unique, distinct and active state of the

brain during sleep. This state was identified in mid-twentieth century [4] and has been termed as rapid eye movement sleep (REMS); interestingly most dreams usually appear during this state [5–7]. Thus, the sleep state has been classified as REMS and non-REMS. As REMS does not normally appear during waking and it appears only after continuation of non-REMS for a variable period, loss of non-REMS usually is accompanied with REMS loss as well. However, as REMS is a distinct state and animals can be deprived almost exclusively of REMS alone to a reasonable extent with minimum disturbance to non-REMS and suitable control experiments can be designed to compensate for the small disturbance in the non-REMS state, we conducted studies by depriving the animals of REMS. Details of methods of REMS deprivation (REMSD) and their advantages and disadvantages have been published elsewhere [8–10].

2.1 REMS in Health and Diseases

Sleep, waking and dreaming during sleep were experienced by individuals and were known to humans ever since the evolution of knowledge; however, the neural mechanism of their regulations was unknown though. Unlike external behavioural manifestations of sleep and waking, there was no visible manifestation of occurrence of dreams until the discovery of dream- and REMS-associated expressions of electrophysiological signals. The REMS is present across species higher in evolution and the total quantity as well as proportion of REMS as compared to total sleep time varies among species [11–14]. The quantity of REMS is maximum in babies and it reduces with ageing [15, 16]. It is expressed more in the preterm babies and in those born immature [17, 18], suggesting that REMS has a role in brain development and maturity [19, 20]. The quantity of REMS has been found to be affected in most of the diseases from simple fever to complex psycho-somatic disorders [21–24]. Experimental REMS loss has been reported to affect psycho-somatic behaviour including memory consolidation [25–29], irritability, concentration, mood and behaviour [8, 30]. Based on these observations, as a unified hypothesis, we had proposed that REMS maintains brain excitability [31, 32]. As explained above the potential gradient across the neuronal membrane, the transmembrane potential, is the underlying cause for manifestation of neuronal excitability, and Na-K ATPase plays a major role in maintenance of transmembrane potential and brain excitability. Hence, we had proposed that REMS loss could affect neuronal and brain excitability by altering Na-K ATPase activity or vice versa and consequently the function of REMS is to maintain brain excitability.

3 Neuronal Excitability and Na-K ATPase

Due to unequal distribution of ions across neuronal membrane the intracellular compartment of neuron is relatively negative with reference to the extracellular space. Variations in the ionic concentration across the neuronal membrane alter the excitability status of neurons that affects neuronal behaviour and functioning.

The Na-K ATPase is a key naturally occurring biomolecule (factor) that simultaneously extrudes $3Na^+$ and imports $2K^+$ at the cost of energy released by hydrolysis of one ATP molecule in each cycle and maintains transmembrane potential gradient for optimum functioning of neurons. The importance of Na-K ATPase in maintaining the ionic gradient is emphasized by the findings that disruption of Na-K ATPase activity caused hyper-excitability and severe alterations in neuronal functioning [33, 34]. Additionally, modulation of Na-K ATPase activity may also affect several other neuronal functions including exchange of Ca^{2+} [35], transcription [36], neurotransmitter release [37], synapse formation [38], neuronal volume [39] and apoptosis [40].

3.1 REMS Loss and Na-K ATPase Activity

The transmembrane potential is the key index to infer about the excitability status of a neuron. Therefore, to comment on changes in neuronal excitability due to modulation of sleep-waking-REMS, ideally recording of intracellular potential needs to be carried out in animals under control and experimental conditions during various stages of sleep, waking, REMS and their losses. However, due to technical limitations it is not possible to record transmembrane (intracellular) potential from the same neuron in freely moving behaving animals and follow it up continuously through days under normal and altered behavioural conditions including sleep loss and REMSD. Hence, we settled for estimation of Na-K ATPase activity as a reflection of excitability level of neurons in the whole brain or in anatomically identified areas in the brain in control and after REMSD.

An enzyme activity (all other conditions remaining unaltered) normally depends on its allosteric modulation and/or transcriptional regulation of its molecules. As there was no study of REMS loss-associated changes in brain Na-K ATPase activity, rats were subjected to REMSD for 2–8 days and suitable control experiments were carried out. At the end of the experiments the enzyme activity was estimated in the rat brain. The Na-K ATPase activity increased in the whole brain as well as in localized brain regions of the experimental rats but not in the brains of rats maintained under various control conditions [10]. We also observed that the enzyme activity was increased first in the brainstem and then the increase spread to other brain regions [10, 41]. A representation for relative changes in Na-K ATPase activity under various conditions in different brain regions is shown in Fig. 16.1. As the Na-K ATPase activity increased after REMSD, it suggested that normally REMS is likely to maintain the Na-K ATPase activity of neurons in the brain and in the absence of REMS the activity was increased. Thus, the results for the first time supported our hypothesis with experimental data; however, it raised the next question that how REMS-loss, a behavioural change, could affect the Na-K ATPase activity.

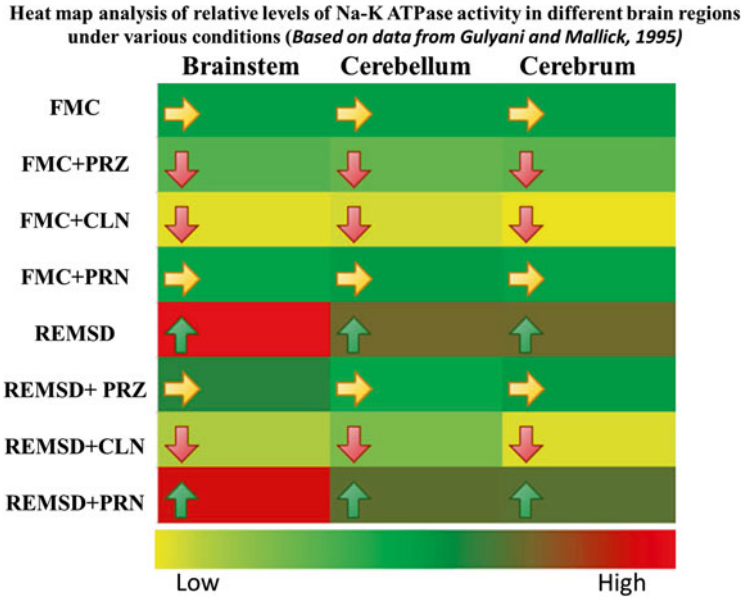

Fig. 16.1 Heat map analysis of relative levels of Na-K ATPase activity in different brain regions under various conditions in vivo (*based on data from Gulyani and Mallick* [41]). Abbreviations: *FMC* free moving control; *FMC+PRZ* FMC treated with prazosin (α1-AR antagonist); *FMC+CLN* FMC treated with clonidine (α2-AR agonist); *FMC+PRN* FMC treated with propranolol (β-AR antagonist); *REMSD* REMS deprived group; *REMSD+PRZ* REMSD treated with prazosin (α1-AR antagonist); *REMSD+CLN* REMSD treated with clonidine (α2-AR agonist); *REMSD+PRN* REMSD treated with propranolol (β-AR antagonist); *Low* lower Na-K ATPase activity compared to respective brain region of FMC group; *High* increased Na-K ATPase activity compared to respective brain region of FMC group

3.2 Mechanism of Action of Na-K ATPase

Na-K ATPase belongs to a P-type ATPase, the class of evolutionarily conserved enzymes that catalyze active transport of cations at the expense of hydrolysis of ATP across the plasma membrane in all mammalian cells. The P-type refers to the unique characteristic of these enzymes in forming a transient, phosphorylated aspartyl residue during the catalytic cycle. The P-type ATPases are divided into five subfamilies P1–P5 (each of which is further divided into subgroups), and they differ from each other in their transported ligands and regulatory pathways. P-type ATPases contain five functional and structurally distinct domains: three cytoplasmic domains, viz. actuator domain (A-domain), nucleotide-binding domain (N-domain) and phosphorylation domain (P-domain) and two membrane-embedded domains, transport domain (T-domain) and class-specific support domain (S-domain). We proposed that REMSD must be inducing some changes in the molecules in the body fluids, which then would affect the Na-K ATPase and increase its activity.

Further, we hypothesized that REMSD would cause changes in the level of one or more biomolecules related to REMS regulation so that such neural circuitry is likely to be disturbed during REMSD resulting in release of factors and/or alteration of biomolecules, which then would increase the Na-K ATPase activity; therefore, it is necessary to understand the neural regulation of REMS.

4 Clue from Our Understanding on Neural Regulation of REMS

The REMS is regulated by the interaction of REM-ON and REM-OFF neurons in the brainstem and these neurons are under the influence of host of other neurons in the brain [42, 43]. In short, the NA-ergic neurons in the locus coeruleus (LC), the REM-OFF neurons, normally cease activity during REMS [44], they continue firing during REMSD [45] and if they are kept active, REMS does not appear [46]. These results suggested that if the NA-ergic neurons cease firing during normal REMS, the excess NA would get washed-off from the neuronal projection sites in the brain. However, instead, if these neurons continue firing, as it has been reported during REMSD [45], the brain (synaptic sites) would get flooded with NA during REMSD. Based on our findings we proposed that cessation of the NA-ergic REM-OFF neurons is a pre-requisite for REMS generation [47] and there would be elevated NA in the brain after REMSD [48, 49].

4.1 Additional Support for Elevated NA in the Brain After REMSD

The enzyme monoamine oxidase (MAO) is responsible for breakdown of the excess NA at the synaptic cleft and thus helps maintaining brain NA level at an optimum level. REMSD has been reported to reduce the MAO in the brain [50]; therefore, it would not be sufficient to break down NA and consequently there would be elevated level of NA in the brain after REMSD. Additionally, it has been found that after REMSD there is increased level of tyrosine hydroxylase (TH), the rate-limiting enzyme for synthesis of NA in the brain [51–53], which would increase NA synthesis in the brain. Finally, as a direct evidence in a recent study we have observed that NA level increased in brain regions after REMSD (manuscript under preparation).

4.2 Elevated NA Increases Na-K ATPase Activity in the Brain after REMSD

Isolated, independent studies showed that NA level and Na-K ATPase activity increased in the rat brain upon REMSD. To confirm if there exists any cause and effect relationship between them we argued that NA antagonist should be able

to prevent REMSD-associated stimulation of the enzyme activity in vivo; NA should stimulate the Na-K ATPase activity in vitro and NA antagonist should prevent such NA-induced stimulation of the enzyme activity in vitro. Indeed it was observed in vivo that the REMSD-induced stimulation of the rat brain Na-K ATPase activity was prevented by α1-adrenoceptor (AR) antagonist, prazosin [41]. Also, NA stimulated the enzyme activity in vitro and the effect was prevented by prazosin [54–56]. These results confirmed that the REMSD-associated elevated NA stimulated the Na-K ATPase activity in the rat brain. However, we needed to confirm the source of NA for inducing such action, particularly if the NA was released from neurons in the brain and was not imported from the periphery.

4.3 Locus Coeruleus NA-Ergic Neuronal Activities Are Responsible for REMSD-Associated Stimulation of Na-K ATPase Activity

The LC is the primary source of NA in the brain [57, 58] and the brain receives most of the NA from these neurons [59–62]. It was already known that under normal condition these REM-OFF neurons cease firing during REMS [63] and they continue firing during REMSD [45]. Further, we and others had also shown using various methods that if these REM-OFF neurons in the LC were kept active or were not allowed to cease firing, REMS was prevented [46, 64, 65]. Therefore, there was substantial evidence that the NA released from these neurons is likely to stimulate the Na-K ATPase activity in the brain; however, it needed to be confirmed. Taking clue from the findings from our other studies it was hypothesized that if the neurons in the LC were not allowed to cease activity, there should be reduced REMS and increase in the Na-K ATPase activity in the rat brain.

To confirm, chronic rats were surgically prepared with bilateral chemitrode (guide cannulae) aiming the LC for microinjection of chemicals and electrodes for recording electrophysiological signals to identify sleep-waking. After recovery from surgical trauma and acclimatization of the rats to the recording environment, under freely moving conditions sleep-wake-REMS were recorded in these rats for 48 h with or without infusing GABA-antagonist picrotoxin bilaterally into the LC. The assumption was that picrotoxin in LC would not allow the inhibitory GABA to inhibit the NA-ergic REM-OFF neurons to cease activity and therefore REMS would not appear. Indeed it was observed that upon picrotoxin infusion the REMS was significantly reduced and the brain Na-K ATPase activity was significantly increased to a level comparable to otherwise normal rats deprived of REMS [64]. This confirmed that the NA released due to non-cessation (activity) of the NA-ergic LC-REM-OFF neurons indeed is responsible for REMSD-associated increased Na-K ATPase activity (schematically shown in Fig. 16.2), which in turn would increase the brain excitability.

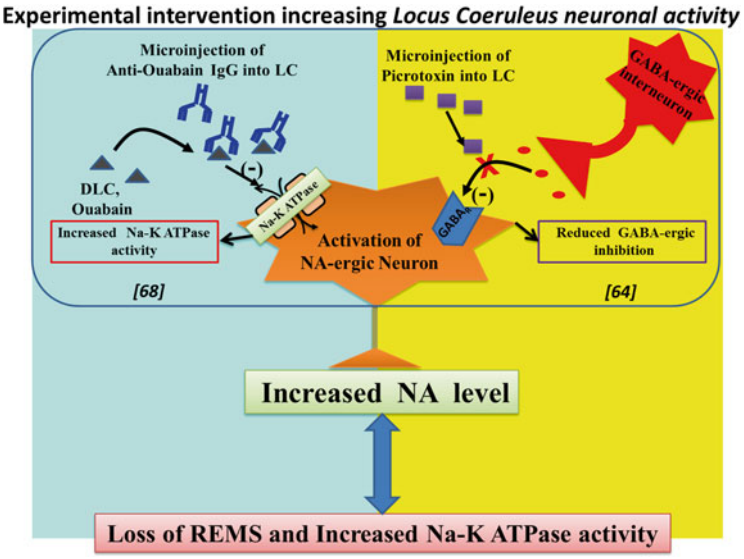

Fig. 16.2 Experimental intervention increasing LC-neuronal activity. The figure represents experimentally supported relationship between LC-neuronal activity, Na-K ATPase activity and REMS. *Left half* shows the application of anti-ouabain antibodies in LC reduces REMS which in turn increases Na-K ATPase activity. *Right half* shows application of picrotoxin increased LC-neuronal activity by blockage of GABA receptor, reduces REMS and increases Na-K ATPase activity. The *numbers in parenthesis* show the citation in the reference list

5 Na-K ATPase Activation-Induced Alterations in Neuronal Excitability and REMS

Our hypothesis is that REMS maintains brain excitability. In support we showed that REMSD increased Na-K ATPase activity, which could be the sole (or at least a significant factor) cause or effect for the altered brain excitability. Our contention was supported by the fact that inhibition of Na-K ATPase activity was reported to induce increased neuronal excitability [34] and elevated intracellular Na^+, that occurs during increased neuronal excitability, is reported to stimulate the Na-K ATPase activity [66, 67]. Nevertheless, to confirm the same in vivo particularly with reference to REMS, we hypothesized that if the Na-K ATPase activity of the neurons in the LC were stimulated, the REM-OFF neurons would be activated and there should be reduced REMS.

It was known that the cerebrospinal fluid contains endobains (naturally occurring anti-Na-K ATPase molecules) and their levels are altered in psychological disorders when REMS is also affected [68, 69]. As a strategy, first antibodies against the naturally occurring endobains were raised [70, 71]. Thereafter, chronic rats were surgically prepared with bilateral cannulae in the LC and electrodes for electrophysiological

sleep-waking recording. After recovery from surgical trauma sleep-waking recording was done under control injection and after injection of the antibodies against endobains into the LC [68]. The antibodies were expected to neutralise the naturally occurring endobains (Na-K ATPase antagonist) around the LC-REM-OFF neurons leading to activation of those neurons and cause loss of REMS (analogous to REMSD condition). Indeed it was observed that the rats injected with the antibodies showed reduced REMS as compared to controls [68], which was comparable to that of activation of LC neurons by electrical stimulation [46] or by microinjection of picrotoxin into the LC [64] (schematically summarized in Fig. 16.2). All these findings taken together confirmed in vivo that REMSD elevated the level of NA in the brain, which in turn increased Na-K ATPase activity in the rat brain. As Na-K ATPase plays a key role in the maintenance of brain excitability and REMS maintains NA level at an optimum level in the brain (by preventing rise of NA level), we proposed that REMS maintains brain excitability [31, 72].

5.1 Allosteric or Transcriptional Modulation of Na-K ATPase

An enzyme activity may be affected by either or both allosteric modulation or by transcriptional regulation. We showed that REMSD modulated both the K_m as well as the V_{max} of the Na-K ATPase in the rat brain [73], suggesting that upon REMSD, the enzyme activity was increased by modulation of both the factors. It led us to propose that short-term effect of REMSD could be mediated by allosteric mechanism, while the long-term REMSD increased the synthesis of the enzyme with or without allosteric regulation. Further, as we discussed above that the REMSD induced effects were mediated by NA, it needed to be evaluated if both the effects were mediated by the elevated level of NA.

5.2 Involvement of AR-Subtype in Mediating the REMSD Associated NA-Induced Increase in Na-K ATPase Activity

Synaptosomal membranes were prepared from normal rat brain and the effects of various doses of NA on the isolated synaptosomal membrane Na-K ATPase activity was estimated in vitro in the presence and absence of various AR-antagonists. It was observed that in such membrane preparation the NA stimulated the Na-K ATPase activity in vitro in a dose-dependent manner and the effects were prevented by pretreating the samples with prazosin (α1-AR-antagonist), but not by propranolol (β-AR-antagonist). These findings confirmed that the NA stimulated the Na-K ATPase in isolation and the effects were mediated by NA acting on α1-AR [56]. We also observed that while NA increased the Na-K ATPase activity in vitro in isolated membrane prepared from normal rat brain, it was ineffective in membrane prepared

from the brain of rats already deprived of REMS [56]. These findings although supported allosteric modulation of Na-K ATPase activity by NA, it did not allow us to comment if NA is involved in transcriptional regulation of the enzyme.

5.3 *Molecular Mechanism of Action of NA-Induced Stimulation of Na-K ATPase Activity*

Our results showed that NA stimulates Na-K ATPase activity by acting on α1-AR. The α1-AR is a member of GPCR family and Na-K ATPase activity is known to be inhibited by the Ca^{2+} [74–76]. Therefore, we attempted to understand if Ca^{2+} plays any role in NA-induced stimulation of the enzyme activity. We observed that the NA acting on α1-AR activated phospholipase-C (PLC) and stimulated the Na-K ATPase activity [56]. As Ca^{2+} is released as a downstream signaling of PLC, the results suggested that some intracellular Ca^{2+} was necessary for NA-induced stimulation of the enzyme activity. In a series of experiments it was observed that in synaptosomal membrane preparation NA stimulated the enzyme in the presence as well as absence of Ca^{2+} in the medium [76–78]. All these results taken together suggest that the latter (Ca^{2+}) is likely to be membrane bound, which got released by the action of NA and stimulated the enzyme activity.

Notwithstanding the above, in other studies we found that REMSD reduced the synaptosomal Ca^{2+} level [77] suggesting that reduced intracellular Ca^{2+} may be the cause of stimulating Na-K ATPase activity. Further, the NA inactivated the L-type calcium channel and prevented the influx of extracellular Ca^{2+} [77]. As a molecular mechanism of action we also observed that the NA and REMSD increased the dephosphorylated form of the enzyme, the active form of the Na-K ATPase [73], suggesting that NA dephosphorylates the enzyme. Taking together all these findings we proposed a model that elevated level of NA (including during REMSD) acting on α1-AR on one hand reduced influx of Ca^{2+}, while on the other hand released some Ca^{2+}, possibly bound to the membrane (may or may not be linked to the enzyme) which then dephosphorylated the Na-K ATPase in a coupled reaction [9]. As all these studies were conducted on isolated membrane preparation, although the results indicated allosteric modulation of NA on Na-K ATPase activity, NA-induced transcriptional regulation is yet to be understood, which is underway. Notwithstanding, the question that follows is that if the NA exerts such harmful effect on the brain, why through evolution NA remained into existence and it continues to play such significant role in a unique manner through evolution especially in relation to REMS. We argued that a critical (optimal) level (like a set point) of NA in the brain is likely to be very important in maintaining normal physiological processes and if that is disturbed, disease or predisposition to disease sets in (Fig. 16.3). However, as the REMS and LC neurons are modulated by several factors (inputs) even during healthy condition, the NA level is likely to vary though within physiological limit, the Na-K ATPase activity would be altered, which in

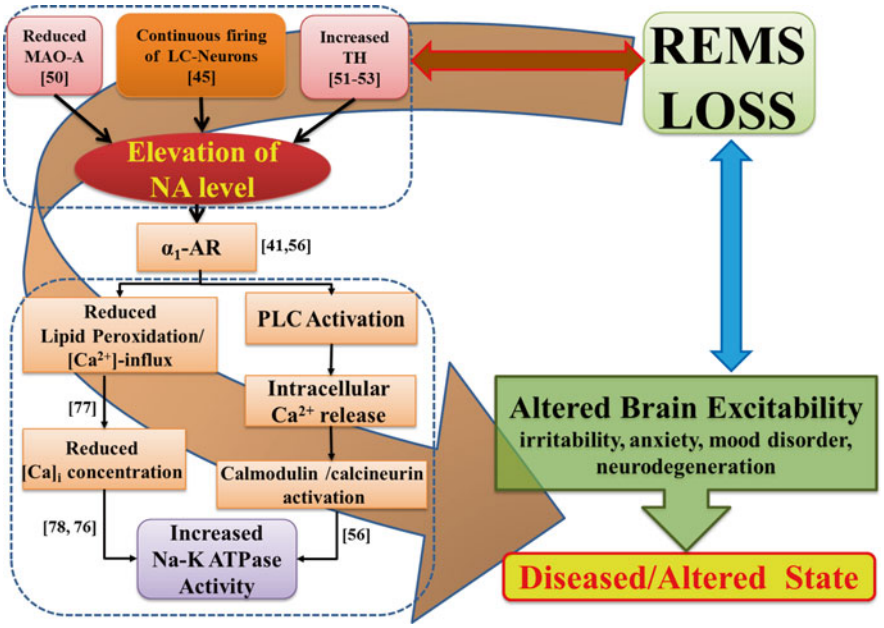

Fig. 16.3 Flow diagram of the intracellular mechanism of REMS loss induced increased brain excitability. REMS loss elevates NA level which in turn increases Na-K ATPase activity and consequently alters brain excitability leading to diseased state. Abbreviations: *LC* Locus coeruleus; *MAO* monoamine oxidase; *TH* tyrosine hydroxylase. *NA* noradrenaline; Ca^{2+} calcium ion. The *numbers in parenthesis* show the citation in the reference list

turn would affect the neuronal excitability. As an explanation we proposed that as glia in the brain is known to have buffering action, it may directly or indirectly play a role in maintaining neuronal excitability.

6 Possible Role of Glia in Homeostasis Maintenance

Glia is known to maintain the milieu of neurons including ionic balance across the neuronal membrane [79, 80]. As NA affects the neuronal Na-K ATPase activity and the glia also possesses Na-K ATPase [81], we evaluated in vivo the effect of REMSD and in vitro the effect of NA on glial Na-K ATPase activity. We observed that in rats upon REMSD the elevated NA influences the neuronal and glial Na-K ATPase in an opposite manner; while the neuronal Na-K ATPase activity was increased, the enzyme activity was decreased in the glia [54]. These results may be supported by our recent in vitro findings that NA downregulated Na-K ATPase in C6 cell line, while it up-regulated the enzyme in the Neuro2a cell line (manuscript under preparation). These findings may be summarised as in the brain NA modulates neuronal

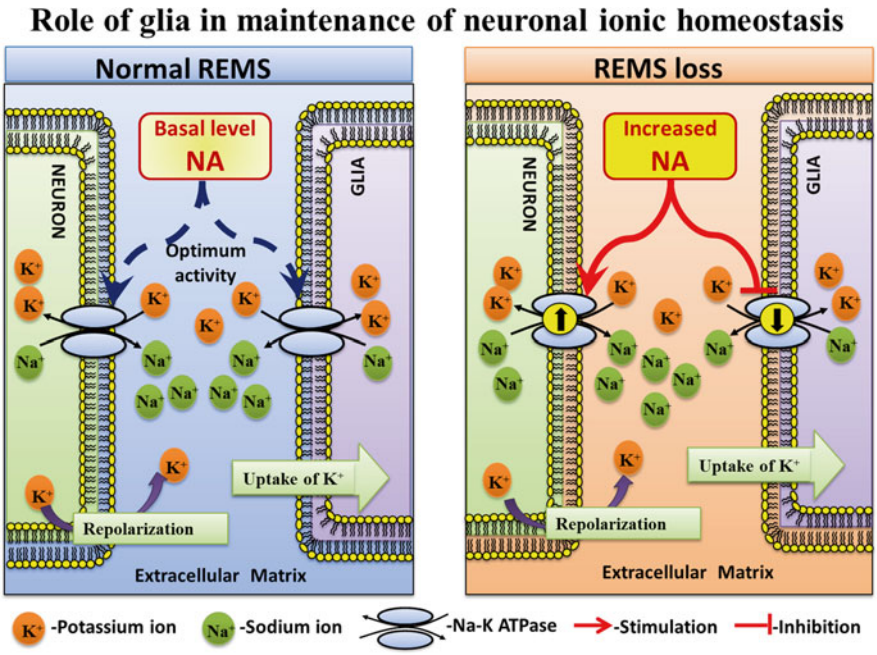

Fig. 16.4 Proposed model of role of glia in maintenance of K^+-homeostasis across neuronal membrane. *Left panel*—in normal REMS condition optimum level of NA is maintained, and thus optimal activity of neuronal and glial Na-K ATPase maintains extracellular ionic homeostasis. *Right panel*—during REMS loss, the elevated NA influences neuronal and glial Na-K ATPase in opposite manner; the Na-K ATPase activity increases in neurons while it decreases in glia [54], possibly as a compensatory effect, which helps maintaining the extracellular ionic homeostasis required for optimal Na-K ATPase activity. (*Dashed line* shows the basal activity, while *solid line* shows altered modulation in Na-K ATPase activity by the elevated level of NA)

and glial Na-K ATPase in an intricate and well-orchestrated manner that helps homeostatic regulation of the neuronal (brain) excitability (Fig. 16.4), which ultimately affects the behaviour.

7 REMS Serves Housekeeping Function for Optimum Brain Function

We know that the LC is the primary site for NA-ergic neurons in the brain and these neurons project throughout the brain [57]. These LC-neurons are maximally active through waking, reduces during non-REMS and cease activity during REMS [82, 83]. Further, these neurons do not cease firing during REMSD [45], if they are kept inactive, REMS appears [84] and if these neurons are not allowed to cease activity or if they are kept active by any means [46, 64, 65], appearance of REMS is

prevented. Such behaviour of the LC-NA-ergic neurons suggests that NA is essential for brain functioning; however, it may have a dose-dependent effect. Therefore, we proposed that normally during waking state, due to continuous activity of the LC neurons, NA is continuously released in the brain. The NA maintains the Na-K ATPase activity of the neurons and glia in an opposite manner and maintains the excitability level of the neurons in the brain at an optimum level for the brain to perform optimally. This is required because during waking the brain is at a higher state of alertness and sensitivity to perform various functions ideally with least inertia. The advantage of NA being associated for such alertness is that the associated increased oxidative damage can be taken care of by the same NA, which has an antioxidant property as well [85–87]. However, it cannot continue uninterrupted possibly because of normal wear and tear and no system can work by sustained activation. Therefore, as compared to waking state during non-REMS the LC neurons slow down activity reducing the level of NA in the brain and finally during REMS, due to cessation of the LC neurons, NA release is stopped. This withdrawal (complete or partial) of NA normally prevents building up of excess NA, helping the synapses (brain) to wash off the built-up NA and its metabolites. This action is a natural mechanism of recuperation of the neurons in the brain, which could be analogous to overcoming refractoriness of the neurons, so that the system is in its optimum level of excitability (activity) to express normal behaviour(s). As the Na-K ATPase activity maintains neuronal (brain) excitability, the fundamental property of neurons, we proposed that REMS serves housekeeping function of the brain, the brain excitability [9].

8 Conclusion

REMS is an instinct behaviour expressed in the higher forms of living species. The LC NA-ergic REM-OFF neurons in the brain are active in all states except during REMS; however, they continue to remain active during REMSD. Therefore, the NA level is withdrawn during REMS; however, during REMSD its level is increased. The NA stimulates and inhibits the neuronal and glial Na-K ATPase activity, respectively, possibly to maintain the ionic homeostasis so that the neuronal and brain excitability is maintained at an optimum level. However, if the NA level is disturbed as in REMS-loss for instance, the Na-K ATPase is affected leading to alteration in neuronal excitability. The latter then affects many, if not most of the physiological processes regulated by the brain; thus REMSD affects almost all the physiological processes. These findings have led us to propose that REMS maintains brain NA level, which maintains brain excitability and thus serves as housekeeping function of the brain.

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Chapter 17
Regulation of Na^+/K^+-ATPase Activity in the Nervous System

Ljiljana M. Nikolic

Abstract The Na^+/K^+-ATPase or sodium pump (NKA) is a membrane-anchored protein responsible for creating and maintaining the Na^+ and K^+ gradients across the plasma membrane of animal cells by using ATP hydrolysis to move three Na^+ out of the cell and two K^+ into the cell. In the nervous system, NKA activity is essential for the proper functioning of neurons and glial cells and for the driving of diverse brain processes. Regulation of NKA activity in the brain is achieved through multitude of complex mechanisms. The purpose of this chapter is to present an overview of mechanisms through which neurotransmitters such as glutamate, dopamine, and serotonin regulate NKA activity in neurons and glial cells. Here, the focus is on the signaling pathways and protein kinases that mediate neurotransmitter effects on the NKA activity. In addition, the chapter considers the regulation of neuronal and glial NKA activity by its direct interacting partners, that is, a diverse plasma membrane, synapse associated, cytoskeleton, and signaling cytoplasmic proteins that form complexes with NKA in the brain. Such complexes not only regulate NKA activity but also enable the pump to function as a signal transducer in the brain. In view of important physiological role that NKA plays in the brain, the perturbed regulation of NKA activity in neurons and glial cells and its association with the onset and progress of nervous system disorders is also addressed.

Keywords Sodium pump • Neuron • Glia • Neurotransmitters • Protein complexes • Nervous system disorders

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1 Introduction

1.1 Overview of Na^+/K^+-ATPase Properties

Na^+/K^+-ATPase or sodium pump (NKA) is ubiquitous membrane-bound protein that uses energy of ATP hydrolysis to move three Na^+ out of the cell and two K^+ into the cell [1], thereby maintaining the Na^+ and K^+ gradients across the plasma membrane of animal cells. This fundamental machinery of the NKA has been known for many years, and recently it was found that $3Na^+/2K^+$ movement is associated with the proton import, which points to a more complex function of NKA [2]. Accumulating evidence from recent years showed that this membrane pump forms complexes with multitude of cellular proteins and is involved in various signal transduction pathways [3]. For example, NKA signaling pathway triggers dendritic growth in developing neurons via induction of a specific program of gene expression [4].

Physiological functions of the NKA in the brain have been mainly deduced from its transporting role. There is general agreement that steep Na^+ and K^+ gradients set by the NKA influence a variety of brain processes including activities of voltage gated ion channels and synaptic activity [5–13]. It has been firmly established that neuronal and glial uptake of K^+ released during neuronal activity, necessary for maintenance of neuronal signaling, occurs primarily by active transport mediated by NKA [6, 14–16]. Activity of NKA can be specifically inhibited by cardiotonic steroids which directly bind to an enzyme and interrupt the transport cycle by plugging the ion pathway from extracellular side [17]. Some of the well-known NKA inhibitors are plant-derived drugs ouabain and digoxin. In addition, endogenous ouabain-like inhibitors were also found to be present in the brain [18–20]. All the transport-related functional properties of NKA reside in the catalytic α subunit which is differentially expressed in the brain [21–28]. In adult mammalian brain the α1 isoform is expressed in both neurons and glia, α3 isoform is characteristic for neurons, and α2 isoform is specific for glial cells.

In essence, the regulation of NKA activity is crucial for the maintenance of neuronal and glial function and thereby preservation of brain stability. In part, brain NKA activity is finely tuned by the presence and a combination of three α subunit isoforms that possess distinct kinetics. However, multiple regulatory events are also involved in the regulation of NKA activity in the brain. An overview, by no means complete, of reported regulatory mechanisms will be given in the following sections focusing on the regulation of NKA activity by neurotransmitters, as well as specific proteins that through direct interaction affect NKA activity in both neurons and glial cells.

2 Regulation of NKA Activity by Neurotransmitters

Numerous studies spanning couple of decades reported that neurotransmitters modulate activity of the NKA. A variety of data indicate that modulation of NKA activity by neurotransmitters is accomplished by controlling the phosphorylation state of the pump.

Regulation of NKA activity is associated with neurotransmitter receptor-mediated modulation of protein kinases activities through secondary messenger signaling cascades. Protein kinases involved in the regulation of NKA activity are protein kinase A (PKA) activated by cAMP, cGMP-activated protein kinase G (PKG), and phospholipid/Ca^{2+}-dependent protein kinase C (PKC) [29–31]. With the exception of PKG, the phosphorylation targets for other two protein kinases are determined to be specific Ser residues in the NKA α subunit. Even though the underlying molecular mechanisms of phosphorylation remain to be firmly established, the direct effect of phosphorylation on the NKA activity most likely arises from an alteration in the pump structure caused by phosphorylation of the Ser residue. Many of the effects of protein kinases on the NKA activity are countered by protein phosphatases. In fact, signaling cascades promoted by protein kinases at the same time involve inhibition of protein phosphatases [29]. The mechanisms of neurotransmitter-mediated regulation of NKA involve modulation of the pump activity already located in the plasma membrane and translocation of NKA to or from plasma membrane. Summarized findings on the regulation of NKA activity by some of the most studied neurotransmitters will be further presented.

2.1 Glutamate

The effects of major excitatory neurotransmitter glutamate on NKA activity have been extensively investigated, since glutamate mediates nearly 80 % of synaptic transmission in the brain [32]. Neuronal receptors and glial transporters for glutamate are proteins that rely on sodium gradients principally established by the NKA. Given that ionotropic glutamate AMPA receptors are primarily sodium channels while NMDA receptors permit sodium and calcium [33, 34], neuronal glutamate receptors are directly coupled to NKA activity (Fig. 17.1a). The stimulatory effect of glutamate was observed in Purkinje neurons, whereby increase in the NKA activity via activation of NMDA receptors was attenuated by blocking the PKG [35]. A pronounced and rapid activation of NKA was observed after incubation of cerebellar neurons with glutamate and activation of NMDA receptors [33]. The observed increase in the NKA activity was associated with the decrease in PKC-mediated phosphorylation of NKA through a mechanism that involves activation of calcineurin. In cultured cerebral neurons activation of ionotropic glutamate receptors has a differential effect on the activity of NKA isoforms as distinguished by isoform sensitivity to ouabain [36]. Thus, glutamate caused marked increase in the activity of highly sensitive NKA isoform, while slight decrease in activity was detected for isoform weakly sensitive to ouabain. Relation between metabotropic glutamate receptors and increase in the NKA activity was established in Purkinje neurons [35]. This relation was found to be mediated by PKC and most probably inositol-triphosphate production, since inhibition of PKC completely abolished the glutamate-induced increase in the NKA activity.

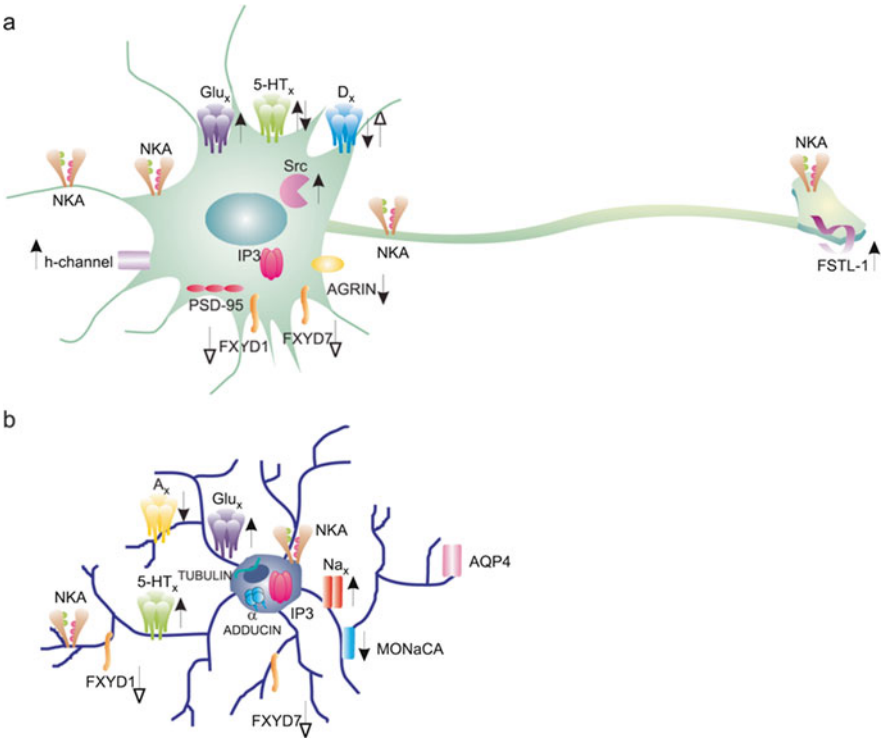

Fig. 17.1 Overview of the neurotransmitters and various cellular proteins that regulate Na^+/K^+-ATPase activity in neurons (**a**) and glial cells (**b**). Na^+/K^+-ATPase interacting plasma membrane, synapse associated, cytoskeleton, and signaling proteins are presented. Known changes in the Na^+/K^+-ATPase activity are depicted by *arrows. Black upward arrows* depict the increase, while *black downward arrows* depict the decrease of Na^+/K^+-ATPase activity. *Open arrows* depict proposed changes in the Na^+/K^+-ATPase activity. *Abbreviations*: *NKA* Na^+/K^+-ATPase; *Glux* glutamate receptors; D_x dopamine receptors; *5-HT* serotonin receptors; A_x adenosine receptors; *PSD-95* postsynaptic density protein; *IP$_3$R* inositol 1,4,5-trisphosphate receptors; *FSTL-1* follistatin-like 1 protein; *MONaKA* modulator of NKA activity; Na_x type of sodium channels; *h-channel* hyperpolarization-activated channels; *AQP-4* aquaporin 4

Glutamate uptake in the brain is performed predominantly by astrocytes through the action of Na^+-dependent glutamate transporters GLAST/EAAT1 and GLT1/EAAT2 (Fig. 17.1b [37]). Administration of glutamate at high levels was found to increase NKA α1 and α2 subunit expression in astrocytes in fetal telencephalic neuron-glia culture [38]. Glutamate-induced increase in NKA activity was also reported in cultured cortical astrocytes as measured by ouabain-sensitive ^{86}Rb uptake [39]. Increased NKA activity observed in astrocytes has been related to the α2 isoform, since the large proportion of augmented pump activity was sensitive to low concentrations of ouabain. Increase in the NKA activity was also observed in cultured human fetal astrocytes after administration of L-glutamate

[40]. The same study detected that, in the presence of glutamate, redistribution of GLAST from the cytoplasm to the astrocytic plasma membrane is accompanied with an increase in the NKA activity. Additional data are needed to further elucidate the physiological significance of regulation of glial NKA by glutamate. Nevertheless, available data suggest that glutamate-induced recruitment of additional NKA capacity in astrocytes may be necessary for keeping the normal extracellular glutamate concentration during the periods of neuronal activity. In addition, the sodium-coupled uptake of glutamate and increased NKA activity in astrocytes stimulate uptake of glucose which is further processed to the lactate [41]. When released from astrocytes, lactate is uptaken by neurons in which it can fuel energy requirements. Recent data revealed that other types of receptors can modulate NKA activity and affect glutamate uptake by astrocytes demonstrating additional complexity of NKA regulation by neurotransmitters. Thus, activation of adenosine A_{2A} receptors has been shown to decrease NKA activity and inhibit glutamate uptake by astrocytes [9].

2.2 Dopamine

The effect of dopamine on the NKA activity has been documented in striatum, a brain region characterized with highly expressed dopamine receptors that are members of a family of G protein-coupled receptors (Fig. 17.1a [42]). Inhibition of NKA activity by dopamine through activation of D1 and D2 dopamine receptors has been demonstrated in isolated neostriatal neurons [43]. Inhibitory effect of dopamine on the NKA activity was also observed in striatum and it was linked to the cAMP signaling cascade and activation of PKA [44]. Another study on striatum showed that dopamine D1 receptor activation inhibits NKA α subunit activity via signaling that is PKA dependent and involves phosphorylation of dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) [45]. Furthermore, it has been shown that dopamine in neostriatal neurons can exert its inhibitory effect through a mechanism that does not involve direct NKA phosphorylation [46]. Study conducted on medium spiny neurons isolated from neostriatum showed that inhibitory effect of dopamine on the NKA activity is linked to the removal of active pumps from the plasma membrane. Thus, it was shown that dopamine induced decrease in the plasma membrane expression of NKA α2 subunit, while no change was observed in total Na^+/K^+ content [47]. Further research on neostriatal neurons revealed that inhibitory effect of dopamine on NKA can be specifically attributed to the D1 type of dopamine receptors [10]. Generally, dopamine exerts inhibitory effect on NKA activity in striatum, at least when it binds to D1 receptors. On the other hand, D2 receptors in striatal neurons may be presumably involved in activation of NKA, since activation of these receptors by dopamine increase activity of sodium channels and by increasing intracellular sodium concentration may stimulate NKA activity [10].

2.3 Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) receptors are characterized by great diversity and except for one member of the family, a 5-HT_3 receptor which is a ligand-gated ion channel, are mostly G protein-coupled metabotropic receptors [48, 49]. In the brain, 5-HT receptor subtypes are distributed in highly distinct pattern. Some of the receptors from 5-HT family are known to modulate ion fluxes and cause alterations in neuronal membrane potential. Modulation of NKA activity by 5-HT in the brain was found to be bidirectional pointing toward the involvement of complex and interconnected signaling pathways (Fig. 17.1a). Early biochemical studies showed that administration of 5-HT increases NKA activity in the cerebral cortex of developing and adult rats [50, 51]. However, 5-HT-induced inhibition of NKA mediated by cAMP cascade was observed in leech tactile sensory neurons [52]. Furthermore, 5-HT inhibited NKA activity via 5-HT_3 receptors in hippocampal CA1 pyramidal neurons as revealed by electrophysiological measurements [11]. Another study showed that 5-HT_{2B} receptors mediate the decrease of NKA activity in 1C115-HT neuronal cell. In these cells 5-HT_{2B} dependent PKC activation promotes phosphorylation of NKA α1 subunit [53]. 5-HT also affects activity of NKA in glia, since glial cells uptake 5-HT via Na^+-dependent transporter [54–56]. Research on glial cells (Fig. 17.1b) showed that 5-HT activates NKA in rat cerebral cortex [57]. Glial NKA activity is modulated by more than one type of 5-HT receptors. Increase in the NKA activity via activation of 5-HT_6 receptors was observed in cerebellum while 5-HT_{1A} receptors mediate NKA increase in cerebral cortex and hippocampus most probably through pathways involving adenylate-cyclase coupled to G protein [12].

3 Regulation of NKA Activity by Direct Interaction with Specific Proteins

Over the past decade, considerable interest has been directed at elucidating the interaction and assembling of NKA in various protein complexes. Formation of such complexes has been shown to be important for the regulation of NKA activity in neurons and glial cells and to enable the pump to function as a signal transducer in the brain (Fig. 17.1a, b). Here, attention is drawn to the data showing NKA assemblies with some plasma membrane, synapse associated, cytoskeleton, and cytoplasmic signaling proteins.

3.1 Plasma Membrane Proteins

NKA often tightly associates with small single-span membrane proteins that belong to the FXYD family [58]. These proteins are identified and named after the sequence of their conserved motif (phenylalanine-X-tyrosine-aspartate

amino acid sequence). Several of FXYD proteins FXYD1, FXYD2, FXYD6, and FXYD7 are expressed in the brain [40, 59–61]. Some of them have been shown to bind to the NKA and modulate its activity. One of the members, phospholemman (FXYD1), was found to interact with NKA α isoforms in a nonspecific manner, since phospholemman expression was observed in both α3-containing neurons and α2-containing glia [60]. FXDY1 protein was found to reduce NKA activity without influencing the pump Na^+ affinity in purified choroid plexus preparation. Another member, FXYD7, is expressed in both neurons and astrocytes and associates specifically with NKA α1 subunit isoform in the brain. When associated with α1 NKA and expressed in *Xenopus* oocytes, FXYD7 reduces the NKA transport function by affecting the affinity of NKA for external K^+ [59]. It can be hypothesized that by acquiring lower K^+ affinity as a result of association with FXYD proteins in the brain, NKA becomes more efficient in extracellular K^+ clearance, thereby ensuring proper neuronal activity. In this way, FXYD proteins are acting as fine modulators of NKA activity and tune the pump activity according to the specific physiological demands. Novel cloned membrane-bound protein, named modulator of NKA activity (MONaKA), was also found to be a binding partner of NKA [62]. The MONaKA protein is highly expressed in astrocytes and by association with NKA inhibits its ATPase and ion transport activities. NKA interaction with some ion channels has also been shown. For example, Na_x channels associate with NKA in glial cells and modulate its function [63]. Na_x channels are characterized as an atypical leak channels that detect changes in extracellular concentration of Na^+. Direct interaction between NKA α1 and α2 subunit isoforms and Na_x channels was found to mediate Na^+-dependent activation of NKA in glia. Increased NKA activity may stimulate anaerobic glycolysis and production of lactate which is important to fuel neuronal activity especially during prolonged firing. Codistribution and functional interaction between NKA and h-channels has been found in neurons [64]. The current generated by NKA and the h-current flowing through hyperpolarization-activated channels (h-channels) participate in generating the resting membrane potential of neurons. Both proteins are codistributed in the soma and spines of mesencephalic trigeminal neurons whereby activation of h-channels by Na^+ influx generates ouabain-sensitive NKA current. NKA also interacts with water channels AQP4 expressed at the end feet of astrocytes. NKA and AQP4 are placed in close proximity at the plasma membrane whereby NH_2 terminus of AQP4 interacts with both α1 and α2 isoforms of catalytic NKA subunit [65]. In addition, the same NH_2 terminus of AQP4 also interacts with main astrocytic metabotropic glutamate receptor 5 (mGluR5) which suggests existence of a macromolecular transporting complex encompassing NKA, AQP4, and mGluR5 in astrocytic membrane. Clearly, further studies are needed to confirm the existence of such complex and to examine its functionality. However, it can be assumed that stimulation of mGLuR5 and uptake of glutamate via Na^+-dependent transporters would increase NKA activity whereby intracellular Na^+ in the domains surrounding NKA would be simultaneously buffered by influx of water through AQP4 channels.

3.2 Synapse-Associated Proteins

Postsynaptic density protein (PSD-95) located beneath the postsynaptic membrane can be regarded as a main protein responsible for distribution of NKA in synapse. The PSD-95 was found to form a complex with NKA α subunit isoforms in dendritic processes of neurons [23, 34, 66]. NKA and PSD-95 are codistributed in high degree at synaptic sites and associate via specific PDZ3 domain of PSD-95 and N-terminus of α NKA. In addition, PSD-95 was also found to bind to the glutamate [67], serotonin [68], and dopamine receptors [69] which are known to functionally interact with NKA. Furthermore, PSD-95 binds to and enhances the current carried through glial Kir 4.1 channels [70], channels that are functionally coupled to the activity of NKA in regulating extracellular K^+ concentration. PSD-95 has also been identified as a binding partner of Kv channels in neurons [71], channels that are functionally coupled with NKA in determination of membrane potential. Such PSD-95 protein organization roughly sketches assembling of NKA with functionally related proteins in synapses which would certainly have effect on the NKA activity.

Another synapse-associated protein that specifically binds to the NKA is agrin. This protein is not only released for the nerve terminals of motor neurons as originally found but also synthesized and released by neurons in the brain [72, 73]. Agrin interacts with α3 NKA subunit isoform via its C-terminus and inhibits NKA activity most likely by displacing the pump from the plasma membrane [74, 75]. In fact, some portion of the α3 NKA is normally inhibited by endogenous neuron-released agrin.

Follistatin-like 1 (FSTL-1) was also found to directly interact with NKA [76]. This glycoprotein is transported to axon terminals via small vesicles and has presynaptic distribution. Secreted FSTL1 increases NKA activity upon binding to the α1 subunit isoform and thereby acts as an NKA agonist. FSTL-1 was found to activate presynaptic NKA which causes hyperpolarization of membrane potential and suppression of synaptic transmission.

As already discussed, NKA activity in the brain is modulated by neurotransmitters. Accordingly, several studies reported that NKA directly associates with neurotransmitter receptors and transporters at synapses. Specific association of NKA α1 subunit isoform and AMPARs was found in dendrites of cortical neurons [34]. Association of astroglial α2 NKA subunit isoform with GLAST and GLT-1 was detected in somatosensory cortex and cerebellum [77, 78]. Direct interaction between D1 receptors and α3NKA has been shown in the postsynaptic areas of dendritic spines in cultured neostriatal neurons [66]. Direct association of adenosine A_{2A} receptors and α2 NKA was recently demonstrated in astrocytes in the cerebral cortex and striatum [9].

3.3 Cytoskeleton Proteins

An interaction of NKA with components of the cytoskeleton has also been documented. This interaction is thought to be important for correct trafficking and targeting of NKA to the specific membrane domains [29]. Some of the cytoskeletal proteins that directly

interact with NKA in the brain are adducin and tubulin. Adducin is highly expressed in the brain and plays an essential role in the assembly and regulation of actin mesh as it caps the fast-growing ends of actin filaments and thereby controls cytoskeleton-membrane interactions [79]. α-adducin specifically binds to the α2-NKA subunit isoform which is predominantly expressed in glial cells [80]. Direct interaction and formation of α-adducin/α2 NKA complex is implicated in the regulation of NKA expression. The NKA activity is also modulated through association and dissociation from tubulin, a dynamic component of microtubules [81–83]. More precisely, the NKA α subunit interacts with the tubulin which is posttranslationally modified through acetylation. Association with acetylated tubulin inhibits NKA activity. As demonstrated in cultured astrocytes, increase in NKA activity induced by L-glutamate is related to the dissociation of acetylated tubulin from NKA.

3.4 Signaling Proteins

NKA is considered to be a transducer of signals from extracellular milieu to the interior of cell as it communicates with certain cytosolic signaling proteins. Src kinase, a cytosolic protein involved in several signaling pathways, has been identified as an NKA binding partner. The family of Src kinases are non-receptor-type tyrosine kinases and five members of this family, Src, Fyn, Lyn, Yes, and Lck, are present at substantial levels in the brain [84, 85]. These Src kinases regulate neuronal excitability and synaptic transmission by modulating activities of ion channels, NMDA, AMPA, and GABA type A neurotransmitter receptors [86–88]. A study on cortical neurons has shown that Src tyrosine phosphorylation also regulates activity of NKA [89]. The administration of several tyrosine kinases inhibitors such as genistein, lavendustin, and herbimycin was found to attenuate the NKA activity as measured by the decrease in the pump current. By testing the effect of several tyrosine kinases present in the brain, Lyn kinase was identified as a specific kinase that promotes increase in the NKA activity as measured by the augmentation of the pump current. Immunoprecipitation and Western blotting assays revealed that Lyn kinase directly interacts with NKA α3 subunit isoform. Lyn kinase appears to directly phosphorylate tyrosine residues of α3 NKA; however, the site for Lyn/NKA interaction needs to be further characterized. Interestingly, inhibitors of Src kinase were found to inhibit glutamate transporter activity in astrocytes [78]. Therefore, it can be suggested that Src kinase is a component of NKA/glutamate transporter complex.

NKA has also been identified as a binding partner of inositol 1,4,5-trisphosphate receptors (IP_3R), a ligand-gated Ca^{2+} channel predominantly located at the membrane of intracellular Ca^{2+} stores such as endoplasmic reticulum [90]. These receptors are target for secondary messenger IP_3 and are responsible for the release of Ca^{2+} from endoplasmic reticulum, whereby released Ca^{2+} controls numerous cell activities including signal transmission and synaptic plasticity in the brain. Increase in the cytosolic Ca^{2+} concentration through activation of IP_3R has been shown to

increase NKA activity in astrocytes but not in neurons maintained in cultures [91]. It has been shown that NKA subunit isoforms, α2 in astrocytes and α3 in neurons, have confined distribution within the plasma membrane which parallel underlying endoplasmic reticulum thereby constituting specialized Ca^{2+} signaling complexes [92, 93]. Furthermore, a well-conserved motif in N-terminal tail of α1 NKA subunit isoform directly binds to the N-terminus of IP_3R in lysates from the whole rat brain [94], while in cultured hippocampal astrocytes NKA was found to associate with IP_3R via ankyrin-B [95]. As IP_3R binds directly to numerous molecules and function as a center of multiple signaling cascades [90], investigating the effect of NKA/IP_3R association on the NKA activity points to a new framework of research that integrates various IP_3R signal complexes.

4 Conclusions

The accumulated findings supported by a wealth of acquired experimental data have revealed the complexity of mechanisms involved in the regulation of NKA activity and pushed forward our understanding of the role this enzyme has in the nervous system. Coupling of NKA activity with neurotransmitter receptors and transporters in both neurons and glial cells through signaling cascades as well as through direct interaction emphasizes the importance of modulation of NKA activity in the regulation of synaptic transmission in the nervous system. Furthermore, as recognized from the experimental data, a well regulation of NKA activity is accomplished through specific interactions with certain cellular proteins that are responsible for trafficking, precise targeting of the pump at the plasma membrane, and fine-tuning of enzyme activity to meet the specific physiological demands. Other interactions, such as NKA interaction with Src kinase and IP_3R, favor the formation of signaling complexes that are able to modify numerous cellular functions. Novel partners and regulatory pathways that modulate NKA activity are most certainly waiting to be revealed, given the importance of the Na^+ and K^+ gradients set by the NKA for the functioning of many proteins in the nervous system.

There is increasing evidence that alterations in mechanisms that underlie the regulation of NKA activity are associated with the onset and progress of certain nervous system disorders. It has been shown that NO-mediated regulatory pathway acting through cGMP is compromised and unable to regulate the NKA activity in mouse model of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease characterized by dysfunction of motor neurons in the spinal cord, brainstem, and cortex. The same study revealed NKA collapses in both neurons and glial cells due to the loss of all three catalytic α subunit isoforms [96]. Recent research also showed that protein complex consisting of α2 NKA and α-adducin is enriched in astrocytes of ALS mouse model while elevated levels of α2 NKA and α-adducin were also observed in the spinal cord of ALS patients [80]. In vivo knockdown of spinal cord α2 NKA/α-adducin complex was found to suppress degeneration of motor neurons and increase life span of ALS mouse. In mouse model of familial hemiplegic migraine type 2, human mutation in gene for α2 NKA subunit

isoform causes retention of NKA protein in the endoplasmic reticulum [97] indicating that trafficking of the enzyme to the plasma membrane is compromised in this disease. Reduced brain NKA activity has been detected in animal models of depression, anxiety- and mania-like behavior [98–101]. In Myshkin mouse model of mania-like behavior, suppression of agrin was found to increase brain NKA activity. This finding implicates the regulation of NKA activity by agrin as a potential therapeutic target for the treatment of behavior-related disorders. More research is needed to establish the importance of different mechanisms that underlie the regulation of NKA activity in the onset and progress of nervous system disorders. Given the advance that has been accomplished, this research is certainly very promising.

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Chapter 18
Regulation of Membrane Na^+-K^+ ATPase in Health and Disease

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Abstract Na^+-K^+ ATPase is primarily localized in the plasma membrane and occurs in the form of α-subunits (α-1, -2, and -3 isoforms) and β-subunits (β-1 and -2 isoforms) mainly. The inhibition of this enzyme by cardiac glycosides such as ouabain has been shown to raise the intracellular concentration of Na^+ and promote the increase in cardiac contractile force as a consequence of increased Na^+-Ca^{2+} exchange. Several studies have observed that Na^+-K^+ ATPase not only serves as a receptor for cardiac glycosides but also acts as a Na^+-pump for maintaining the electrolyte homeostasis as well as a signal transducer for the formation of reactive oxygen radicals and cellular growth. Different hormones and endogenous factors such as marinobufagenin are considered to regulate the Na^+-K^+ ATPase activity through protein kinase-induced phosphorylation of phospholemman, a membrane protein which is tightly associated with this enzyme. Stimulation of Na^+-K^+ ATPase by catecholamines and phosphorylation of phospholemman seems to be an adaptive mechanism for the prevention of Ca^{2+}-overload-induced arrhythmias whereas excessive increase in the plasma levels of marinobufagenin or high concentrations of cardiac glycosides are considered to induce cardiac dysfunction associated with depression of the Na^+-K^+ ATPase activity.

Keywords Cardiac Na^+-K^+ ATPase • Cardiac glycosides • Marinobufagenin • Phospholemman • Cardiac dysfunction • Signal transduction • Protein kinase-induced phosphorylation • Na^+-pump ATPase

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1 Introduction

Since the discovery of Na^+-K^+ ATPase in 1957 [1], extensive studies have examined the role of this enzyme in both health and disease conditions [2–10]. It is now well known that Na^+-K^+ ATPase or Na^+-pump ATPase is intimately involved in the transport of Na^+ and K^+, and thus maintains the electrochemical gradient across the cell membrane [2, 8, 11, 12]. Cardiac glycosides have been demonstrated to inhibit the Na^+-K^+ ATPase activity and increase the intracellular concentration of Na^+, which then increases the cytoplasmic concentration of Ca^{2+} through Na^+-Ca^{2+} exchanger and thus produces the positive inotropic action in the heart [3, 13, 14]. In addition to serving as receptor for cardiac glycosides for the treatment of heart failure [3], Na^+-K^+ ATPase is considered to act as a signal transducer for promoting cellular growth and thus may participate in the pathogenesis of cancer [15, 16]. While this enzyme has been suggested to be involved in cell survival, complete inhibition of Na^+-K^+ ATPase by ouabain was associated with cell death due to necrosis [17]. Likewise, ouabain at low doses has beneficial effects in the heart whereas at high concentrations, it exerts cardiotoxic actions [3]. Such actions of ouabain may be related to the high and low affinities of ouabain binding sites on Na^+-K^+ ATPase [18]. This chapter focuses on the structural and functional properties of Na^+-K^+ ATPase with particular emphasis on cardiac membranes. Some evidence has also outlined to indicate the involvement of Na^+-pump ATPase as a signal transducer. Furthermore, it is planned to describe some of the regulatory mechanisms for changes in the Na^+-K^+ ATPase activity.

2 Structural and Functional Properties

Although Na^+-K^+ ATPase is considered to be primarily localized in the cell membrane, the functional density of Na^+-K^+ pump current in the t-tubules was found to be 3–3.5-fold higher than that in the sarcolemma of myocardium [19]. Several studies have been carried out to examine the biochemical properties of partially purified Na^+-K^+ ATPase [20–23] from the tissue as well as that of the enzyme present in the sarcolemmal membrane. Treatment of the sarcolemmal membrane with phospholipase C and trypsin was observed to depress the Na^+-K^+ ATPase activity [22]. It was shown that Na^+-K^+ ATPase undergoes conformational changes during its activation by Na^+ and K^+, which subserve the possible mechanism underlying the active transport of cations [21]. The inhibition of the enzyme by ouabain, unlike that by Ca^{2+}, was allosteric in nature; Na^+-K^+ ATPase was also depressed by different inhibitors of sulfhydryl groups [23]. It should be mentioned that initial attempts for the solubilization and purification of Na^+-K^+ ATPase from the cardiac muscle by the use of both ionic and nonionic detergents yielded the enzyme with low activity in the range of 20–30 μmol P_i/mg protein/h [14, 24–26]. On the other hand, when the purification of Na^+-K^+ ATPase from cardiac tissue was carried out by the treatment with deoxycholate and NaI, the activity was found to be in the range of 140–160 μmol P_i/mg

protein/h [23, 27]. The fact that detergents and chaotropic salts did not alter the key properties of Na^+-pump ATPase from those seen in native sarcolemmal enzyme indicates that Na^+-K^+ ATPase is an integral membrane protein.

The Na^+-K^+ ATPase is made up of α- and β-subunits and undergoes E_1–E_2 transition, a characteristic feature of the P-type ATPases [28–32]. The occurrence of three α-subunit isoforms (α_1, α_2, and α_3) and two β-subunit isoforms (β_1 and β_2) has been reported. It was suggested that these isoforms of the enzyme confer evolutionary advantage and regulation of gene expression during development in a tissue-specific manner [33]. The differential expression of Na^+-K^+ ATPase in different cells or tissues appears to be due to its structural heterogeneity [34]. By X-ray microdialysis of frozen cryosections, the Na^+-K^+ pump was identified to be present in the apical and basal cell membranes [35]. A monoclonal antibody, anti-BSP-3, directed against brain cell surface protein [36] recognized mouse Na^+-pump and immunoprecipitated Na^+-K^+ ATPase in microsomal fraction of the kidney; basolateral cell surface of polarized cells was stained by BSP-3 antibody. The α-subunit (MW 84–120 kDa) was considered to play a vital role in the catalytic activity of Na^+-pump ATPase. Sheep kidney Na^+-K^+ ATPase β-subunit (MW 55 kDa) cDNA was cloned and it was shown that p-subunit amino acid sequence was structurally similar to Kdp c subunit of Na^+-K^+ ATPase in *E. coli* [37]. The β-subunit of the Na^+-pump has been shown to play a critical role in cellular resistance to cardiac glycosides [38]. Decreasing the α-helix conformation of plasma membrane proteins was found to inhibit the Na^+-pump due to high concentrations of Ca^{2+} [39]. The amino acid sequence of the catalytic subunit of Na^+-K^+ ATPase (α-isoform) was derived from its cDNA [40]. Expression of the entire mouse cDNA coding for α-subunit conferred ouabain resistance in monkey CV-1 cells whereas deletion of the C-terminal of the α-subunit cDNA resulted in impairing this property [41]. Thus the behavior of Na^+-K^+ ATPase activity is considered to be determined by the composition of its isoforms.

Substitution of amino acids, Gln-Ala-Ala-Thr-Glu-Glu-Glu-Pro-Gln-Asn-Asp-Asn-ò Arg-Ser-Ala-Thr-Glu-Glu-Glu-Pro-Pro-Asn-Asp-Asp, in the N-terminal at extracellular domain of the α-subunit of the Na^+-K^+ ATPase in HeLa cells conferred ouabain resistance [42, 43]. The three α-subunit isoform gene structures have been characterized; all three isoforms have similar exon-intron structure [44]. Upregulation of β-subunit modulates the number of K^+-pump formation and translocation to the membrane surface in addition to regulating the α-β heterodimer formation [45]. Vascular smooth muscle Na^+-K^+ ATPase α-subunit isoform is regulated by alternative splicing of single transcript [46]. Since γ-thio ATP was found to be bound to ATP binding site in the Na^+-pump ATPase and mediate the inhibitory effect, addition of ATP was observed to remove this inhibition [47]. The activity of inactivated enzyme was also restored by incubating with a sulfhydryl reactive agent [48]. Substitution of amino acids α_1 C113 → Y and α_1 C113 → F of the Na^+-pump ATPase renders ouabain resistance compared to the wild type [49]. It has been shown that the Na^+-pump activity is regulated in part by a liver fatty acid binding protein by increasing the plasma membrane fluidity [50]. Substitution of amino acid Y317→ C in α_1-subunit ecto-domain H_3–H_4, caused an increased ouabain resistance in Madin-Darby canine

kidney cell line by affecting the ouabain binding to α-subunit [51]. Amino acid residues 496-HLLVMKGAPER-506 form the fluorescein isothiocyanate (FITC) binding domain in the catalytic α-subunit of the Na^+-pump ATPase. 494-PRHLL-498 is the most critical sequence for nucleoside binding [52]. Transfection of HeLa cells with double mutants generated by the amino acid substitutions at Asp111 and Arg122 in the H_1–H_2 extracellular domain of sheep Na^+-pump α-subunit rendered ouabain resistance to the cells. It was suggested that H_1–H_2 extracellular domain of Na^+-pump α-subunit possesses the functional unit of the Na^+-K^+ ATPase [43].

The yeast Na^+-K^+ ATPase is made up of α- (catalytic), β- (structural), and putative γ-subunit. Presence of α-β-subunit complex alone confers the ouabain-inhibitable Na^+-pump activity whereas the γ-subunit is redundant [47]. The Cys104 in H_1 transmembrane domain of α-subunit of Na^+-pump possesses the cardiac glycoside binding site [53]. The α-subunit hydrophobic domain H_1 and H_2 serves as signal-anchor type II whereas H_2–H_4 confers "halt" transfer signal. The membrane insertion property of Na^+-pump is conferred by amino-terminal possessing signal/anchor type II/halt transfer sequences [54]. The region corresponding to Gly554 → Pro785 in the cytoplasmic domain of α-subunit is essential for complex formation [55]. Substitution of amino acid at D369 → N porcine kidney Na^+-K^+ ATPase α-subunit caused an 18-fold increase in ATP binding by net reduction in negative charge in the phosphorylation site D369 → N [56]. Based on hydropathy plot, different numbers of transmembrane domains, varying from six [57], seven [58], and eight [40], have been proposed for the α-subunit. The H_1–H_2 and H_3–H_4 domains were orienting to the extracellular face and FITC [ATP] binding site, facing the cytoplasm [34]. Both N-terminus and C-terminus in the α-subunit are present in the cytoplasm; N-terminus is the most divergent whereas the H_1–H_2 and H_3–H_4 domains are shown to participate in ouabain binding. Amino acid residues Asp 369 and Lys 501 were labeled by FITC whereas Cys356, Asp 710, and Lys 719 were identified as ATP binding region of α-subunit [28, 40, 59]. It is pointed out that the α-subunit isoform distribution is well conserved across the species [28]. The β-subunit has been shown to mediate the membrane insertion and its transmembrane domain is also conserved across the species. The extracellular domain of different isoforms of β-subunit has different glycosylation sites. Six cysteine residues are critical for the disulfide bridge formation in all β-isoforms [60]. It has been conclusively demonstrated that the stoichiometry of Na^+-pump ATPase is 3 Na^+ (efflux):2 K^+ (influx) and is similar in epithelial and excitable cells [28].

3 Signal Transduction by Na^+-K^+ ATPase

It has become clear that the inhibition of Na^+-K^+ ATPase by cardiac glycosides affects cation fluxes and produces direct actions on cardiac contractility, electrical excitability, and conduction [61]. Synchronized modulation of Na^+-K^+ pump ATPase has been

shown to elicit membrane potential hyperpolarization in the cardiac cells [62]. It should also be noted that transmural gradient in the cardiac Na^+-K^+ pump has been observed to generate a transmural gradient on Na^+-Ca^{2+} exchange [63]. There is a functional communication between Na^+-K^+ ATPase and ATP-sensitive K^+ channels [64]; the activation of K^+-ATP channels by the inhibition of Na^+-K^+ ATPase has been shown to occur in the membrane [65]. It has been reported that the binding of ouabain to Na^+-K^+ ATPase converts this enzyme to a signal transducer and initiates different gene regulatory pathways through the activation of tyrosine kinase and Ras/Raf/MEK/MAPK pathway as well as the increased production of reactive oxygen species in the cardiomyocytes [66]. Not only did the inhibition of Na^+-K^+ ATPase activate tyrosine kinase-mediated signaling, ouabain was also found to increase mitochondrial production of reactive oxygen species and regulate the intracellular concentration of Ca^{2+} [67]. Furthermore, the inhibition of Na^+-K^+ ATPase resulted in an impairment of mitochondrial Ca^{2+}-retention, increased oxidative stress, and enhanced oxidative phosphorylation [68]. In fact, redox-activated protein kinases including PKA, PKC, and CaMKII have been shown to affect Na^+ and Ca^{2+} transporter as well as cationic channels [69]. It was interesting to observe that phosphorylation of sarcolemmal Na^+-K^+ ATPase (α-subunit) due to CaMKII caused a significant reduction in the activity of this enzyme [70]. These results clearly indicate that different protein kinases and redox-related system are intimately involved in the signal transduction mechanisms when Na^+-K^+ ATPase activity is inhibited by cardiac glycosides.

In order to examine the role of Na^+-K^+ ATPase inhibition due to cardiac glycosides in producing the increase in intracellular Ca^{2+} in cardiomyocytes, a pharmacologic approach was employed to understand the involvement of different signal transduction mechanisms [71–73]. The results were found to support the view that in addition to sarcolemmal Na^+-Ca^{2+} exchanger, sarcolemmal L-type Ca^{2+}-channels and store-operated Ca^{2+}-channels may be involved in raising the intracellular concentration of Ca^{2+} upon the inhibition of Na^+-K^+ ATPase. Furthermore, both sarcolemmal Na^+-H^+ exchanger and Na^+-channels may play a critical role in increasing the ouabain-induced increase in the concentration of Ca^{2+} in cardiomyocytes [71, 72]. These results are interpreted to suggest that the inhibition of sarcolemmal Na^+-K^+ ATPase markedly affects the function of other membrane proteins. The involvement of sarcoplasmic reticulum in raising the intracellular concentration of Ca^{2+} due to the Na^+-K^+ ATPase inhibition was also tested by incubating cardiomyocytes with agents that affect the sarcoplasmic reticulum Ca^{2+} stores. Treatments with caffeine, ryanodine, and cyclopiazonic acid attenuated the ouabain-induced increase in the levels of intracellular Ca^{2+}. Inhibitors of CaMKII, PKA, and inositol-3-phosphate receptors were also observed to depress the ouabain-induced increase in the intracellular Ca^{2+} in cardiomyocytes [73]. These results show that the participation of sarcoplasmic reticulum in raising the intracellular concentrations due to the inhibition of Na^+-K^+ ATPase by ouabain is of indirect nature and may involve multiple signal transduction mechanisms in cardiomyocytes.

4 Regulation of Na^+-K^+ ATPase

Various hormones, prostaglandins, and neuropeptides have been shown to exert regulatory effect on Na^+-K^+ ATPase in a tissue-specific manner. For example, catecholamines were found to stimulate the Na^+-K^+ ATPase activity in the heart through the activation of β-adrenoceptors and PKA mechanisms [74, 75]. Angiotensin II showed biphasic effects on renal Na^+-K^+ ATPase as the enzyme was stimulated at its low concentrations and inhibited by its high concentrations [76]. The stimulatory effect of angiotensin II was associated with increased activities of MAP kinase, tyrosine kinase, and NADPH oxidase. Estradiol-induced expression of Na^+-K^+ ATPase catalytic isoforms in vascular tissue was mediated through the formation of nitric oxide [77]. A neuropeptide, myomodulin, was observed to depress the Na^+-K^+ ATPase activity in heart interneurons [78]. Different purine nucleosides, inosine, guanosine, and adenosine, were found to stimulate Na^+-K^+ ATPase activity in a dose-dependent manner in erythrocytes [79]. Marinobufagenin, an endogenous ligand of α-1 subunit of Na^+-K^+ ATPase, has been reported to depress the myocardial enzyme activity [80]. Likewise, thromboxane B2, a product of thromboxane A2 during the synthesis of prostaglandin, was shown to inhibit the Na^+-K^+ ATPase activity in the heart [81]. Thus the activity of Na^+-K^+ ATPase is regulated by a wide variety of hormones and endogenous factors and seems to play an important role in the adaptation and maladaptation of different organ function in the body.

The regulation of Na^+-K^+ ATPase is considered to be affected mainly by phosphorylation and dephosphorylation of phospholemman, a small transmembrane protein which is associated with the enzyme [82–85]. Phospholemman is a member of the FXYD family of proteins and is a major substrate for different protein kinases [86, 87]. In its dephosphorylated form, phospholemman exerts an inhibitory action on Na^+-K^+ ATPase whereas it is stimulatory in the phosphorylated form. Phospholemman phosphorylation and modulation of Na^+-K^+ ATPase due to catecholamines and ischemia in the heart has been suggested to function in a manner similar to the interaction of phospholamban and Ca^{2+}-pump ATPase in the sarcoplasmic reticulum [88, 89]. It should be mentioned that both PKA and PKC have been shown to phosphorylate phospholemman at conserved serine residue (Ser 68) in its cytoplasmic domain and induce major changes in the protein conformation and thus alter the Na^+-K^+ ATPase activity [86]. Furthermore, phospholemman phosphorylation and Na^+-K^+ ATPase stimulation has been suggested to be an integral part of sympathetic fight-or-flight response for limiting Ca^{2+}-overload-induced arrhythmias as a consequence of attenuated rise in the intracellular concentration of Na^+ [83].

Na^+-K^+ ATPase is regulated by different hormones not only in physiological conditions, but this enzyme is also a target of regulation by various factors under diseased situations. Extensive work has indicated marked changes in the Na^+-K^+ ATPase activity in diverse cardiovascular diseases [4–7, 9]. In view of the ability of Na^+-K^+ ATPase to maintain cell volume [90], it is possible that changes in the enzyme activity may alter cellular function by inducing changes in cell volume. During cardiac hypertrophy and transition to heart failure due to hypertension, a decrease in α-1 isoform and an increase in α-3 isoform in cardiac Na^+-K^+ ATPase are associated with

an increase in the plasma levels of endogenous ligand, marinobufagenin [91]. In fact, depressed cardiac Na^+-K^+ ATPase activity and associated reduction in Na^+-K^+ ATPase α-1 isoform in heart failure were seen upon the infusion of marinobufagenin [92]. Different hypertrophic stimuli have been reported to alter the enzyme activity by affecting the gene expression of Na^+-K^+ ATPase β-1 and α-3 isoforms [93]. The inhibition of Na^+-K^+ ATPase and the development of cardiac dysfunction in endotoxemia were associated with myocardial TNF-α protein expression via calcium/mTOR signaling [94]. A modest diet-induced increase in serum cholesterol was found to increase the sensitivity of sarcolemmal Na^+-K^+-pump to intracellular Na^+, whereas a large increase in cholesterol levels decreased the sensitivity to Na^+ [95]. Some investigators have also observed the arrhythmogenic role of Na^+-K^+ ATPase in human heart failure and have suggested its involvement as an important modulator of excitability and refractoriness in human atria [96, 97]. Such results provide evidence that Na^+-K^+ ATPase is regulated by diverse mechanisms during the development of heart disease. Different diseases including diabetes [98] have been shown to alter the properties of this enzyme and thus can be seen to regulate the Na^+-K^+ ATPase.

5 Conclusions

From the foregoing discussion, it is evident that the membrane Na^+-K^+ ATPase not only serves as a Na^+ pump for maintaining the homeostasis of electrolytes in the cell but also forms an essential component of the signal transduction mechanisms. Various hormones and endogenous factors have been shown to regulate the Na^+-K^+ ATPase activity through phosphorylation and dephosphorylation of phospholemman, a membrane protein which is associated with this enzyme. Although α-isoforms (α-1, -2, and -3 subunits) and β-isoforms (β-1 and -2 subunits) of the Na^+-K^+ ATPase are tissue specific, their role in the regulation of enzyme activities has been suggested in the development of disease processes. A shift in the Na^+-K^+ ATPase isoform composition seems to occur during the transition of cardiac hypertrophy to heart failure. Particularly, the plasma level of the endogenous ligand, marinobufagenin, has been shown to increase in heart failure due to hypertension which may phosphorylate phospholemman, change the composition of Na^+-K^+ ATPase isoforms, and depress the enzyme activity.

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Chapter 19
Redox Regulation of the Na^+-K^+ ATPase in the Cardiovascular System

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Abstract The Na^+-K^+ ATPase is called "the oldest pump" as it has been the first of P-Type ATPases family to be discovered. This α/β heterodimeric molecule has an essential role in membrane transport of ions and organic molecules, and in cardiac myocytes, plays a key role in excitation–contraction coupling. Due to dynamic changes in the complex in vivo milieu, the pump function is tightly regulated in order to adapt to changing needs. Accumulating evidence has formed a consensus view that pump regulation is mediated by changes in phosphorylation of the FXYD1 protein that associates with the pump. However, this view is challenged by the lack of putative phosphorylation sites on the whole family of FXYD proteins that are expressed in tissue-specific manner. Moreover, the proposed functional effects of the phosphorylation, e.g. via β adrenergic signalling, are at odds with the role of the pump in clinical conditions like heart failure, and the clinical efficacy of drugs that block β_1 adrenergic signalling. Regulation of the pump function via oxidative post-translational modification has emerged as an alternative with glutathionylation of β_1 pump subunit and FXYD1 playing a dynamic regulatory role via receptor-coupled signalling in a variety of clinical conditions. In this chapter we briefly review structure and function of the Na^+-K^+ pump, and discuss in detail its regulation by redox pathways in the heart and critical regulatory role of FXYD1 proteins. We present the emerging role of redox regulation in the vasculature, where such signalling can have broad effects on cellular processes including vascular proliferation.

Keywords Na^+-K^+ ATPase • Na^+-K^+ pump • Oxidative signalling • Reactive oxygen species • Glutathionylation • FXYD1 • Beta adrenergic signalling • Angiotensin II • Phosphorylation

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1 Introduction

1.1 Function of the Na^+-K^+ Pump

The Na^+-K^+ pump was the first of the family of P-type ATPases to be discovered [1]. P-type ATPases function to hydrolyze ATP to translocate ions across the cell membrane against their transmembrane gradients. The Na^+-K^+ pump is expressed in all eukaryotic cells and hydrolyzes an ATP molecule to export $3Na^+$ ions in exchange for the import of $2K^+$ ions [2]. The Na^+-K^+ ATPase cycle is described by the Post–Albers scheme [3, 4]. The Na^+-K^+ ATPase cycles through two major conformational states, E1 and E2, which are characterised by differences in their interactions with Na^+-K^+ and ATP [5]. Na^+ and ATP bind with high affinity to the E1 conformation of the enzyme, during which phosphorylation occurs [6]. Three Na^+ ions are then occluded while the enzyme remains phosphorylated. The enzyme in the E1-P($3Na^+$) conformation loses its affinity for Na^+ while the affinity for K^+ is increased. Therefore, $3Na^+$ ions are released to the extracellular medium and $2K^+$ ions are bound. The binding of K^+ induces a spontaneous dephosphorylation of the E2-P conformation. The dephosphorylation of E2-P leads to the occlusion of $2K^+$ ions, forming the E2($2K^+$) conformation [6]. Intracellular ATP promotes the release of K^+ from the E2($2K^+$) conformation to finish the cycle back to the E1ATPNa^+ conformation. These three main reactions form the full catalytic cycle of Na^+-K^+ ATPase, which is illustrated in Fig. 19.1.

The $3Na^+$:$2K^+$ exchange ratio results in a net outward current and produces an electrochemical gradient across the cell membrane [2] which provides the energy for various active secondary co- and counter-transport processes, including Na^+:H^+ exchange, Na^+:Ca^{2+} exchange, Na^+ dependent Cl^- transport [7, 8] and the transport of organic molecules such as glucose. The Na^+-K^+ pump thus has a central role in the establishment and maintenance of homeostasis in the intracellular milieu. The essential function of the pump in maintaining the transmembrane Na^+ gradient plays a key role in excitation–contraction coupling in cardiac myocytes through modulating effects on intracellular Ca^{2+} via Na^+:Ca^{2+} exchange, which transports one Ca^{2+} ion in exchange for three Na^+ ions [9]. Relatively small changes in intracellular Na^+ can have significant effects on contractile force [10]. Of pathophysiological significance, the relationship between intracellular Na^+ and contractility is distinctively different in the normal heart compared to the failing heart in which increased $[Na^+]_i$ is believed to contribute to contractile decline and arrhythmias [11, 12]. These adverse effects occur in part because Na^+/Ca^{2+} exchange increases cytosolic Ca^{2+}. Ca^{2+}-induced diastolic Ca^{2+} releases from the sarcoplasmic reticulum then reduces the amount available for release in systole [11, 13]. Raised $[Na^+]_i$ is also thought to contribute to the heart failure phenotype by reducing mitochondrial Ca^{2+} uptake, which in turn increases the production of reactive oxygen species (ROS) [14]. An inhibitory oxidative modification of mitochondrial ATP synthase [15] then reduces the energy supply [16].

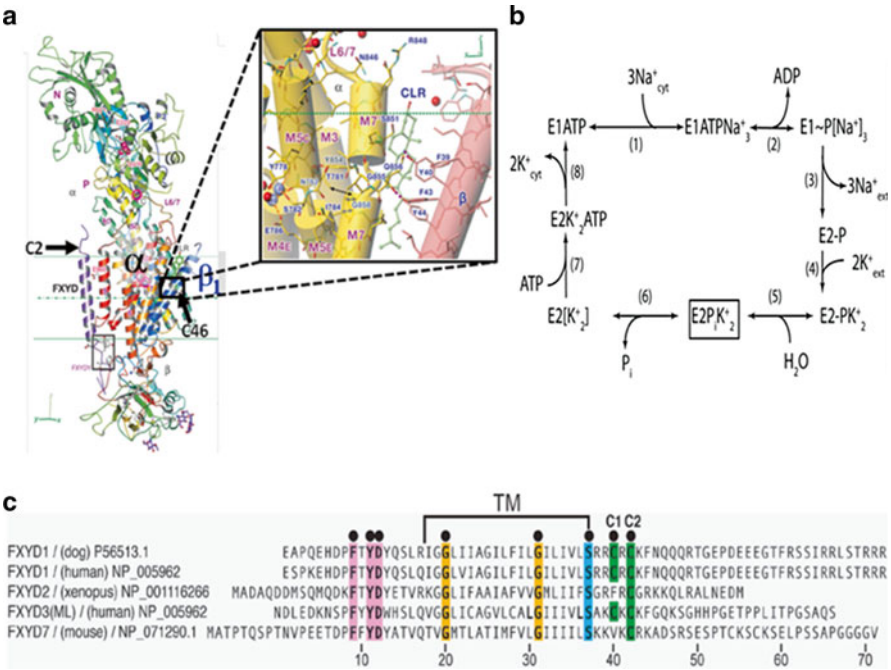

Fig. 19.1 The structure and catalytic cycle of the Na^+-K^+ pump. (**a**) The three-dimensional structure of the α/β/FXYD complex in a conformation analogous to $E2PiK_2^+$. Transmembrane domains are between the two *unbroken green lines*. The β subunit and the FXYD proteins are single-transmembrane-spanning while ten helices of the α subunit span the membrane. Reactive cysteine residues in β_1 subunit (C46) and FXYD (C2) are indicated. The expanded section illustrates the proximity of the glutathionylation site to hydrogen bonds between β- and α subunits (*broken lines*). (**b**) Albers–Post scheme for Na^+-K^+ pump catalytic cycle. When three Na^+ ions have been bound to the E1 conformation (*1*) the cytoplasmic access gate is closed and locked with phosphorylation of the α subunit (*2*), causing occlusion of Na^+ ($[Na^+]_3$) within the molecule. A gate opens to the outside and Na^+ is released (*3* and *4*) when its binding affinity decreases with E1P → E2P change. K^+ is bound (*5*), the gate is closed and the resultant conformational change of the pump stimulates its dephosphorylation (*6*). The $E2PiK_2^+$ product state of dephosphorylation (shown in the *box*) is the conformation for which the three-dimensional crystal structure is known. (**c**) Sequence alignment of FXYD1–3 and 7. Numbering corresponds to the FXYD1 sequence and begins at 1 after the signal peptide (not shown). Conserved residues are marked using *filled circles*, with conserved cysteine residues labelled C1 and C2. TM indicates the transmembrane domain

1.2 Structure of the Na+-K+ Pump

The Na^+-K^+ pump is a heterodimer comprised of a large α subunit and a smaller β subunit. The α subunit has ten transmembrane helices [17] and includes the cytoplasmic catalytic domain (ATP-binding and phosphorylation sites) and the extracellular ouabain binding site [18]. The major isoform, α_1, is expressed in nearly every tissue.

In contrast, the expression of α_2, α_3 and α_4 isoforms is restricted to specific tissues. The β subunit is a single membrane-crossing protein with its amino terminus in the cytoplasm. It is required for folding, membrane insertion and membrane delivery of the catalytically active α subunits [18], and has highly conserved glycosylation sites and disulfide bridge-forming cysteine residues [19]. In addition to this chaperone-like function, the β subunit is essential for enzyme activity and influences the transport properties of mature sodium pumps. There is strong evidence for a modulatory function of the β subunit for ion translocation, cation transport and K^+ occlusion [19]. Currently, three different Na^+-K^+ ATPase β isoforms have been identified, which are distributed in a tissue-dependent manner. Like the α_1 subunit, the β_1 subunit is expressed in nearly every tissue.

A third subunit, termed γ, is part of the FXYD family of small, single transmembrane proteins, named after their signature extracellular FXYD sequence [20]. They are expressed in a tissue-specific manner [21] and associate with the Na^+-K^+ pump in all tissues. Despite a close association, FXYD proteins are not an integral part of the catalytic and ion transporting function of the Na^+-K^+ pump. However, it is firmly established that the presence or absence of FXYD proteins modulates Na^+-K^+ pump function [22, 23]. The three-dimensional structure of the α/β/FXYD complex in a conformation analogous to $E2PiK_2^+$ is shown in Fig. 19.1.

2 Regulation of the Cardiac Na^+-K^+ Pump

2.1 Dependence of Pump Function on Ligands and Membrane Voltage

$[Na^+]_i$ is a major determinant of pump activity [24], with Na^+ levels of 70–100 mM causing maximal pump activation [25]. $[K^+]_i$ regulates Na^+-K^+ pump activity by competing with intracellular Na^+ for binding to the E1 form [26], which results in pump inhibition. Extracellular K^+ stimulates pump activity. However, the concentration of K^+ required for half-maximum pump activity is variable (1.5–2.7 mM) [27] with saturation occurring at a concentration of 10 mM of extracellular K^+ [28]. Extracellular Na^+ inhibits the release of Na^+ from the E2 conformational form, thus modulating pump function [28, 29].

The pump turnover rate is also dependent on the membrane voltage [30]. The major voltage-dependent steps can be interpreted in terms of the "access channel" model. According to this, the translocation of charge through the membrane is a result of the location of the Na^+ and K^+ binding sites within the electrical field [30]. Major steps in the pump cycle that are voltage-dependent include the release of Na^+ ions and the binding of the extracellular K^+ ions at the external pump site [31–34].

2.2 Regulation of the Cardiac Na+-K+ Pump by Signalling Pathways: Historical Controversies

Receptor-coupled signalling cascades regulate the activity of the pump in response to changing needs. Effects of the adrenergic receptor-coupled activation of protein kinases A and C (PKA and PKC) on pump function have been extensively studied, but remarkably, the functional consequences of such signalling on the cardiac pump have remained a matter of controversy [35]. According to a multitude of studies performed on cardiac myocytes, a "consensus view" has emerged that, unlike is seen in other tissues, PKA mediates pump *stimulation* in the heart. The most recent of these studies have attributed the stimulation to phosphorylation of Ser68 in the cytoplasmic terminal of FXYD1, which relieves an inhibitory effect of FXYD1 on the pump [35]. In striking contrast to this consensus view, the work from the Rasmussen Laboratory with whom we collaborate has shown that PKA activation with exposure of isolated cardiac myocytes to the adenylyl cyclase activator forskolin in vitro causes Na^+-K^+ pump *inhibition*. We have shown this to be mediated by the activation of PKC and NADPH oxidase in a downstream pathway that is shared with Ang II [36, 37]. As previously reviewed, differences in experimental protocols between the studies [35, 36] and potential experimental sources of error in Na^+-K^+ pump studies in cardiac myocytes, in particular specific details of how the voltage clamp technique was applied to measure the electrogenic pump current [36], may have contributed to the discrepancies. Moreover, reconciling the effects of protein kinase-dependent signalling on the pump that are attributed to phosphorylation with the structure of the pump and the expression pattern of its subunits in different tissues poses a challenge. A regulatory role for phosphorylation of FXYD is difficult to reconcile with the fact that only FXYD1, expressed in the heart, has functional phosphorylation sites [23] which cannot account for the regulation of the pump by this mechanism in other tissues. An ideal consensus motif for PKA phosphorylation on the α_1 pump subunit exists, and despite an expected poor access of PKA to the site [38], it can undergo phosphorylation. However, functional effects and molecular mechanisms have been difficult to establish [39]. An amino acid mutation designed to mimic phosphorylation of the PKA motif seemed to have no functional effect in *Xenopus* oocytes, a result that was consistent with molecular dynamics simulations. In contrast, simulated phosphorylation of the wild-type amino acid suggested that phosphorylation decreases Na^+ binding affinity and hence should *inhibit* the pump [39]. PKC can phosphorylate subunits despite the absence of a consensus motif for this family of kinases, but the functional significance is uncertain [39].

2.3 Redox Signalling and Role of Glutathionylation in Regulation the Cardiac Na+-K+ Pump Function

Since phosphorylation of the Na^+-K^+ pump molecular complex cannot readily account for effects of the protein kinase-dependent regulation of the Na^+-K^+ pump function, we have explored the role of oxidative post-translational modifications in

kinase-dependent pump regulation in collaboration with the Rasmussen Laboratory. Oxidative modifications can affect the structure and function of proteins in a manner analogous to phosphorylation [40]. They seemed a plausible alternative because chemical oxidants can inhibit Na^+-K^+ ATPase in membrane fragments [41] as well as pump activity in cardiac myocytes [42].

Protein glutathionylation (protein-GSS) was considered as a plausible candidate for mediating this redox regulation, due to its stability and reversibility. Because of the classic role of Ang II in activation of NADPH oxidase [43], we examined the effects of Ang II on pump function and a role for protein-GSS. Exposure of myocytes to Ang II increased the co-immunoprecipitation of the membranous $p22^{phox}$ subunit of NADPH oxidase with the cytosolic $p47^{phox}$ subunit in myocyte lysate, consistent with the translocation of $p47^{phox}$ to the cell membrane that is required for activation of NADPH oxidase [44]. It also increased co-immunoprecipitation of the Na^+-K^+ pump molecular complex with $p47^{phox}$. Ang II decreased the electrogenic Na^+-K^+ current (I_p) measured in voltage clamped cardiac myocytes. The decrease in I_p was abolished by blocking translocation of $p47^{phox}$, and hence NADPH oxidase activation, and by blocking εPKC activation [44]. These results are consistent with PKC-dependent phosphorylation of $p47^{phox}$ necessary for its translocation. The Ang II-induced activation of oxidative signalling was associated with glutathionylation of the β_1 subunit of the Na^+-K^+ pump (β_1-GSS) [45]. Mutational studies of Na^+-K^+ pumps expressed in *Xenopus* oocytes identified C46 as the reactive residue in the β_1 subunit [45] and, consistent with the NADPH oxidase-dependence of Ang II-induced inhibition of I_p in cardiac myocytes [44], there was a causal relationship between $ONOO^-$-induced β_1 subunit glutathionylation and pump inhibition [45]. The position of C46 in the three-dimensional structure of the pump is illustrated in Fig. 19.1.

Previous has also examined whether β_1 AR-dependent signalling causes downstream oxidative modification of the Na^+-K^+ pump. Forskolin was used in vitro to activate adenylyl cyclase that is coupled to the β_1 AR as an alternative to using a receptor agonist. This approach was used to overcome issues related to imperfect selectivity of the available agonists [46]. Forskolin was shown to activate NADPH oxidase via PKA- and PKC-dependent pathways and, via this signalling cascade and resulting β_1 subunit glutathionylation, inhibit I_p of cardiac myocytes [36]. Glutathionylation of C46 of the β_1 Na^+-K^+ pump subunit thus has fulfilled the criteria suggested by Gallogly et al. [47] for being a regulatory mechanism of pump function. However, the in vivo significance of these findings in physiology and pathophysiology remained to be examined. Given the substantial differences in redox status between in vitro and in vivo settings [48, 49], and since the in vitro effects of Forskolin on the pump were transient and waned after 30 min of exposure [36], it was imperative to examine the kinase-dependent Na^+-K^+ pump regulation in vivo. Thus the effects of PKA and PKC on pump regulation in the heart were examined through the modulation of β_1 AR- and Ang II receptor-coupled signalling in vivo. The study protocol was specifically designed to also address the technical issues in multiple studies, all performed in vitro, which shaped the consensus view of PKA-dependent pump stimulation [35]. We have performed the first in vivo examination of the effects of PKA/PKC-dependent signalling on the cardiac Na^+-K^+

pump, and have comprehensively demonstrated that, in contrast to previous reports [35], β_1 AR-coupled, PKA-mediated signalling inhibits the cardiac Na^+-K^+ pump via redox-dependent mechanisms [50].

This finding has broad implications in cardiac physiology. Due to an expected increase in myocyte $[Ca^{2+}]_i$ with a rise in $[Na^+]_i$ secondary to PKA-mediated oxidative pump inhibition, it is expected to augment the β_1 AR-coupled, Ca^{2+}-dependent facilitation of excitation–contraction coupling [51]. Moreover, in contrast to the suggested phosphorylation-dependent stimulation of the pump, a PKA-mediated decrease in pump current would prolong the action potential duration and hence prolong voltage-dependent Ca^{2+} influx [51]. The PKA-dependent Na^+-K^+ pump inhibition therefore should act in synergy with other mechanisms to increase cardiac contractility with β_1 adrenergic stimulation in the normal heart.

This newly proposed scheme also has important implications for understanding pathophysiology and treatment of heart failure. It readily integrates the diverse paradigms of adverse effects of high $[Na^+]_i$ [52], oxidative stress and cellular energy deficiency in heart failure [53]. Historically, the clinical use of "β blockers" has been based on small clinical trials and not through a bench-to-bedside approach [54], and therefore, the mechanisms for the efficacy of this class of drugs are poorly understood. Our scheme integrates redox signalling into β_1 AR-coupled signal transduction and is in good agreement with the clinical efficacy of β_1 AR blockade in heart failure. Based on this data, and the scheme that has evolved, β_1 AR blockade is expected to reverse oxidative inhibition of the sarcolemmal Na^+-K^+ pump and be beneficial in heart failure.

2.4 Activation of β_3 ARs and Redox Regulation of the Na^+-K^+ Pump

Three different β ARs are expressed in human cardiac myocytes with a rank order of β_1 AR > β_2 AR > β_3 AR. They are mainly activated by norepinephrine released from sympathetic nerve fibres that form a network around the myocytes, but a circulating pool of norepinephrine and other catecholamines also contributes to adrenergic signalling [55]. β_1 AR is classically known to be coupled to the stimulatory G protein (Gs), which activates adenylyl cyclase and cyclic AMP synthesis. The β_2 AR is coupled to Gs as well as the inhibitory G protein (Gi)-mediated signalling, whereas the β_3 AR in cardiac myocytes is coupled to Gi proteins [56]. The downstream pathway activated by β_3 AR includes NOS, NO-activated guanylyl cyclase, and cyclic GMP synthesis [57]. In contrast to the Na^+-K^+ pump inhibition in response to β_1 AR-coupled signalling, we have shown with the Rasmussen Laboratory that selective stimulation of β_3 ARs in vitro increases the I_p of cardiac myocytes from normal rabbits [58]. As expected from activation of the Na^+-K^+ pump, acute intravenous administration of a β_3 AR agonist has opposite effects on cardiac performance in sheep with and without heart failure, consistent with the known differential effects on excitation–contraction coupling with changes in $[Na^+]_i$

from low and high baseline levels [58]. The β_3 AR is upregulated in human heart failure [59]. This has been widely considered to be maladaptive. However, when seen in light of the β_3 AR-dependent Na^+-K^+ pump activation, human studies actually suggest that β_3 AR agonists might be beneficial, as reviewed [60]. The in vivo effects of β_3 AR stimulation on redox regulation of the pump, both under normal physiological and pathophysiological conditions, are not known. In a preliminary report, we show that this modification mediates inhibition of the Na^+-K^+ pump in cardiac myocytes in diabetes. This is of pathophysiological significance given the central role of the pump in maintenance of the homeostasis of the intracellular milieu, and in processes such as excitation–contraction coupling amongst others, thus contributing to pathogenesis of diabetic cardiomyopathy. In vivo β_3 AR activation abolished diabetes-induced increase in eNOS-GSS, shown to mediate uncoupling of the enzyme, thus promoting maintenance of the coupled state of eNOS in cardiac myocytes. This effect that was independently supported by a significant increase in eNOS-GSS in myocardium of β_3 $AR^{-/-}$ mice and is consistent with a previous report of a marked increase in eNOS uncoupling in β_3 $AR^{-/-}$ mice shown by eNOS monomerization with pressure-overload induced hypertrophic cardiomyopathy [15].

2.5 FXYD Proteins and Redox-Dependent Na^+-K^+ Pump Regulation

While phosphorylation of FXYD1 is implicated in the regulation of the cardiac myocyte Na^+-K^+ pump, there are no known functional phosphorylation sites on FXYD2–7. In contrast, two cysteine residues in the cytoplasmic terminal, named C1 and C2 in Fig. 19.1, are conserved in the seven-member mammalian family (Fig. 19.1). While most cysteine residues in proteins do not undergo oxidative modifications, C1 and C2 are good candidates for susceptibility to glutathionylation, because they are flanked by the basic amino acids lysine and arginine [61].

FXYD1, native to cardiac myocytes, and other FXYD proteins that we expressed in *Xenopus* oocytes were susceptible to glutathionylation, with the exception of FXYD2. Mutagenesis identified C2 but not C1 as reactive, with the reactivity of C2 depending on flanking basic amino acids. C2 is flanked by two basic amino acids in FXYD1 but only one in FXYD2 (Fig. 19.1) and mutation of the non-basic amino acid to a basic one made FXYD2 susceptible to glutathionylation, similar to other members of FXYD family [61]. In addition to the importance of surrounding basic amino acids in the 2D protein structure, surrounding residues in the 3D structure also play a critical role. Indeed, the 3D structure of the Na^+-K^+ pump suggests that proximity to basic amino acids in the α subunit might account for the differences in reactivity between C1 and C2 [61].

A reactive cysteine in the C2 position of FXYD proteins was critical for the reversal of glutathionylation of C46 of the β_1 subunit and Na^+-K^+ pump inhibition induced by chemical oxidants or exposure of myocytes to Ang II. This conclusion

was independently supported by results obtained in *Xenopus* oocytes expressing FXYD proteins with and without a reactive C2 (see Bibert et al. for details) [61]. Of importance for receptor-coupled signalling, a decrease from baseline glutathionylation of C46 and an increase in I_p induced by a β_3 AR agonist was also dependent on a reactive C2 [61].

3 The Na+-K+ Pump Cycle and Redox-Dependent Regulation of Pump Function

3.1 Susceptibility of C46 in β_1 Na+-K+ Pump Subunit to Glutathionylation

Since GSH is hydrophilic and strictly cytosolic, glutathionylation of C46 is counter-intuitive in view of its location in the transmembrane segment (Fig. 19.1), with its sulfhydryl group facing the lipid bulk phase. The three dimensional structure that indicates this location is known in only one of the Na+-K+ pump's conformations and we subsequently showed that susceptibility to glutathionylation of C46 depends on the conformational states the pump undergoes in its catalytic cycle (Fig. 19.1) [62]. The β subunit forms many contacts with transmembrane segments 7 (αM7) and 10 of the α subunit [63] with polar residues lining the interface between the subunits from the cytoplasm to C46 [64] and, using molecular dynamics simulations, Thøgersen and Nissen [64] demonstrated that minor structural changes in the pump molecular complex are likely to cause a membrane deformation that yields a hydrophilic environment for C46. This might explain the conformation-dependence of access for GSH.

There are no neighbouring basic amino acids that would promote the low p*K*a of the sulfhydryl group to promote glutathionylation of C46 in the primary sequence. However, a cluster of four arginines and one lysine near the C terminus of αM10 is ~15 Å from the side chain of C46 in the known crystal structure [63] and might move in response to Na+ binding. Such movement and membrane deformation allowing access of the sulfhydryl group of C46 can provide an environment promoting glutathionylation of C46. Correlation between conformation-dependent access for trypsin to digest the β_1 subunit and the C terminus of αM10 [65] would seem consistent with such speculations.

Speculations about changes in p*K*a of C46 during the catalytic cycle are based on the tacit assumption that glutathionylation must always be accounted for by physicochemical properties of the glutathionylated cysteine residue. However, in intact cells, glutathionylation of proteins can be catalysed by glutathione *S*-transferase (GST) [66]. Similarly, deglutathionylation is not necessarily only described in physicochemical terms. Deglutathionylation of proteins is selectively catalysed by glutaredoxin 1 (Grx1). Grx1 co-immunoprecipitates with FXYD1 and the β_1 pump subunit in cardiac myocyte lysate [61] and addition of recombinant

Grx1 to the lysate reverses β_1 subunit glutathionylation induced by oxidative stress [61]. When included in patch pipette solutions, recombinant Grx1 also counteracted oxidative stress-induced inhibition of I_p [45]. We have found that translocation of Grx1 may contribute to the in vivo deglutathionylation that occurs with blockade of the β_1 AR [50]. A balance between opposing effects of GST and Grx1 may be important in determining the level of glutathionylation of the Na^+-K^+ pump in a manner reminiscent of the roles kinases and phosphatases have in determining phosphorylation of proteins. Differential access of GST and Grx1 to the Na^+-K^+ pump in its different conformations may contribute to conformation-dependence of glutathionylation in cells.

3.2 *Glutathionylation and Na^+_i/K^+_i-Dependence of Na^+-K^+ Pump Turnover*

A monensin-induced increase in $[Na^+]_i$ renders the β_1 Na^+-K^+ pump subunit resistant to glutathionylation in intact myocytes [62], and an Ang II-induced increase in oxidative stress inhibits I_p of voltage clamped myocytes when $[Na^+]$ in patch pipette solutions is near physiological intracellular levels but not when it is high or when pipette solutions are K^+-free [62]. The in vivo relevance of this is highlighted by the dependence of an increase in I_p on $[K^+]$ in pipette solutions when myocytes are studied ex vivo after treatment of rabbits with an ACE-inhibitor [67]. Corresponding results have been obtained in diabetes, known to be associated with oxidative stress. Diabetes induced experimentally in rabbits caused a decrease in I_p that was dependent on the pipette $[K^+]$ as was reversal of the decrease when the rabbits had been treated with an Ang II receptor antagonist [68].

The dependence of oxidative Na^+-K^+ pump inhibition on $[Na^+]_i$ and $[K^+]_i$ is consistent with the susceptibility of the β_1 subunit to glutathionylation in different conformational states of the pump. Binding of Na^+ occurs to Na^+-K^+ pump species in the E1 conformation (Fig. 19.1b), a confirmation that is highly susceptible to glutathionylation [62]. Since Na^+ binds in competition with K^+, kinetically incompetent, susceptible E1 species that have bound K^+ accumulate when $[K^+]_i$ is high while a high $[Na^+]_i$ has the opposite effect, i.e. it is expected to decrease the abundance of E1 species and hence decrease glutathionylation. Such a dependence of glutathionylation on $[Na^+]_i$ and $[K^+]_i$ has important consequences for pump function.

Glutathionylation-dependent Na^+-K^+ pump inhibition could become self-amplifying if an increase in $[Na^+]_i$ were to increase oxidative stress. However, the increase in the $[Na^+]_i$: $[K^+]_i$ ratio with pump inhibition should reduce susceptibility to glutathionylation and hence eliminate the risk of self-amplifying pump inhibition abolishing all function during oxidative stress. Although less abundantly expressed than pumps with β_1 subunits, pumps with β_2 or β_3 subunits should provide some additional back-up function because these subunits are not susceptible to glutathionylation [45]. Na^+_i- and K^+_i-dependence of β_1 subunit glutathionylation

is also expected to mediate receptor-coupled, protein kinase-dependent regulation of Na^+-K^+ pump function in a manner that might traditionally have been attributed to effects on ligand binding sites. For example, the Ang II-induced pump inhibition at low- but not high $[Na^+]_i$ [62] we referred to above that might have been due to effects of Ang II-dependent signalling on Na^+ binding can also be accounted for the inverse relationship between $[Na^+]_i$ and the susceptibility of C46 in β_1 subunits to glutathionylation. This relationship would effectively mimic a change in the pump's Na^+ affinity.

4 Regulation of the Na^+-K^+ Pump in the Vasculature

It is well established that in the vasculature, the Na^+-K^+ pump participates in the modulation of contractility in vascular smooth muscle cells (VSMCs) and hence in vascular tone [69–72]. This is attributed predominantly to its coupling with the Na^+/Ca^{2+} exchange, with inhibition of the Na^+-K^+ pump increasing $[Na^+]_i$. This, in turn, activates the reverse mode of the Na^+/Ca^{2+} exchanger, increasing intracellular Ca^{2+} ($[Ca^{2+}]_i$) and, subsequently, contractility of VSMCs [73–76]. Despite the physiologically important role of the Na^+-K^+ pump in vascular function, mechanisms for the regulation of its activity in health and disease are poorly understood.

A role for redox regulation of the Na^+-K^+ pump could not be extrapolated from the cardiac myocytes to the VSMCs given the tissue-specific expression of the pump subunits, differential susceptibility of pump subunits to glutathionylation, and possible differences in the signalling pathways coupled to the Na^+-K^+ pump in VSMCs vis-à-vis cardiac myocytes. Therefore, we examined whether glutathionylation of the β_1 Na^+-K^+ pump subunit and oxidative Na^+-K^+ pump inhibition occur in VSMCs, and whether this contributes to altered vascular function in response to Ang II-induced NADPH oxidase activation. We have reported that glutathionylation of the Na^+-K^+ pump's β_1 subunit occurs in the vasculature. Moreover, Ang II increases glutathionylation of the β_1 Na^+-K^+ pump subunit and decreases the activity of the pump in VSMCs in an NADPH oxidase-dependent manner [77]. This novel oxidative pathway is also observed in both rabbit and human vessels. The significance in regulation of the pump activity in vessels is suggested by the associated reduction in K^+-induced vasorelaxation, an index of Na^+-K^+ pump function, in aortic rings exposed to Ang II in vitro, and the increase in K^+-induced vasorelaxation by disruption of the renin-angiotensin system by ACE inhibition in vivo [77].

The effects of the oxidative pathway on the pump function in VSMCs we have shown are expected to mediate alterations in vascular contractility through the effects of $[Na^+]_i$ on $[Ca^{2+}]_i$ [73, 76]. Since Na^+-K^+ pump is also implicated in mediating the effects of NO in vascular smooth muscle relaxation [78], redox regulation of the pump may have significance for altered regulation of vascular tone in pathophysiological conditions such as diabetes mellitus and hypertension that are characterised by neurohormonal abnormalities and increased oxidative stress.

A technical challenge in delineating the contribution that β_1-GSS and pump inhibition make to Ang II-induced changes in vascular tone is the direct effect of ouabain, the specific inhibitor of the Na^+-K^+ pump extensively used to measure the function of the pump, in causing vasoconstriction. One approach to overcome this issue, especially in vivo, is the careful use of transgenic mouse technologies. In order to understand the physiological impact of glutathionylation of C45 (reactive cysteine in mice), the cysteine residue can be mutated to a non-glutathionylable residue, and this way, a "knock-in" mouse is created where the endogenous protein is replaced by the mutant version of the protein. This amino acid replacement strategy has the advantage that no interference exists with the endogenous protein, as seen in knock out (KO) systems, and that the mutant protein is regulated and, presumably, expressed in the same manner as the wild type protein. The knock-in methodology can, in principle, provide a unique opportunity to examine the specific role of β_1-GSS in vascular function and in regulation of systemic blood pressure in a variety of experimental designs, such as Ang II infusion or disease states, like diabetes.

Our ex vivo data supports an important role for FXYD proteins in the regulation of vasculature tone. We show that oxidative inhibition of the Na^+-K^+ pump was dramatically increased in a FXYD1 KO model, as suggested by decreased K^+-induced relaxation. Furthermore, pre-incubation of VSMCs with recombinant FXYD protein reduced Ang II-induced β_1-GSS and prevented Ang II-induced pump inhibition. FXYD1 also protected against Ang II-induced altered vascular tone in intact aortic rings ex vivo, consistent with effect of FXYD1 to relieve redox-mediated Na^+-K^+ pump inhibition.

Whilst a critical role for FXYD1 in promoting relief of oxidative Na^+-K^+ pump inhibition is supported by our report, the pathophysiological significance of this role remains to be elucidated. Inhibition of the Na^+-K^+ pump at baseline in the FXYD1 KO model indicates that under pathophysiological conditions, such as Ang II-induced oxidative stress or in diabetes, these mice might have an augmentation of adverse cardiovascular remodelling due to a lack of FXYD to counter-balance oxidative pump inhibition.

In addition to mediating acute alterations in vascular tone, ROS play important roles in signalling promoting VSMC growth [79]. Since the Na^+-K^+ pump is increasingly recognised as a signalling molecule that is coupled to Src kinase-dependent pathways [80], redox regulation of the Na^+-K^+ pump can have a role in signalling that regulates VSMC proliferation. Modulation of these effects by FXYD1 to inhibit the effects of chronic oxidative stress on vascular proliferation has the potential to make this protein a promising target for treatment of ROS-mediated vascular dysfunction in a variety of disease states.

5 Conclusions

Redox modification of Na^+-K^+ pump through glutathionylation of its β_1 subunit is integral in physiological regulation of the pump in heart and vasculature. Change in β_1 subunit glutathionylation in either direction mediates functional stimulation or

inhibition of the pump in response to receptor-coupled stimuli. In disease conditions with oxidative stress, such as heart failure, Na^+-K^+ pump function is inhibited through an increase in β_1 subunit glutathionylation. Targeting the redox inhibition of the pump, e.g. by utilising the associated FXYD1 protein, is a rational approach to treat a variety of cardiovascular pathologies.

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Chapter 20
Regulation of Na^+-K^+-ATPase in Pulmonary Vasculature

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Abstract Na^+-K^+-ATPase plays important role in maintaining pulmonary vascular tone as well as homoeostasis. Two different isoforms of Na^+-K^+-ATPase has been identified in ovine pulmonary artery. α-1 subunit of Na^+-K^+-ATPase is responsible for the regulation of basal tone of pulmonary artery in sheep. Arachidonic acid inhibits Na^+-K^+-ATPase in ovine pulmonary artery via 20-HETE production and protein kinase C pathway. Whereas, lipoxygenase has a secondary role in arachidonic acid-induced inhibition of Na^+-K^+-ATPase in this particular vasculature. BAY 41-2272 a NO-independent activator of sGC induces cGMP-independent vasodilation of sheep pulmonary artery through stimulation of sodium pump which is the primary target for the vasodilation of this vasculature. Eicosapentaenoic acid inhibits functional Na^+-K^+-ATPase through decrease in the protein expression of the α-1 subunit of sodium pump in pulmonary artery but this attenuation is independent of cGMP production. Sodium nitroprusside induced vasodilation is the result of the link between increased intracellular cGMP and activated sarcolemmal Na^+-K^+-ATPase in canine pulmonary arterial smooth muscle cells. High salt diet leads to electrical changes in rat pulmonary artery which may be due to the opening of K^+ channels and activation of sodium pump. H_2O_2, xanthine, and xanthine oxidase stimulate sodium pump activity of bovine pulmonary arterial endothelial cells. Regulation of sodium pump expression or activity and trafficking occurs by activation of dopamine receptors in various tissues including lungs and vascular beds. In conclusion, Na^+-K^+-ATPase is regulated in pulmonary vasculature of different species like ovine, canine, bovine and human by different signaling pathways.

Keywords Na^+-K^+-ATPase • Sodium pump • Ovine • Canine • Bovine

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1 Introduction

Sodium pump plays an important role in the regulation of smooth muscle contractility and tone in several vascular beds [1–3]. It also regulates pulmonary arterial smooth muscle tone [4, 5]. Inhibition of sodium pump by hypothalamic inhibitory factor an endogenous inhibitor of the enzyme with ouabain like property causes pulmonary hypertension in rats [6]. Pulmonary arterial sodium pump also contributes to monocrotaline-induced pulmonary hypertension [7]. Stimulation of sodium pump leads to relaxation of the vascular smooth muscle through the membrane hyperpolarization which is the result of decrease in intracellular Ca^{2+} influx through the voltage-gated Ca^{2+} channels. One more mechanism for the relaxation of vascular smooth muscle has been explained by the stimulation of the sodium pump through Na^{+}–Ca^{2+} exchange mechanism which is also responsible for the reduction in the intracellular Ca^{2+} in the cell. On the other hand, inhibition of the plasmalemmal sodium pump leads to contraction in smooth muscle.

2 Regulation of Na^{+}-K^{+}-ATPase in Ovine Pulmonary Vasculature and Its Signal Transduction Pathways

Two isoforms of Na^{+}-K^{+}-ATPase in sheep pulmonary vasculature have been identified by using polyclonal western blot technique. These isoforms are α-1 and α-2 subunit of sodium pump. Functionally both the isoforms are characterized by the use of concentration-response curve to ouabain-induced inhibition of the Na^{+}-K^{+}-ATPase. The curve is biphasic in nature representing low ouabain sensitive α-1 isoform and the high oubain sensitive α-2 isoform. Approximately, molecular size of both the subunits is 110 kDa. Ouabain at 1 μM inhibits sodium pump and leads to increase in basal tone of sheep pulmonary artery. 5-hydroxy tryptamine does not regulate sodium pump in this vasculature. It has been also reported that protein kinase G inhibitor KT5823 has no effect on sodium pump in this vessel [5]. Even cGMP is unable to stimulate the Na^{+}-K^{+}-ATPase in ovine pulmonary artery. Chanda and coworkers also reported that low affinity isoform of sodium pump (α-1) regulates the basal tone of sheep pulmonary vasculature. Endothelium plays an important role in regulation of vascular sodium pump in different vessels but in sheep pulmonary artery endothelium has no effect on the pump (Fig. 20.1).

Arachidonic acid and its metabolites play an important role in the pathogenesis of pulmonary hypertension in different animals. Endothelin-induced pulmonary hypertension in sheep is mediated by cyclooxygenase products of arachidonic acid [8] while, in sheep fetal pulmonary circulation, 20-Hydroxyeicosatetraenoic acid (20-HETE), a cytochrome P450 metabolite of the fatty acid is responsible to maintain high pulmonary vascular resistance [9]. 15-Hydroxyeicosatetraenoic acid (15-HETE), a lipooxygenase product of arachidonic acid has been shown to enhance pulmonary artery contraction in female rabbits [10]. Various metabolites of arachidonic acid

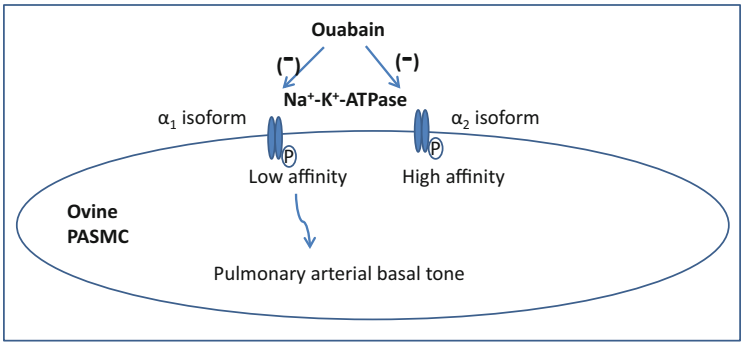

Fig. 20.1 Ovine PASMC express two isoforms of Na^+-K^+-ATPase (α_1 and α_2). Isoform (α_1) having low affinity towards ouabain has important role in maintaining pulmonary arterial basal tone

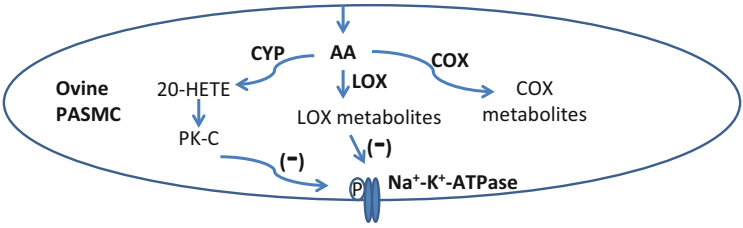

Fig. 20.2 Arachidonic acid (AA) from membrane phospholipids, gets metabolized by three pathways in pulmonary arterial smooth muscle cells (PASMC). Cytochrome P450 ω-hydroxylase (CYP) produces 20-HETE in ovine PASMC, which activates protein kinase C (PK-C). PK-C inhibits Na^+-K^+-ATPase by phosphorylation. AA through metabolism by lipoxygenase (LOX) pathway also inhibits Na^+-K^+ ATPase. COX metabolites do not have any effect on Na^+-K^+ ATPase

cause hypoxic pulmonary vasoconstriction in rats [11] and rabbits [12]. Multiple signaling mechanisms have been implicated in the constriction of pulmonary artery smooth muscles by arachidonic acid and its metabolites.

As shown in Fig. 20.2 arachidonic acid inhibits functional activity of the Na^+-K^+-ATPase in the vascular smooth muscle of the sheep intralobar pulmonary artery [13]. Arachidonic acid slightly increases basal tone of sheep pulmonary vasculature. Arachidonic acid inhibits sodium pump through 20-HETE production. Lipoxygenase pathway has a secondary role in sodium pump inhibition by arachidonic acid. However, cyclooxygenase pathway has no role in arachidonic acid-induced inhibition of sodium pump. Singh and coworkers also reported that protein kinase C is involved in the inhibition of Na^+-K^+-ATPase by arachidonic acid/20-HETE in sheep pulmonary artery. It is important to consider the role of Na^+-K^+-ATPase in the maintenance of ovine pulmonary arterial tone and inhibitory effect of arachidonic acid on this enzyme is responsible in understanding the role of the fatty acid in pulmonary hypertension [13].

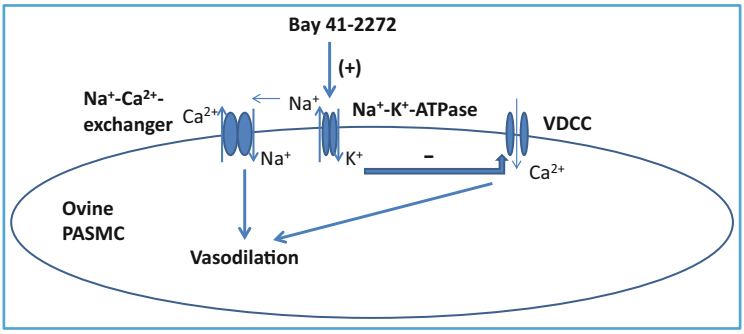

Fig. 20.3 Bay 41-2272 stimulates Na^+-K^+-ATPase in cGMP-independent manner, which in turn activates Na^+–Ca^{2+} exchanger to stimulate Ca^{2+} efflux and inhibit voltage-dependent Ca^{2+} channel (VDCC) to attenuate Ca^{2+} influx, leading to vasodilation

Impairment in NO-cGMP signaling has been implicated in the pathogenesis of pulmonary hypertension. Direct stimulation of sGC by pyrazolopyridines such as riociguat (BAY 63-2521), BAY 41-2272 and BAY 41-8543 provides a therapeutic strategy for the management of pulmonary hypertension. BAY 41-2272 induces vascular relaxation in sheep pulmonary artery [14]. It is an NO-independent stimulator of soluble guanylyl cyclase which leads to increase in intracellular cGMP. BAY 41-2272 has a potential against cardiovascular disorders [15]. It has been seen that BAY 41-2272 produces relaxation through cGMP dependent as well as cGMP-independent signaling pathway. BAY 41-2272 induces cGMP-independent vasodilation of sheep pulmonary artery through stimulation of sodium pump which looks like the primary target for the dilation of this vasculature (Fig. 20.3). However, sodium pump is not stimulated by protein kinase A in this pulmonary vasculature [14].

Eicosapentaenoic acid is one of the omega-3 fatty acids and is useful in prevention of cardiovascular diseases. Eicosapentaenoic acid has vasodilatory effect in various vascular beds including ovine pulmonary artery. This vasorelaxation is endothelium-dependent as well as -independent in nature [16]. Eicosapentaenoic acid has vasoconstrictor potential in rabbit pulmonary vascular bed [17]. On the other hand, eicosapentaenoic acid shows inhibition of the sodium pump in the sheep pulmonary vasculature at high concentration. Eicosapentaenoic acid inhibits functional Na^+-K^+-ATPase through decrease in the protein expression of the α-1 subunit of sodium pump in pulmonary artery but this attenuation is independent of cGMP production [18].

3 Regulation of Na^+-K^+-ATPase in Canine Pulmonary Vasculature

Sodium pump plays an important role in sodium nitroprusside-induced vasorelaxation in canine pulmonary artery. However, in hypoxic condition, sodium pump has no role in sodium nitroprusside-induced vasorelaxation. Sodium pump activity is

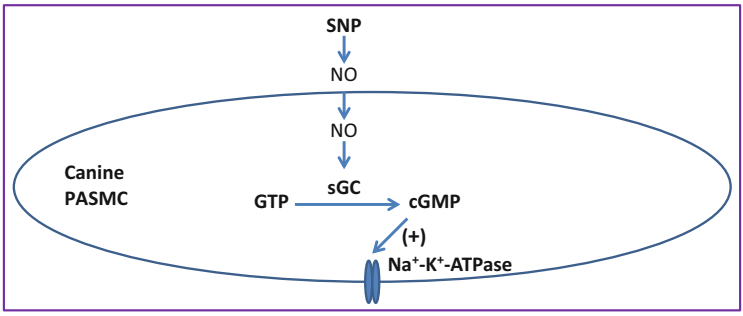

Fig. 20.4 Sodium nitroprusside (SNP) releases nitric oxide (NO) which activates soluble guanylyl cyclase (sGC) causing increase in i/c cGMP level. Intracellular cGMP stimulates Na^+-K^+-ATPase by an undetermined mechanism

resistant to hypoxic condition in canine pulmonary vessel [19]. Apart from this sodium nitroprusside-induced vasodilation is the result of the link between increased intracellular cGMP and activated sarcolemmal Na^+-K^+-ATPase in canine pulmonary arterial smooth muscle cells (Fig. 20.4). This link establishes a fact that sodium pump has a role in cGMP-mediated vasodilation in these smooth muscle cells [20].

4 Regulation of Na^+-K^+-ATPase in Bovine Pulmonary Vasculature and Its Signaling Pathway

U46619 inhibits the sodium pump activity in bovine pulmonary smooth muscle cells. The inhibition occurs through two different parallel pathways: one is mediated by glutathionylation of the sodium pump and the other by augmenting the inhibitory activity of the 70 kDa inhibitor protein of sodium pump. U46619 as an inhibitor is responsible for irreversible inhibition of sodium pump in an isoform specific manner during treatment of the cells with U46619 but at the same time phospholemman deglutathionylates the sodium pump which leads to pump activation [21]. A new protein inhibitor of sodium pump has been identified which has a molecular weight of 15.6 kDa. This protein inhibitor is present in cytosolic fraction of bovine pulmonary artery smooth muscle cells and exhibits differential affinity toward $\alpha_2\beta_1$ and $\alpha_1\beta_1$ subunits of Na^+-K^+-ATPase. It has been noticed that α-2 subunit is more sensitive than α-1. This protein inhibitor interacts reversibly with the E1 site of the enzyme. This interaction blocks the phosphorylated intermediate formation at the enzyme. Thus the protein inhibitor leads to an alteration in the confirmation of the Na^+-K^+-ATPase [22].

Low dose of ouabain (10 nM) increases intracellular calcium and leads to stimulation of m-calpain activity and further proteolytically activates protein kinase C alpha (PKCalpha) in caveolae (signalosomes) of the cells in the pulmonary artery smooth muscle cells of bovine. The activation of PKCalpha increases the bovine pulmonary arterial smooth muscle cell proliferation through Go/G1 to S/G2-M

phase transition. This data confirms the signaling pathway of low dose of ouabain-mediated pulmonary artery smooth muscle cell proliferation and this signaling pathway plays a vital role in pulmonary artery smooth muscle cell proliferation [23]. Protein kinase C acts as the endogenous regulator in these endothelial cells. However, effects of hydrogen peroxide (H_2O_2) as oxidant are not mediated by activation of PKC or by changes in the expression or phosphorylation of alpha1 subunit of the sodium pump [24].

5 Regulation of Na^+-K^+-ATPase in Rat Pulmonary Vasculature and Its Signal Transduction Pathway

It has been observed that high salt diet leads to electrical changes in rat pulmonary artery which may be due to the opening of K^+ channels and activation of sodium pump [25]. Endothelium Influences beta-adrenoceptor-mediated mechanical and electrical functions in rat pulmonary artery. Isoprenaline-induced hyperpolarization involves activation of K^+ channels and sodium pump of smooth muscle cells possibly in parallel but mutually dependent on the presence of endothelial cells in pulmonary arteries of rat [26]. Protein kinase C mediates phosphorylation of phospholemman, a homologue of Na-K-ATPase γ subunit, when it is associated with the α-2 subunit of sodium pump. However, phosphorylation of phospholemman by protein kinase A occurs when it is associated with the α-1 isoform of sodium pump in the caveolae of pulmonary artery smooth muscle cells. Phospholemman is purified from the caveolae and reconstituted into the liposomes which are used to unravel the mechanism of regulation of α-2 subunit of sodium pump by protein kinase C. However, phosphorylated phospholemman does not change the affinity of the sodium pump for the sodium and even after phosphorylation by protein kinase C in the caveolae of pulmonary artery smooth muscle cells [27]. Inhibition of α-2 subunit of sodium pump by ouabain (a sodium pump inhibitor) plays a crucial role in modulating the Ca^{2+} influx regulatory components in the caveolae microdomain for marked increase in intracellular Ca^{2+} in the pulmonary smooth muscle, which may be important for the manifestation of pulmonary hypertension [28]. In rat pulmonary artery, spermine NONOate produces cyclic GMP-independent relaxation partially through the activation of Na^+-K^+-ATPase, sarco-endoplasmic reticulum Ca^{2+}-ATPase, and calcium-activated potassium channels in rat pulmonary artery [29].

According to an observation, inhibition of sodium pump with ouabain leads to blockade of ACh-induced relaxation persisting in the presence of inhibition of cyclooxygenase (COX), nitric oxide synthase (NOS) and soluble guanylate cyclase (sGC) in pulmonary artery in hypoxia-induced pulmonary hypertension [30]. One more study of the same group shows that inhibition of sodium pump abolishes NS309 (SK_{ca} and IK_{ca} channels opener)-induced relaxation mediated by KCa2 and KCa3.1 in rat small pulmonary arteries. The low concentrations of K^+ (1–6 mM) induced endothelium-independent relaxations are abolished in the presence of ouabain [31]. Inhibition or depletion of PKC did not prevent H_2O_2-induced increases in pump

activity. Sodium pump activity is regulated by a protein kinase C in bovine pulmonary artery endothelial cell. Acute lung injury occurs due to presence of oxidant in many cases and these oxidants are the causative agents for injury of pulmonary vascular endothelium. The injury occurs due to oxidant leads to alteration in the function of sodium pump. H_2O_2, xanthine, and xanthine oxidase stimulate sodium pump activity of bovine pulmonary arterial endothelial cells and this effect is prevented by catalase. This increase in sodium pump activity of bovine pulmonary arterial endothelial cells by H_2O_2 is mediated by increased intracellular sodium and an increased rate of sodium pump turnover. Further, it has been suggested that the increased pump activity may acts as an early marker of endothelial cell perturbation [32].

Hypoxia has no influence on function of sodium pump in rat pulmonary artery endothelial cells [33]. Chronic pulmonary artery occlusion for 14 days increases alveolar fluid clearance through α-1 subunit of sodium pump overexpression in rats [34]. Reversible temperature-sensitive alterations in lung fluid balance have been observed in rat lung preparations which are exposed to hypothermic perfusion. Hypothermia induces pulmonary edema formation, which is rapidly cleared upon re-warming by activation of ouabain-sensitive sodium pump [35]. Apart from this, active sodium transport through alveolar epithelium keeps alveoli of the lungs relatively dry and hyperoxia increases epithelial permeability which leads to pulmonary edema. However, hyperoxia leads to complex and nonparallel changes in Na^+-K^+-ATPase antigenic protein, hydrolytic activity and unidirectional active sodium resorption in rats [36]. Nitric oxide exposure and sulfhydryl modulation alter L-arginine transport in cultured pulmonary artery endothelial cells occurs partially through sodium pump [37].

6 Regulation of Na^+-K^+-ATPase in Human Pulmonary Artery

Human plasma and urine contain an endogenous bufodienolide vasoconstrictor marinobufagenin-like immunoreactive Na^+-K^+-ATPase inhibitor. It causes concentration-dependent vasoconstriction in human pulmonary artery [38]. This is clearly important to investigate whether an elevated plasma concentration of the cardiotonic steroids such as endogenous ouabain, digoxin, marinobufagenin, and telocinobufagin may have a role in pulmonary hypertension and other cardiovascular disorders [39].

7 General Signaling Pathways in Pulmonary System

Dopamine, a GPCR agonist is an endogenous regulator of Na^+-K^+-ATPase. Regulation of sodium pump expression or activity and trafficking occurs by activation of dopamine receptors in various tissues including lungs and vascular beds [40].

However, dopamine receptor-mediated regulation of sodium pump activates a large range of cellular responses which include endocytosis or exocytosis, phosphorylation or dephosphorylation of α isoform of sodium pump, and different signaling pathways. These pathways include phosphatidylinositol (PI)-phospholipase C (PLC) or protein kinase C (PKC), PI3K, adaptor protein 2, cAMP/protein kinase A (PKA), tyrosine phosphatase, and mitogen-activated protein kinase (MAPK) or extracellular signal-regulated protein kinase (ERK). These signaling mechanisms are responsible for the cross talk between DA receptors and sodium pump activity [40].

It is well established that basolateral Na^+-K^+-ATPase plays a critical role for removal of alveolar pulmonary edema fluid. This depends on active ion transport across the alveolar epithelium of lungs. Na^+ enters through the sodium channels which are present on the luminal epithelium and extruded into the lung interstitium by basolaterally located sodium pump thereby establishing a local osmotic gradient to reabsorb water fraction of the edema fluid from the airspaces of the lungs. There are several regulators for the resolution of alveolar edema across the tight epithelial barrier. These regulators are cyclic adenosine monophosphate (cAMP)-dependent mechanisms through adrenergic or dopamine receptor stimulation as well as various several others cAMP-independent mechanisms which include thyroid hormone, dopamine, glucocorticoids and growth factors [41]. In a study, it has been observed that high-frequency jet ventilation is a successful treatment for the pulmonary edema after seawater drowning. The mechanism of successful high-frequency jet ventilation treatment is due to better correction of hypoxemia and improvement in the rehabilitation of sodium pump activity in the rabbit's lungs [42].

8 Conclusions

The understanding of signaling mechanisms regulating Na^+-K^+-ATPase in pulmonary arterial cells, endothelial cells and epithelial cells is of immense importance in the therapeutic management of lung diseases such as pulmonary hypertension and pulmonary edema. Further, the signaling pathways in the regulation of Na^+-K^+-ATPase explain the basic physiological mechanisms in the control of pulmonary circulation and alveolar fluid clearance.

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Chapter 21
Exercise-Induced Regulation of the Na, K-Pump in Skeletal Muscles

Carsten Juel

Abstract Ion gradients across the skeletal muscle membrane undergo pronounced changes during intense muscle contractions. These changes influence excitability and muscle performance. The ion changes are counteracted by the activity of the Na, K-pump. Regulation of the Na, K-pump in association with muscle activity is therefore important for muscle function. This short review focuses on exercise-induced acute changes in Na, K-pump activity in skeletal muscles. The Na, K-pump is dependent on the intracellular Na^+ concentration, which is influenced by muscle activity. Exercise changes the number of functional pumps in the outer membrane (translocation). In addition, the Na, K-pump activity is influenced by hormones, purines, nitric oxide and exercise-induced oxidative stress, which increases subunit S-glutathionylation. Some of these regulatory changes are mediated by changes in the interaction between the Na, K-pump α and β subunits and the regulatory protein phospholemman (FXYD1). Thus, exercise-induced acute regulation of the Na, K-pump in skeletal muscles is a multifactorial process.

Keywords Na, K-ATPase • Muscle ion changes • Hormones • Purines • Glutathionylation • Nitric oxide • Phospholemman

1 Introduction

1.1 Ion Shifts, Importance of the Pump

Ion gradients across the skeletal muscle membrane undergo pronounced changes during intense muscle contractions. These changes are caused by the repetitive action potentials invading sarcolemma and T-tubules. Many studies have focused on

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the extracellular accumulation of K^+, which has been shown to be dependent on the intensity of the work done by the muscle. Changes in the K^+ gradient likely modulate excitability, which may influence sarcoplasmatic reticulum Ca^{2+} release. Changes in K^+ are therefore one of the underlying mechanisms for skeletal muscle fatigue. It is difficult to design a study that could offer direct proof for K^+ as a fatigue factor. However, many studies have suggested a link between K^+ accumulation and a reduction in force. Muscle activity also increases the intracellular Na^+ concentration, which likewise may decrease muscle excitability. However, the effect of Na^+ shifts in association with muscle activity and muscle fatigue is less-often studied. The ion-shifts during muscle activity are counteracted by the activity of the Na, K-pump. The regulation of the pump during muscle activity is therefore highly important for muscle function.

The Na, K-pump is active in the resting muscle to counteract the passive fluxes of Na^+ and K^+ due to membrane leakage. Another mechanism that challenges the pump is the extra Na^+ efflux due to pH regulatory transport systems, such as the Na^+/H^+ exchange and the Na^+-bicarbonate co-transport. pH regulation accounts for 30–40 % of the Na, K-ATPase activity in the resting muscle [1].

In conclusion, muscle activity is associated with a large increase in Na^+ and K^+ fluxes due to action potential activity and increased activity in pH-regulating transport systems. Therefore, muscle activity puts an extra load on the Na, K-pump, which can be accelerated up to 50-fold. The acute regulation of the Na, K-pump during muscle activity is a multifactorial process. These processes are the topic of the present review.

2 Methods to Quantify the Na, K-Pump Activity

Labelling with radiolabelled ouabain has long been used to quantify the number of Na, K-pump proteins in skeletal muscle. This technique is also suitable for quantifying long-term changes in the number of pumps, for instance in association with training and age. However, this technique cannot detect acute changes in the activity of existing pumps. The same holds for the western blotting technique, which is, however, able to measure isoform content and changes in isoform distribution.

The activity of the pump has been quantified using indirect methods, such as ion flux measurements and changes in muscle ion content. Direct measurements of Na, K-pump activity in skeletal muscle are associated with great difficulties. This is due to the presence of other ATPase, especially the Ca^{2+}-ATPase. One compromise is to use the K^+ stimulated 3-O-methylfluorescein phosphatase assay (3-O-MFP method), which uses 3-O-MFP as an artificial substrate [2]. This method is advantageous because the ATPase activity can be measured in muscle homogenates since the method is specific and not sensitive to other ATPase, such as the Ca^{2+}-ATPase. However the measurements are carried out without any Na^+ present; the method is therefore insensitive to changes in pump activity mediated by Na^+ affinity changes. A more direct quantification of the Na, K-pump activity is obtained using an assay that directly

quantifies the release of inorganic P from the hydrolysis of ATP. These measurements can be carried out without Na^+ and with different Na^+ concentrations, and consequently, the method can be used to quantify Na^+-sensitive ATPase activity and changes in Na^+ affinity (K_m for Na^+). However, it is necessary to reduce the background ATPase activity. This is done by membrane purification using centrifugation steps [3].

3 Effect of Exercise

A number of studies in humans have shown that the maximal in vitro Na, K-pump activity quantified with the 3-O-MFPase technique is reduced with exercise. This phenomenon has been referred to as exercise-induced inactivation of the pump [4, 5]. The exercise protocols included both submaximal and fatiguing exercises of different durations. Similar experiments in rats have shown conflicting results.

Experiments in rat models with the ATPase assay technique have clearly shown that 30 min of exercise increases the maximal in vitro Na, K-pump activity [6]. In humans, exhaustive exercise performed for a duration of 4 min has been shown to result in an increased Na, K-ATPase activity after exercise [7]. In contrast, intermittent exercise of 30 min duration has been demonstrated to *reduce* the maximal Na, K-pump activity [8].

These contradictory results are partly due to the different techniques used. One obvious difference is that the 3-O-MFPase technique is insensitive to affinity changes, whereas the Na, K-ATPase assay reveals changes in both V_{max} and K_m (affinity). It is also obvious that a number of underlying mechanisms could be involved and the possibility exists that these mechanisms could be both stimulatory and inhibitory. The following chapters describe a number of proposed underlying mechanisms for changes in pump activity during muscle activity.

4 Ion Sensitivity of the Pump

The Na, K-pump is sensitive to the intracellular Na^+ concentration. Therefore, exercise-induced changes in the internal Na^+ concentration will affect the pump rate, a simple and appropriate regulatory mechanism. The K_m for the Na^+-dependent activity of the Na, K-pump varies with the interval, 6–14 mM, if quantified in rat muscle homogenates with a Na, K-ATPase assay. The K_m is dependent on the Na, K-pump protein isoform composition [3]. The functional pump is a heterodimer comprising one α and one β subunit. The K_m for sodium is lower (affinity higher) in heterodimers with α1 and β1 subunits compared to heterodimers with α2 and β2 subunits. In rat muscles, α1 and β1 are the most abundant in oxidative muscles, and consequently, the pumps in these muscles have a higher affinity for Na^+ compared to in glycolytic muscles [3]. In human skeletal muscles, the distribution of the Na, K-pump isoform seems to be independent of the muscle fibre types (unpublished).

The intracellular Na^+ in intact muscles is approximately 15 mM, which is close to the K_m for Na^+. Consequently, the Na, K-pump is sensitive to small muscle activity-induced changes in cellular Na^+. The sodium-dependent activation of the pump is already important at the onset of exercise. The affinity of the Na, K-pump is high for K^+ (the K_m is low compared to the K^+ plasma concentration). The pump is therefore considered to be saturated with K^+, and changes in K^+ concentrations considered without importance for regulation during muscle activity.

It has been suggested that low frequency muscle activation (excitation) is associated with increased Na, K-pump activity that is not dependent on intracellular Na^+ increases [9]. Any underlying mechanism for such excitation-induced Na, K-pump activation has not been suggested. It could be argued against that even low frequency stimulation produces a local Na^+ influx, which, although not measurable, could locally activate the pump. Changes in pH during muscle activity are considered unimportant for Na, K-pump regulation, but may affect the whole-body ion balance via other mechanisms [10].

5 Hormones

Catecholamines are released into the blood during exercise. In vitro experiments with isolated muscles have demonstrated an increased Na^+ efflux, K^+ influx, and hyperpolarisation during incubation with epinephrine and norepinephrine [11]. Similar effects have been obtained with the beta agonists isoproterenol and salbutamol. The effects could be mimicked by cAMP derivatives, suggesting a role for adenylate cyclase, and probably for protein kinase A. Although most of the original experiments were done in vitro, it is generally accepted that catecholamines stimulate the Na, K-pump during muscle activity. Insulin has also been demonstrated to increase pump activity by increasing the turnover number of the protein. But insulin level is not related to exercise intensity. Insulin is, therefore, not considered to have a regulatory role during muscle activity.

6 Translocation of Pump Subunits

It has been suggested that exercise (and insulin) induce a translocation of Na, K-pump proteins from an intracellular store to the plasma membrane [12–15], which could contribute to the acute increase in Na, K-pump activity in association with muscle activity. These studies were carried out using rat models. Studies in humans with purified sarcolemmal membranes have also reported an increased membrane content of Na, K-pump proteins after exercise [16]. However, the mechanism has also been questioned [17]. The problem is that it is impossible (with the ouabain labelling technique) to detect an intracellular store of pumps where pump proteins could be translocated to the outer membrane. More recent studies have confirmed an

increased number of pumps in purified outer membranes after exercise. In addition, it was reported that the pump proteins are associated with caveolin-3, a marker protein for caveolae, and that the association changes with exercise [18]. Therefore, the most likely model for translocation is that the pumps are recruited from caveolae close to the surface membrane instead of from intracellular stores. The translocation process is reversible with a half time of approximately 20 min [19]. The signalling mechanism underlying subunit translocation in skeletal muscles is partly unknown, but the involvement of AMP kinase has been suggested [20].

7 Effect of Purines

Purines, such as ATP and ADP, were first demonstrated to restore force in rat muscle depressed with an elevated K+ concentration [21]. A later study confirmed that purines also increase the Na, K-pump activity in purified rat muscle membranes, i.e. with no intact cells present. It was therefore concluded that the pathway leading to pump activation includes protein–protein interaction. Studies with agonists and antagonists revealed that the stimulatory effects of purines are mediated by two independent mechanisms: a P2Y receptor mediated increase in Na, K-pump capacity (V_{max}) and a P2Y receptor independent phosphorylation of phospholemman (FXYD1) and α1 subunits. This phosphorylation induces Na^+ affinity changes [22]. It was suggested that purines released during muscle activity may contribute to the exercise-induced upregulation of the pump, which has been reported in rat muscles [6]. However, similar studies with muscle membranes from humans muscle failed to demonstrate a stimulatory effect of purines on the Na, K-pump [23]. The difference between rat and human muscles may be related to differences in P2Y receptor isoform distribution or differences in the suggested protein–protein interaction. Alternatively, any stimulatory effects of purines are overruled by other inhibitory mechanisms.

8 Oxidative Stress—Glutathionylation

Reactive oxygen species are generated in skeletal muscle during activity [24, 25]. Oxidative stress may lead to chemical modification of a number of muscle proteins that are important for skeletal muscle function. The induced oxidative modifications involve the formation of reversible disulphide bonds between glutathione and reactive cysteine thiols (a phenomenon called S-glutathionylation). Glutathionylation has been demonstrated to increase contractile apparatus Ca^{2+} sensitivity in rat and human skeletal muscles [26]. In addition, it has been reported that glutathionylation of Na, K-pump proteins may lead to modifications in pump function in the heart muscle [27–29]. Glutathionylation of the Na, K-pump in heart muscles has been reported to involve the β1 isoform [28, 30], the α subunits [31, 32] and the regulatory protein PLM (FXYD) [33].

The involvement of reactive oxygen species in Na, K-pump inhibition after exercise is supported by the finding that hypoxia increases the reduction in pump function in human skeletal muscles [4]. Studies with rat muscle membranes have demonstrated the existence of a basal glutathionylation of both α and β units of the Na, K-pump [34]. In addition, experimentally induced glutathionylation with oxidised glutathione was demonstrated to reduce the maximal in vitro activity of the Na, K-pump. Thus there seems to be a correlation between the level of glutathionylation and inhibition of Na, K-pump activity. One consequence of these findings is that the maximal Na, K-pump activity cannot be simply calculated from the number of Na, K-pump proteins (for instance, as determined by the binding of radiolabelled ouabain); the degree of glutathionylation must also be taken into account. Na, K-pump subunit glutathionylation has also been suggested to be of importance in human skeletal muscles. In a study with muscle samples from human subjects, exercise and β2-adrenergic stimulation lead to an unexpected decrease in the in vitro maximal Na, K-pump activity. Glutathionylation was suggested to cause the reduced Na, K-pump activity [8].

9 Nitric Oxide (NO)

Skeletal muscle possesses nitric oxide synthase, and NO has been shown to be released during skeletal muscle activity. A number of studies have shown that NO stimulates the Na, K-pump in cardiac myocytes [35, 36]. The studies in cardiac myocytes have suggested a number of possible mechanisms for NO-dependent activation of the Na, K-ATPase. The classical signalling pathway involves guanylate cyclase, the generation of cGMP and activation of cGMP sensitive kinases. Other kinases (PKCε) may be involved and changes in Na, K-pump affinity may be mediated by phosphorylation of the regulatory subunits phospholemman (FXYD1) [36, 37]. Another possibility is NO-dependent posttranslational modifications, such as protein S-nitrosylation (and S-glutathionylation) of Na, K-pump subunit proteins or proteins involved in regulatory mechanisms that are important for the pump [37, 38]. The effects of glutathionylation are described above. Based on the findings above, it is obvious to assume that NO also stimulates the Na, K-pump in skeletal muscles, but there is a lack of direct evidence.

10 Regulation Mediated by FXYD

The FXYD family consists of a number of isoforms with a tissue-specific distribution. Skeletal muscle expresses the FXYD1 isoform, also named phospholemman (PLM). PLM coexpression with the Na, K-pump subunits decrease the Na^+ affinity (increased K_m for Na^+), but the interaction with the subunits is dependent on the degree of PLM phosphorylation [39]. It has been demonstrated that exercise

translocates the PLM protein to the plasma membrane (probably from caveolae) and increases the association with the α1 subunits [40]. However, the effect of exercise on the Na, K-pump is an increased V_{max}, which is explained by simultaneous exercise-induced PLM phosphorylation [41]. As mentioned above, the FXYD1 subunit may also be the target for a number of regulatory mechanisms, such as glutathionylation and hormone- and NO-induced phosphorylation.

11 Conclusions

The table below summarises the exercise-related mechanisms of importance for regulation of the Na, K-pump in skeletal muscles. Factors involved in long-term changes are not included.

Effect of exercise	Effect on the Na, K-pump	Mechanism
Increased level of hormones (adrenaline)	Stimulation	Phosphorylation of α and FYYD subunits (increased Na^+ affinity)
Release of purines (ATP, ADP)	Stimulation (in rat)	Phosphorylation of α and FYYD subunits (increased Na^+ affinity)
Increased glutathionylation	Inhibition	Block of α and β subunits
Nitric oxide (NO)	Stimulation?	Phosphorylation of α and FYYD subunits (increased Na^+ affinity)
Translocation of pumps	Increased capacity	Increased number of active pumps

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Chapter 22
Advances in the Understanding of Renal Proximal Tubular Na^+/K^+ ATPase Regulation by Parathyroid Hormone and Dopamine

Syed J. Khundmiri, Rebecca D. Murray, and Eleanor D. Lederer

Abstract Na^+/K^+ ATPase activity is highly regulated in the renal proximal tubules by several hormones including PTH and dopamine. Both parathyroid hormone (PTH) and dopamine decrease Na^+/K^+ ATPase activity and expression by similar yet distinct signaling mechanisms. The role of PTH in regulation of Na^+/K^+ ATPase in renal proximal tubules is not very well studied. In contrast, dopamine regulation of Na^+/K^+ ATPase is extensively studied. This chapter focuses on the differential regulation of Na^+/K^+ ATPase by PTH and dopamine in renal proximal tubule cells.

Keywords Na+/K+ ATPase • PTH • Dopamine • NHERF1 • Proximal tubules • Signaling

1 Introduction

Na^+/K^+ ATPase is a ubiquitous enzyme that maintains the intracellular Na^+ and K^+ concentrations and electrochemical gradients across the plasma membrane in all animal cells [1]. The electrochemical gradients formed provide the driving force for various cellular functions, including membrane potential from potassium gradients, sodium-dependent vectorial transport of metabolites across the plasma membrane, and neuronal signaling [2]. The Na^+/K^+ ATPase is a heteromeric enzyme made up of two essential subunits, a catalytic α subunit and a β subunit, and an optional γ subunit [3–5]. Four α subunits (α1-4), and three β subunits (β1-3), and at least seven

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isoforms of γ or FXYD subunits have been described in the literature [6–8]. Using Xenopus oocytes, Ackerman and Geering demonstrated that (1) the α and β subunits assemble in the endoplasmic reticulum, (2) the β subunit is essential for the stability of α subunit structure, and (3) both the subunits depend on each other to exit the endoplasmic reticulum and be inserted into the plasma membrane [9]. In kidney tissues, only the α1 isoform of the α subunit has been shown to be expressed [10, 11]. In kidney epithelial cells, the function of Na^+/K^+ ATPase is extremely important both for energizing active ion transport across the apical membranes as well as regulating total body sodium homeostasis. As such, the activity of Na^+/K^+ ATPase is regulated throughout the nephron by the action of several hormones, including parathyroid hormone, dopamine, angiotensin II, aldosterone, and corticosteroids. The aim of this chapter is to review the regulation of Na^+/K^+ ATPase by PTH and dopamine in the renal proximal tubules. Regulation of Na^+/K^+ ATPase by other hormones is reviewed elsewhere in the book by Katyare et al. and Yingst.

2 Regulation by PTH

Binding of PTH to the PTH receptor results in the activation of protein kinase A (PKA) and C (PKC) signaling. Mandel and colleagues in the early 1990s were the first to describe acute regulation of Na^+/K^+ ATPase by PTH in renal proximal tubular cells [12–14]. Mandel's laboratory demonstrated that PTH directly inhibits Na^+/K^+ ATPase without changing the mitochondrial oxygen consumption. They further demonstrated that analogues of PTH, such as PTH1-34 (activating both PKA and PKC) and PTH3-34 (activating only PKC), decrease Na^+/K^+ ATPase-mediated ion transport through the activation of PKC-PLC pathway. McDonough and colleagues demonstrated acute PTH treatment results in inhibition of Na^+/K^+ ATPase function in animal models [15]. In a series of publications, our laboratory demonstrated some of the molecular mechanisms by which PTH regulates Na^+/K^+ ATPase activity, expression, and phosphorylation. We first dissected the signaling pathways involved in the inhibition of Na^+/K^+ ATPase activity and expression by PTH in opossum kidney proximal tubule cells, a cell culture model for the study of renal proximal tubules. We demonstrated that PTH inhibits Na^+/K^+ ATPase activity and increases serine phosphorylation of the Na^+/K^+ ATPase α1 subunit in a biphasic manner. The short-term inhibition was mediated through a PKC-PLA2 dependent pathway and the long-term by PKA-PLA2 dependent mechanisms. Our results from this study also demonstrated that PTH-stimulated mitogen activated protein kinase (MAPK)/extracellular regulated kinase (ERK) plays an essential role in the activation of PKC and subsequent regulation of Na^+/K^+ ATPase by PTH [16]. In collaboration with Bertorello's laboratory we next demonstrated that activation of the PKC-ERK pathway results in phosphorylation of Ser11 in the rodent Na^+/K^+ ATPase α1 subunit and Ser16 in the Na^+/K^+ ATPase α1 subunit of other species (the location of serine differs due to different translation initiation sites in rodents versus other species) on the Na^+/K^+ ATPase α1 subunit. This phosphorylation of the Na^+/K^+

ATPase α1 subunit triggers clathrin-mediated endocytosis of Na^+/K^+ ATPase α1 subunit into early and late endosomes [17]. We then identified the specific PKC isoforms activated by PTH during the regulation of Na^+/K^+ ATPase. Our data demonstrated that PTH activates two PKC isoforms, PKC α and βI in opossum kidney proximal tubule cells. However, inhibition of PKC α by a specific peptide inhibitor blocked PTH-mediated regulation of Na^+/K^+ ATPase [18]. Our laboratory then completed the mapping of the signaling pathway involved in the regulation of Na^+/K^+ ATPase by PTH. Binding of PTH to its receptor activates ERK in a Src kinase-, PLC-, and calcium-dependent but PLA2-independent manner. We showed that activation of ERK then results in the activation of PLA2. The by-products of PLA2 activation, 20-HETE and arachidonic acid then activate PKCα, which upon activation translocates to the plasma membrane and associates with Na^+/K^+ ATPase. This association produces phosphorylation of Na^+/K^+ ATPase at Ser11/16 by PKCα, promoting endocytosis of Na^+/K^+ ATPase [19].

In recent years our laboratory focused on identifying proteins associated with Na^+/K^+ ATPase α1 subunit and the role of the associated proteins in the hormonal regulation of the Na^+/K^+ ATPase α1 subunit, including regulation by PTH. Immunoprecipitation studies followed by proteomic analysis revealed that sodium-hydrogen exchanger regulatory factor-1 (NHERF1) associates with the Na^+/K^+ ATPase α1 subunit. This came as a surprise to us, because at that time, NHERF1 expression had only been found in the apical membrane and not the basolateral membrane. NHERF1 was initially discovered as an accessory protein required for regulation of sodium-hydrogen exchanger 3 (NHE3) in renal proximal tubule [20], although it has since been demonstrated to regulate several more ion transport processes in the proximal tubule. NHERF1 expresses two canonical PDZ binding domains (PDZ1 and PDZ2) and a C-terminal ezrin-binding domain (EBD), and associates with several ion transporters and transmembrane G-protein coupled receptors in renal proximal tubules and intestinal membranes [21]. Our data demonstrated that lack of the C-terminal EBD prevents PTH-mediated phosphorylation, endocytosis, and inhibition of Na^+/K^+ ATPase α1 subunit. However, lack of the EBD of NHERF1 does not affect the membrane expression and basal activity of Na^+/K^+ ATPase [22]. In a seminal study, Mahon and Segre demonstrated that NHERF1 associates with the PTH receptor and is absolutely required for activation of PLC and downstream signaling by PTH [23]. Based on these studies, our laboratory focused on the role of NHERF1 in the regulation of Na^+/K^+ ATPase α1 subunit. Using NHERF1-deficient opossum kidney proximal tubule cells (OKH), we demonstrated that NHERF1 is essential for regulation of Na^+/K^+ ATPase α1 subunit by PTH. Transfection of NHERF1 into OKH cells completely restored the regulation of Na^+/K^+ ATPase α1 subunit by PTH. Our laboratory also demonstrated that under basal conditions, Na^+/K^+ ATPase α1 subunit associates with NHERF1. Upon treatment with PTH, this association decreased and association with PKCα increased, resulting in phosphorylation, endocytosis, and inhibition of Na^+/K^+ ATPase α1 subunit. We went on to demonstrate that the PDZ1 domain mediates this association between Na^+/K^+ ATPase α1 subunit and NHERF1 [24]. Weinman and his colleagues demonstrated that PTH phosphorylates two residues, Ser77 and Thr96, within the

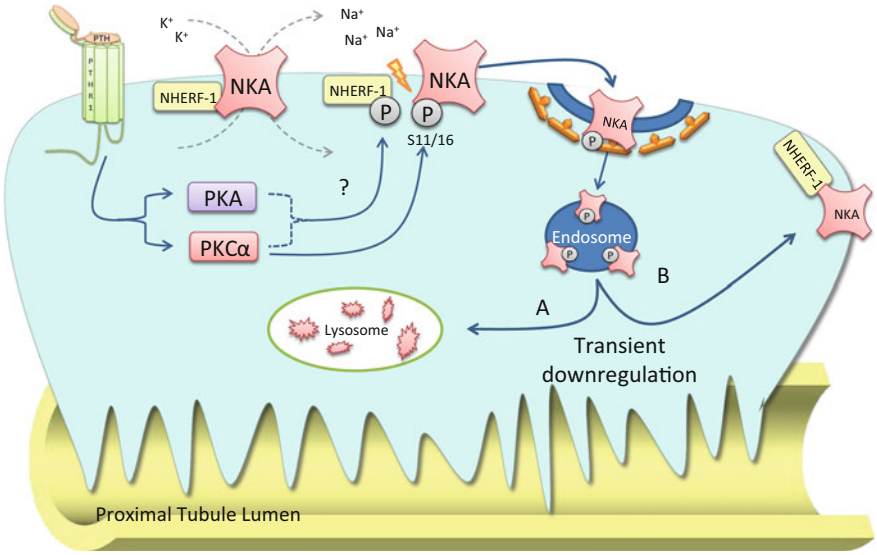

Fig. 22.1 Regulation of Na^+/K^+ ATPase by PTH: Na^+/K^+ ATPase basolateral membrane expression and function are regulated through its interaction with NHERF-1. PTH stimulation of the PTH receptor activates both PKA and PKCα, which together result in the phosphorylation of NHERF-1 and disrupt its association with Na^+/K^+ ATPase. PKCα also phosphorylates residue S11/16 on Na^+/K^+ ATPase, promoting the incorporation of Na^+/K^+ ATPase into clathrin-coated pits, where it is internalized to endosomes. From there, Na^+/K^+ ATPase is either (*A*) degraded in the lysosomes, or (*B*) dephosphorylation and recycled back to the cell surface, resulting in a transient downregulation of Na^+/K^+ ATPase from the basolateral membrane by PTH

PDZ1 domain of NHERF1. Taken together, we propose that PTH-activated PKCα first phosphorylates NHERF1, allowing it to dissociate from the Na^+/K^+ ATPase α1 subunit. This is followed by the subsequent phosphorylation of Na^+/K^+ ATPase α1 subunit at Ser11/16. Phosphorylation of the Na^+/K^+ ATPase α1 subunit then allows association with AP2 and clathrin heavy chain, followed by endocytosis through clathrin-dependent mechanisms (Fig. 22.1). Further studies are required to address this hypothesis.

3 Regulation by Dopamine

Dopamine is a natural catecholamine that is required for the synthesis of epinephrine and norepinephrine. Dopamine also acts as a neurotransmitter and is a strong natriuretic hormone. In fact under conditions of high salt intake almost 50 % of the natriuretic response is due to the action of dopamine [25]. Dopamine acts through two subclasses of G-protein coupled receptors D1 like and D2 like receptors [26]. Mutations in D1R results in about 25 mmHg increase in blood pressure [27]. Renal dopamine is produced mainly from S1 and S2 segments of the proximal tubules and

to a lesser extent from dopaminergic nerves [28]. In proximal tubule cells L-3,4 dihydroxyphenylalanine (L-DOPA) is converted to dopamine by the action of L-amino acid decarboxylase (AAAD) [29] and is not converted to norepinephrine due to the lack of dopamine β-hydroxylase. Unlike neural cells, in renal proximal tubule cells tyrosine is not converted to L-DOPA due to the lack of expression of tyrosine hydroxylase [29]. Therefore, the L-DOPA required as a substrate for dopamine synthesis is transported into the proximal tubule cells by Na+-independent and pH-sensitive type 2 L-type amino acid transporter (LAT2) [30]. Dopamine synthesized from L-DOPA is then released both in the luminal and peritubular sides and acts in an autocrine/paracrine fashion. Dopamine released thus binds to its receptor(s) to inhibit the activities of NHE3 and Na^+/K^+ ATPase respectively [31, 32]. The action of dopamine on these salt transporters is highly compartmentalized. In the luminal side dopamine almost exclusively stimulates cAMP generation and activates PKA while in the peritubular side it activates PLC-PKC pathway [33].

Studies from the laboratories of Aperia and Pedemonte demonstrated that dopamine signals through activation of protein kinase C to inhibit Na^+/K^+ ATPase activity [34, 35]. Bertorello's laboratory in a series of studies demonstrated that dopamine-mediated activation of PKC results in phosphorylation of Na^+/K^+ ATPase α1 subunit at Ser18/23 (rodents/other species) [36, 37]. They further demonstrated that this phosphorylation is important for endocytosis of Na^+/K^+ ATPase α1 subunit [37]. The inhibition of Na^+/K^+ ATPase by dopamine is dependent upon several signaling molecules including PI3 kinase, 14-3-3ζ, AP-2, and dynamin and on the intracellular sodium concentrations [38–49]. The human isoform of Na^+/K^+ ATPase α1 subunit is shown to be similarly phosphorylated at the same serine residues as the rat isoform by dopamine [50]. Different PKC isoforms exert opposite effects on the activity of Na^+/K^+ ATPase. Activation of PKCβ increases the activity of Na^+/K^+ ATPase while activation of PKCζ inhibits the activity of Na^+/K^+ ATPase [35]. Our laboratory demonstrated that dopamine inhibits the activity and phosphorylation of Na^+/K^+ ATPase through activation of PKC in a pertussis toxin dependent but ERK independent pathway [16]. We showed that dopamine activates PKCβ and PKCζ but Na^+/K^+ ATPase α1 subunit associates only with PKCζ. Bertorello's laboratory demonstrated that Na^+/K^+ ATPase α1 subunit associates with exogenously expressed PATJ, a PDZ protein [51]. Weinman and colleagues demonstrated that dopamine phosphorylates NHERF1 [52, 53]. Based on these studies, our laboratory hypothesized that NHERF1 plays an essential role in the regulation of the Na^+/K^+ ATPase α1 subunit by dopamine. Using OKH cells we demonstrated that lack of NHERF1 prevents dopamine-mediated inhibition and endocytosis of the Na^+/K^+ ATPase α1 subunit. We showed that upon treatment with dopamine, NHERF1 dissociates from Na^+/K^+ ATPase α1 subunit and PKCζ associates with Na^+/K^+ ATPase α1 subunit. We further demonstrated that contrary to PTH, dopamine-mediated inhibition and endocytosis of the Na^+/K^+ ATPase α1 subunit requires the PDZ2 domain of NHERF1 [54]. Thus, in some ways similar to the action of PTH, dopamine first phosphorylates NHERF1, resulting in its dissociation from the Na^+/K^+ ATPase α1 subunit, which then allows phosphorylation of

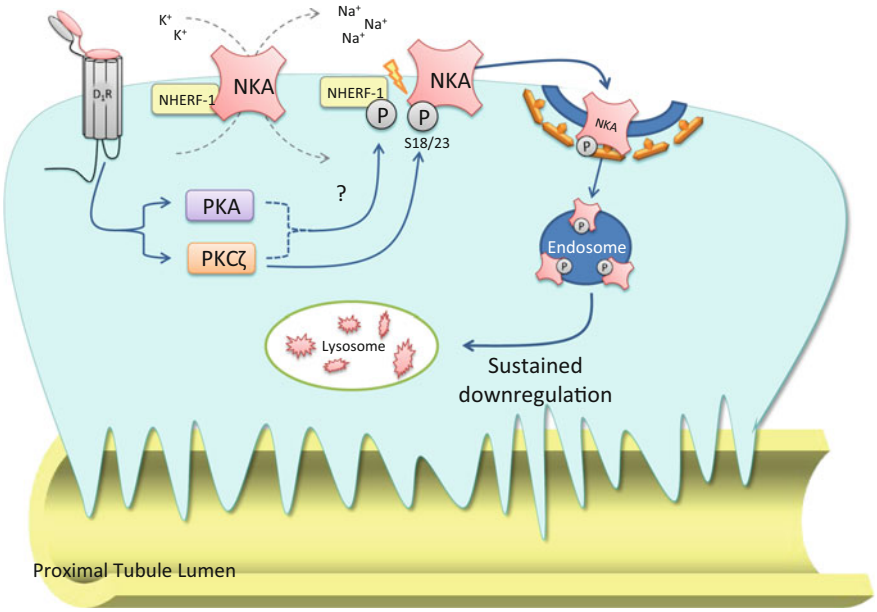

Fig. 22.2 Mechanism of dopamine-mediated Na^+/K^+ ATPase downregulation: Dopamine stimulation of the D_1 receptor activates both PKA and PKCζ, resulting in the phosphorylation of NHERF-1 and disrupting its association with Na^+/K^+ ATPase. PKCζ phosphorylates residue S18/23 on Na^+/K^+ ATPase, promoting the incorporation of Na^+/K^+ ATPase into clathrin-coated pits, where it is internalized to endosomes followed by degradation in the lysosomes, resulting in a sustained downregulation of Na^+/K^+ ATPase from the basolateral membrane by dopamine

the Na^+/K^+ ATPase α1 subunit at serine 18/23. Phosphorylation of Na^+/K^+ ATPase α1 subunit then triggers clathrin-mediated endocytosis of Na^+/K^+ ATPase α1 subunit through binding to 14-3-3ζ and AP-2 (Fig. 22.2).

The action of dopamine is mediated through G-protein coupled receptors (GPCRs). In mammals two types of dopamine receptors have been described, the D1R like receptors (D1R and D5R) and D2R like receptors (D2R, D3R, and D4R) [29, 55, 56]. The D1 like receptors couple to G_0, G_s, $G_{\alpha q}$, and $G_{\alpha 12/13}$ and stimulate adenylate cyclase activity [57–60]. D5R can directly stimulate phospholipase C (PLC) activity [61] but D1R require D2R for stimulation of PLC activity [62]. The D2 like receptors couple to G_0 and $G_{\alpha i}$ and activates PLC-PKC pathway [55, 63, 64]. All of the dopamine receptors are expressed, however, differentially along the nephron segments. All of the dopamine receptors are expressed in the proximal tubule. In medullary thick ascending limb D1, D3, and D5Rs are expressed while in the cortical thick ascending limb only D3R is expressed. In the distal convoluted tubules only D1R and D3R are expressed. The cortical collecting duct expresses all dopamine receptors except D2R. Dopamine regulates sodium transport all along the nephron segments [55, 65, 66]. It specifically inhibits the activity of several ion transporters including NHE1 [67], NHE3 [68–71], sodium–phosphate cotransporter

[72–76], sodium and chloride bicarbonate exchangers [77, 78], Na^+/K^+ ATPase [41, 45, 51, 54, 79–89], NCC [90], and epithelial sodium channel [91–95] in the kidney tubules. The activity of NKCC2 is stimulated by dopamine in the medullary thick ascending limb. However, overall sodium transport in this segment of the nephron is decreased because of the inhibition of Na^+/K^+ ATPase activity [96]. The D2 like receptors are important in the regulation of potassium channel activity in the nephron segments [97].

The expression of dopamine receptors is highly regulated. In conditions of high salt ingestion, dopamine receptors are sensitized and translocate to the plasma membrane following dephosphorylation by protein phosphatase 2A. In conditions of low salt intake the receptors are desensitized and pulled from the plasma membrane through phosphorylation and endocytosis [98–101]. Acutely, the receptors are regulated by the action of G-protein coupled receptor kinases (GRKs). To date seven different GRKs have been described in the literature [102]. The role of GRK4 has been demonstrated to be critical in development of hypertension and regulation of dopamine receptors [103, 104]. In fact in humans with salt-sensitive hypertension three different haplotypes of GRK4 have been described. Most patients with hypertension express 65L/142V/486A haplotype of GRK4 [105]. For a detailed discussion, the readers are directed to an excellent review article by Jose et al. [59].

Dopamine-mediated regulation of Na^+/K^+ ATPase α1 subunit and sodium excretion has been shown to be dependent upon interaction with other hormones/receptors involved in the regulation of sodium transport. Stimulation of dopamine receptors has been shown to inhibit release of catecholamines [106]. In contrast, inhibition of β-adrenergic receptors increase membrane translocation of D1R and increase natriuresis by inhibiting the activities of Na^+/K^+ ATPase and other sodium transporters [107]. The most important interaction of dopaminergic system in development of hypertension and salt sensitivity is through regulation by and of renin–angiotensin system (RAS) [108]. Under low salt dietary conditions, angiotensin II decreases dopamine production [109] and may increase salt reabsorption by stimulating the activities of Na^+/K^+ ATPase and other sodium transporters. Conversely, under high dietary salt conditions, dopamine decreases angiotensin II type 1 receptors (AT1R) in the plasma membrane [110–112] that may increase natriuresis by inhibiting the activities of Na^+/K^+ ATPase and other sodium transporters. Both D1 and D2 like dopamine receptors negatively interact with AT1R [113–115]. Interestingly, activation of D1R increases AT2R expression in the plasma membrane to increase salt excretion [116]. Activation of both D1R and D3R has been demonstrated to inhibit renin release from macula densa by inhibition of cyclooxygenase II [117]. Activation of D3R increases the activity of angiotensin converting enzyme II (ACE2) that synthesizes angiotensin 1-7 and increase natriuresis and vasodilation [118]. In normotensive rat models like Wistar Kyoto rats AT1R and D1R heterodimerize and inhibit each other's functions. In salt hypertensive SHR rats, the ability of D1R to heterodimerize with AT1R is impaired [110]. In normotensive rat models D3R activation results in inhibition of AT1R while in salt hypertensive models D3R activation increases AT1R expression

in plasma membrane [115]. The regulation of this interaction has been demonstrated to be dependent upon GRK4. Wild-type GRK4 activates D1R translocation to the plasma membrane but prevents AT1R translocation to the plasma membrane. In contrast, GRK4 variants increase AT1R phosphorylation and expression in the plasma membrane but inhibit D1R translocation through increased phosphorylation and endocytosis from the plasma membrane (Fig. 22.3) [59]. Interested readers are directed to an excellent review on interaction of dopamine receptors with RAS and other hormones that regulate sodium transport by Zeng and Jose [104]. Thus, dopamine through binding to its receptors and regulation of other hormone receptors cooperatively regulate Na^+/K^+ ATPase activity and membrane expression.

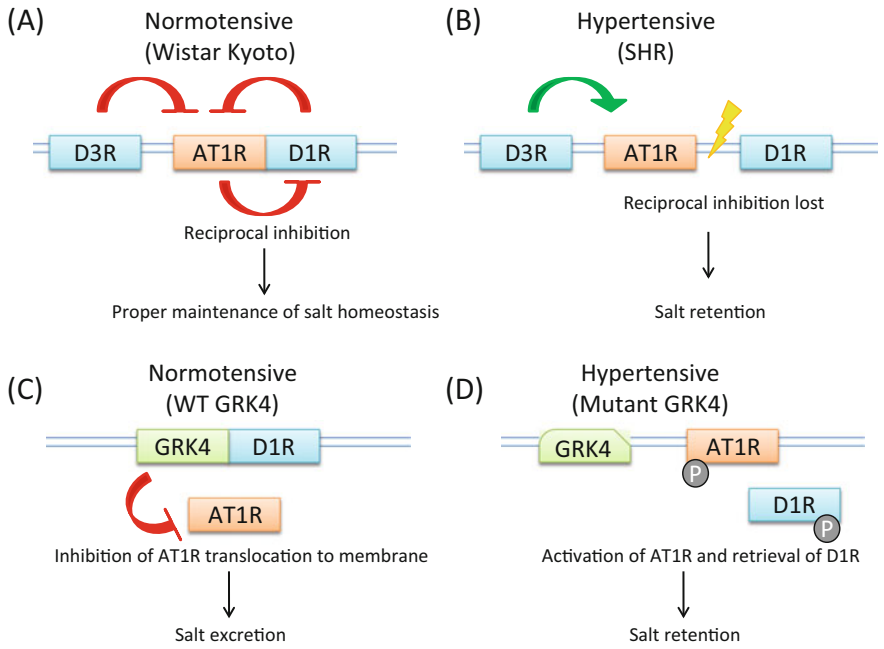

Fig. 22.3 Cooperativity between dopamine and angiotensin receptors: (**a**) In normotensive animals D1R, D3R, and AT1R associate and reciprocally regulate each other to maintain salt homeostasis. (**b**) In salt sensitive animals reciprocal inhibition is lost to increase the activity of AT1R and increase salt reabsorption and Na^+/K^+ ATPase activity. (**c**) In GRK4 wild-type expressing animals, GRK4 inhibits translocation of AT1R and promotes D1R translocation to plasma membrane to inhibit Na^+/K^+ ATPase activity and increase salt excretion while in (**d**) expression of mutant GRK4 inhibits D1R translocation and promotes AT1R translocation to the plasma membrane to increase Na^+/K^+ ATPase activity and increase salt reabsorption

4 Conclusions

In summary, both PTH and dopamine play important roles in the regulation of renal Na^+/K^+ ATPase and salt reabsorption. PTH acutely inhibits Na^+/K^+ ATPase activity. However, sustained PTH desensitizes the effects of PTH on Na^+/K^+ ATPase activity and membrane expression. Not much is known about the role of PTH in the pathogenesis or perpetuation of essential hypertension or on the regulation of Na^+/K^+ ATPase. However, secondary hyperparathyroidism as seen in patients with chronic kidney disease may be an important contributing factor in the regulation of blood pressure and salt homeostasis in these patients. Dopamine plays a more important role in the regulation of Na^+/K^+ ATPase and salt homeostasis. The role of dopamine in regulation of renal Na^+/K^+ ATPase is extensively studied. The role of different dopamine receptors and GRK4 is well established. Other factors may also contribute in regulation of sodium homeostasis in general and Na^+/K^+ ATPase in particular. For example, oxidative stress [119] and inflammation [120], regulation of the other hormones by dopamine (natriuretic peptide, prolactin, angiotensin II, and insulin) may contribute to the regulation of Na^+/K^+ ATPase by dopamine [121, 122]. The differences in the regulation of Na^+/K^+ ATPase by PTH and dopamine may be due to the differences in the signaling cascades activated by these hormones. While the effects of PTH are ERK-dependent, dopamine-mediated regulation of Na^+/K^+ ATPase is ERK-independent. They activate different isoforms of PKC, PKCα by PTH and PKCζ by dopamine. The differences may also in part be due to the handling of Na^+/K^+ ATPase α1 subunit after endocytosis. Some studies have shown that Na^+/K^+ ATPase α1 subunit recycles back to the plasma membrane after PTH treatment while others have demonstrated degradation of Na^+/K^+ ATPase α1 subunit in lysosomes/peroxisomes after dopamine treatment. Further studies are required to determine the role of PTH and dopamine in the regulation of total body sodium homeostasis in general and Na^+/K^+ ATPase in particular especially in old age and chronic kidney diseases.

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Chapter 23
Regulation of Na,K-ATPase in Epithelial–Mesenchymal Transition and Cancer

Zhiqin Li and Sigrid A. Langhans

Abstract Na,K-ATPase is an ion pump that creates an electrochemical gradient across the plasma membrane. In addition, Na,K-ATPase functions as a receptor and a signaling scaffold and its β-subunit has cell adhesion function. Many of the signaling pathways modulated by Na,K-ATPase have been linked to cell growth, apoptosis, cell adhesion, and motility. Changes in Na,K-ATPase function and expression have been reported in various cancers, even early during tumor development. Epithelial–mesenchymal transition (EMT) in which epithelial cells undergo a shift from a well-differentiated polarized epithelial phenotype to a fibroblastic, mesenchymal phenotype is one of the earliest steps in tumor progression. EMT can be induced by growth factors that activate signaling pathways to trigger an intricate network of transcriptional regulators. Interestingly, some of the transcription factors induced during EMT are known regulators of Na,K-ATPase expression. Here we summarize some of the best characterized EMT-inducing pathways, the transcription factors modulated by these signaling pathways and discuss how they may affect Na,K-ATPase subunit expression.

Keywords Na,K-ATPase α-subunit • Na,K-ATPase β-subunit • Transcription • Cancer • Transforming growth factor-beta • Epithelial–mesenchymal transition (EMT)

1 Introduction

Na,K-ATPase is an ubiquitous protein well known for its ion transport function which is critical for maintaining sodium and potassium homeostasis. The enzyme consists of a catalytic α-subunit, an essential β-subunit involved in the enzyme's membrane transport, translation and regulation of sodium and potassium affinity

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and an optional, tissue specific subunit belonging to the FXYD proteins [1, 2]. Four α-subunit isoforms (α_1-α_4) and three β-subunit isoforms (β_1-β_3) have been identified so far [3]. Na,K-ATPase also serves as a receptor for cardiac glycosides, is a signaling molecule and has cell adhesion function. Abnormal expression of Na,K-ATPase subunits and altered enzyme activity play an important role in the initiation and progression of cancers, especially in carcinoma, the most common type of cancer which originates from epithelial cells. Epithelial cells exhibit an apical–basal polarity characterized by the apical and basolateral domains of the plasma membrane, which are two structurally and functionally different regions. In most tissues, the apical membrane domain faces towards the lumen or the outside of the body, the basolateral domain contacts with the basement membrane and the adjacent cells. The apical and basolateral domains have distinct molecular composition and are separated by different cell junctions, such as tight junctions, adherens junctions, and desmosomes [4]. Apical–basal polarity is fundamental to the asymmetric distribution of cell fate determinants and the correct orientation of mitotic spindles in epithelial stem cells [5]. Loss of cell polarity can lead to defects in asymmetric cell division, thereby inducing tumor initiation [6]. Furthermore, epithelial cells tightly attach to one another and form epithelial sheets to cover the surface of organs and line the cavities throughout the body. The continuous epithelial layer forms a physical barrier, preventing tumor metastasis and invasion. During carcinoma progression, epithelial cells lose their apical–basal polarity and cell–cell adhesion; reorganize their cytoskeleton network; and adopt a fibroblast-like phenotype. This process is called epithelial-mesenchymal transition (EMT).

2 The Role of EMT in Cancer

EMT was first recognized in embryogenesis as a natural developmental process for numerous tissue and organ formations. EMT can also be activated during wound healing and tissue fibrosis [7]. However, the understanding of EMT has advanced greatly since it was recognized that activation of EMT is a critical mechanism of tumor invasiveness and metastasis. Cells undergoing EMT acquire tumor cell properties such as increased motility, invasiveness, resistance to therapy, and generation of stem-like cancer cells that are thought to be critical in cancer progression [8, 9]. In addition, some cells can gain the ability to sustain proliferative signaling by producing growth factor ligands, by increasing receptor levels at the cell surface resulting in hyper-responsiveness to otherwise limiting amounts of growth factors or by constitutive activation of downstream signaling pathways [10].

Several well-known interconnected signaling pathways that can initiate EMT have been identified [11]. These include transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), Wnt, and Sonic hedgehog (Shh). Hypoxia and some activated oncogenes like Ras, ErbB2/Her2, and mutant p53 have also been associated with EMT [12]. Many of these pathways share common downstream signaling effectors

forming EMT-inducing networks that converge on EMT-related transcription factors. These transcription factors either repress the expression of epithelial cell markers (such as E-cadherin, cytokeratins, ZO-1, and laminin-1) or activate genes associated with a mesenchymal phenotype [such as N-cadherin, fibronectin, vimentin, and α-smooth muscle actin (α-SMA)] [7]. As a result, the junctional complexes between epithelial cells are disrupted, the basement membrane and extracellular matrix (ECM) underlying the cells are degraded, the cytoskeleton networks are reorganized. Epithelial cells lose their apical–basal polarity and cell–cell adhesion and acquire the capacity of invasiveness and migration and render the cells resistant to senescence and apoptosis.

A hallmark of the EMT process is the loss of E-cadherin expression. E-cadherin is a calcium-dependent cell–cell adhesion glycoprotein that is required for proper establishment of apical–basal polarity in epithelial cells. The protein is composed of three major domains: a single transmembrane domain, a large extracellular five-cadherin-repeats domain, which mediates homophilic cellular interactions; and a highly conserved cytoplasmic domain, which anchors to the actin cytoskeleton through association with α-, β-, γ-catenins and p120 catenin. E-cadherin is the key component of adherens junctions, but also regulates the formation of other cell junctions, such as tight junctions and desmosomes [13, 14]. Na,K-ATPase acts synergistically with E-cadherin to maintain epithelial junctions and polarity [15–17].

3 Transcriptional Control of EMT

Molecular changes during EMT occur at multiple levels, including epigenetic gene regulation, alternative splicing, protein transport, and transcriptional regulation of target genes. Several key transcription factors that promote EMT directly regulate the expression of both cell adhesion and cell polarity complexes, with *CDH1* encoding E-cadherin being a major target gene. Transcription factors that repress E-cadherin and drive the EMT process include the members of the Snail, Zinc finger E-box binding homeobox (ZEB) and Twist families (Fig. 23.1) [18–20].

3.1 Transcription Factors

The Snail family belongs to the zinc-finger transcription factors and consists of three members in vertebrates (SNA1, SNA2, and SNA3), with SNA1 being the best characterized transcription factor that mediates EMT. SNA1 functions as a transcriptional repressor binding to E-box consensus sequences (CAGGTG) on the promoter region of E-cadherin and other target genes involved in cell polarity and development like the Crumbs, Par, and Scribble complexes [21, 22]. The promoter of the Na,K-ATPase β_1-subunit gene *ATP1B1* contains four E-boxes and a noncanonical E-box and SNA1 has been shown to bind to the noncanonical E-box to repress β_1-subunit expression [23].

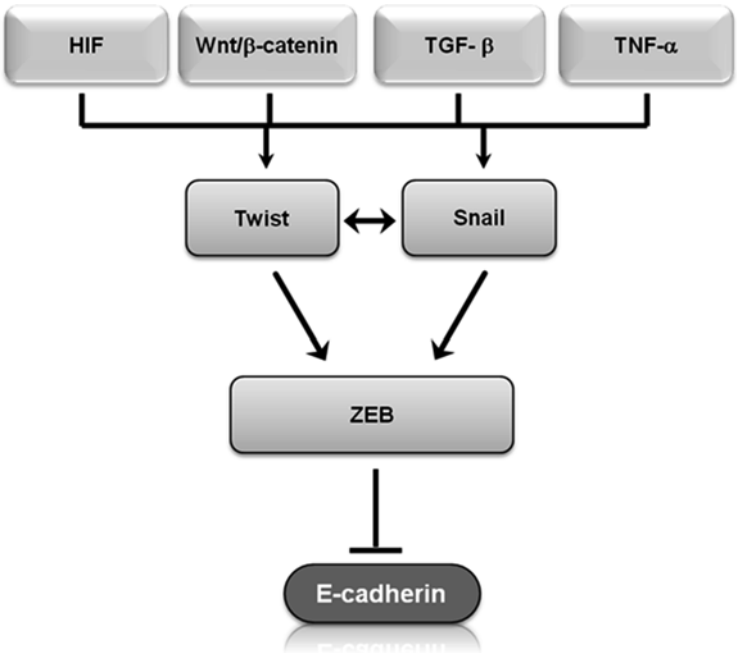

Fig. 23.1 Transcription factors in epithelial–mesenchymal transition (EMT)

ZEB1 is one of two members of the vertebrate ZEB family and acts downstream of Snail and Twist in EMT [20]. ZEB transcription factors bind to E-boxes on the promoter of target genes (CACCT and CACCTG) to activate gene transcription [24]. A consensus ZEB1 binding sequence has been found in the promoter of rat Na,K-ATPase α_1-subunit gene *Atp1a1* and ZEB1 can activate gene transcription in various cells [25–27]. ZEB binding sites were also found on the *FXYD1* promoter [26].

The Twist family has two members: Twist1 and Twist2. The Twist transcription factors have a basic Helix-Loop-Helix (bHLH) domain that mediates DNA binding and homo/hetero-dimerization and a twist box at the C-terminus that is involved in transcription activation or repression [20]. Twist1/2 represses E-cadherin expression independently of Snail, likely through interactions with other repressors [28]. However, there is no evidence, yet, to show that Twist regulates the transcription of Na,K-ATPase.

Ultimately, most signals induce EMT through activation of these transcription factors to repress epithelial genes and upregulate mesenchymal genes and genes involved in extracellular matrix remodeling, cytoskeletal reorganization and cell movement [19]. These EMT-activated transcription factors repress epithelial gene expression by direct binding to the conserved E-Box on the promoter regions of target genes. They also increase mesenchymal and other EMT related genes through indirect activating intracellular signaling pathways [19]. Adding to the complexity of transcriptional regulation, cross-regulations occur at the transcriptional and posttranscriptional levels among EMT-activated transcription factors (Fig. 23.1), and Snail's functions are independent of EMT in cell survival, stem cell function and immune regulation [29].

3.2 Epigenetic Mechanisms

Epigenetic reprogramming is an important contributor to the initiation and progression of EMT [30] and indeed Snail represses E-cadherin expression by forming a corepressor complex with histone deacetylases (HDAC)-1 and -2 [31]. Changes in histone acetylation and even more so, histone and DNA methylation, have important implications in the dysregulation of gene expression during EMT [32]. Methylation is also important in the regulation of Na,K-ATPase expression. Conserved CpG islands that remain unmethylated were found in *ATP1B1* and *Atp1b2* promoter regions [33, 34] but *ATP1B1* promoter was hypermethylated in tumor samples with clear-cell renal cell carcinoma [33]. Higher methylated CpGs were also found in the *Atp1a3* promoter regulating the transcription of the Na,K-ATPase α_3-subunit [35]. Methylation is also involved in the transcriptional regulation of FXYD1 and the FXYD1 promoter contains methylated cytosines and a predicted CpG island [36]. However, at this point it is not known whether epigenetic modifications during EMT indeed result in deregulated Na,K-ATPase subunit levels.

3.3 MicroRNA Regulation of EMT

MicroRNAs (miRNAs) are small noncoding RNA molecules that are important players during EMT through their key roles in posttranscriptional regulation of gene expression and influence multiple signaling pathways [37, 38]. Some examples include miR-138 that targets EMT-related genes vimentin and ZEB2 [39], miR-200 that targets E-cadherin, ZEB1 and ZEB2 [40] and miR-30a that promotes TGF-β-induced EMT through targeting Snail1 [41]. miRNAs also regulate the expression of Na,K-ATPase. For example, miR-92 directly targets Na,K-ATPase β_1-subunit [42]. miR-192 selectively suppresses Na,K-ATPase β_1-subunit expression, but has no effect on the α_1-subunit [43]. Notably, miR-192 also targets ZEB2, which indicates that miRNAs which target genes in EMT might also regulate Na,K-ATPase.

4 Na,K-ATPase, Transforming Growth Factor (TGF)-β Signaling and EMT

4.1 Na,K-ATPase in EMT and Cancer

Na,K-ATPase is a multifunctional protein that not only functions as an ion pump but also as a signaling scaffold and a cell-adhesion molecule. Both pump-dependent and pump-independent functions of Na,K-ATPase contribute to maintaining the epithelial phenotype of normal cells [16, 17, 44–52]. Loss of Na,K-ATPase function or subunit expression in cancer may contribute to tumor progression in multiple ways

including reduced cell aggregation [17, 48, 49, 52–54], altered epithelial junctions and polarity [16, 17, 47] and increased cell motility and invasiveness [17, 55]. Altered expression patterns of Na,K-ATPase have been observed in many human cancers. In human carcinoma, the β_1-subunit expression is reduced in many cases, including renal [56] and lung [57] cell carcinomas, androgen-dependent human prostate cancer cells [58], prostatic adenocarcinoma [59], and urothelial carcinoma [60]. The β_2-subunit is also reduced in human renal, lung, and hepatocellular carcinomas [57] and glioma [61]. In contrast to the β-subunit, the expression of α-subunits varies among different types of cancer tissues. The α_1-subunit is either increased or decreased in non-small-cell lung cancer depending on reports [62, 63], decreased in human colorectal adenocarcinomas [64], advanced prostatic adenocarcinoma [59], gastric cancers and urothelial carcinoma [60], or does not change as reported in human renal, lung, and hepatic tumors [56, 57]. The α_3-subunit was upregulated, but α_2- and α_4-subunit were decreased in human colorectal adenocarcinomas [64]. Moreover, the expression levels of α_1- and β_1-subunit have been reported as predictors of clinical outcomes in patients with bladder cancer, renal clear-cell carcinoma and lung cancer [60, 63, 65]. Patients with high α_1- and low β_1-subunit expression had a high risk for early recurrence in bladder cancer [60], high levels of α_1-subunit were associated with poor survival in patients with renal clear-cell carcinoma [65], and higher α_1-subunit levels had a significant survival advantage in lung adenocarcinoma [63]. Interestingly, in ovarian cancer, low β_1-subunit levels were associated with resistance to the chemotherapeutic oxaliplatin [66], suggesting that the role of Na,K-ATPase in cancer is not only limited to changes associated with EMT.

The functions of Na,K-ATPase in EMT progression are still not fully understood. Some studies point to a role of Na,K-ATPase in the formation and maintenance of epithelial cell polarity. Na,K-ATPase functions as a cell adhesion molecule [17, 48, 49, 52–54] and α_1- and β_1-subunit co-localize with adherens junctions in Madin Darby canine kidney (MDCK) cells [52]. The β_1-subunit forms trans-dimers between adjacent cells or cis-dimers within the same membrane, which is important for initiation and maintenance of cell junctions [50, 53]. Overexpression of the β_1-subunit increased cell–cell adhesion and inhibited cell motility in Moloney Sarcoma virus-transformed MDCK cells (MSV-MDCK) [17, 55]. The α_1-subunit indirectly associates with E-cadherin and the spectrin/actin cytoskeleton via ankyrin, an actin binding protein, which stabilizes adherens junctions [50]. In MSV-MDCK cells that express low levels of E-cadherin and β_1-subunit, simultaneous overexpression of E-cadherin and the β_1-subunit rescued the epithelial phenotype but expression of either protein alone was not sufficient to induce tight junctions and epithelial polarity and to inhibit invasiveness and motility of these cells [17]. In breast cancer cells, knockdown of the β_1-subunit increased proliferation, migration, and invasion [42]. The β_2-subunit, initially known as the adhesion molecule on glia (AMOG), plays an important role in cellular adhesion and migration in the central nervous system [61, 67]. Loss of the β_2-subunit has been implicated in glioma migration and invasion [68]. In polycystic kidney disease, abnormal expression of the β_2-subunit could be the origin of the apical mislocalization of Na,K-ATPase [69] suggesting that abnormal expression of the β_2-subunit and/or mislocalization of the Na,K-ATPase is not

sufficient to disrupt epithelial integrity. On the other hand, impaired Na,K-ATPase activity leads to the loss of tight junctions and epithelial polarity. Inhibition of Na,K-ATPase either by ouabain or K^+ depletion not only prevented the formation of tight junctions and desmosomes, thus disrupting cell polarity [16]; but also increased the permeability of tight junctions [45, 70]. Thus, it appears that structural loss of Na,K-ATPase alone may not explain the role of Na,K-ATPase in EMT but rather disruption of the intricate interplay between Na,K-ATPase activity, cell–cell adhesion, and signaling has to occur for changes in Na,K-ATPase to contribute to EMT.

4.2 TGF-β Signaling and EMT

Among all signaling pathways that can trigger EMT, TGF-β is a major inducer [28]. The TGF-β superfamily comprises TGF-βs, bone morphogenetic proteins (BMP), activins, and other related proteins. These secreted proteins regulate numerous physiological processes, including cell differentiation, proliferation, development, and survival. Interestingly, Na,K-ATPase is one of the target molecules of TGF-β signaling [47, 71–73]. The TGF-β receptor complexes at the cell surface consist of two type II and two type I transmembrane receptors and binding of TGF-β family proteins to the tetrameric receptor complex enables type II receptors to phosphorylate and activate type I receptors. Two major signaling mechanisms which mediate TGF-β induced EMT are SMAD-dependent and SMAD-independent pathways (Fig. 23.2). SMAD-dependent signaling involves phosphorylation and activation of SMAD2 and SMAD3 by the type I receptor, that then bind to SMAD4 to form a trimeric SMAD complex. The SMAD complex translocates into the nucleus and associates with other transcription factors to modulate the expression of target genes. The inhibitory SMADs, SMAD6 and SMAD7, can compete for the same binding sites on type I receptor for SMAD2 and SMAD3 and inhibit TGF-β signaling [74–76]. TGF-β also signals through SMAD-independent pathways with MAPK, phosphoinositide-3 kinase (PI3K), and Rho-GTPases being the three major SMAD-independent pathways that promote EMT. The TGF-β receptor can phosphorylate associated ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) to activate TGF-β-activated kinase 1 (TAK1), which phosphorylates MKK3/6 and MKK4, leading to activation of p38 MAPK and JNK [74–76]. The MAPK pathways may regulate EMT related gene expression directly or by cooperating with other transcription factors, such as SMAD complexes. TGF-β also activates PI3K, and then phosphorylates and activates Akt, leading to activation of mammalian TOR complex 1 (mTORC1) and mTORC2, which increases translation efficiency and cell size. In addition, TGF-β can activate Rho-like GTPase, like RhoA, resulting in actin reorganization. TGF-β-induced RhoA activation also regulates EMT-related gene expression including α-SMA [28, 77]. SMAD-dependent and -independent pathways cooperate and interplay in EMT initiation and progression, and either blocking SMAD-dependent signaling or selectively inhibiting SMAD-independent pathways dramatically decreases TGF-β-induced EMT [11, 28].

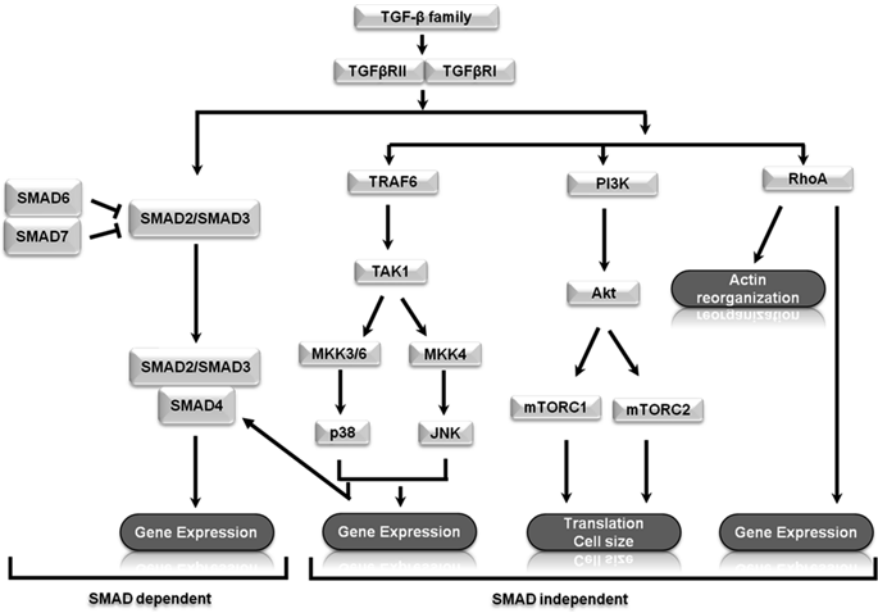

Fig. 23.2 TGF-β signaling in epithelial–mesenchymal transition (EMT)

4.3 Transcriptional Regulation of Na,K-ATPase in TGF-β-Induced EMT

Na,K-ATPase is a target of TGF-β signaling in various cell types. In renal epithelial cells, TGF-β_1 repressed β_1-subunit levels when undergoing EMT [47, 72]. However, in LLC-PK1 cells, a porcine kidney epithelial cell line, TGF-β_1 appeared to decrease β_1-subunit expression at the posttranslational level since no significant reduction in β_1-subunit mRNA levels were found under the experimental conditions [47]. This would be consistent with studies in human renal clear-cell carcinoma tissues, in which no changes in β_1-subunit mRNA levels were found despite a drastic reduction in β_1-subunit expression in these tumors [56]. Nevertheless, recent studies revealed that *ATP1B1* promoter was hypermethylated in tumor samples obtained from clear-cell renal cell carcinoma suggesting that *ATP1B1* is epigenetically silenced by promoter methylation in these tumors [33]. Further studies will be required to address these findings.

In human retinal pigment epithelial cells, TGF-β_2 selectively decreased β_1-subunit expression and knockdown of β_1-subunit resulted in a mesenchymal cell morphology and induced fibronectin and other EMT markers [71]. In these cells TGF-β_2 decreased the transcription of β_1-subunit through two transcription factors: Smad 3 and hypoxia inducible factor (HIF) [71]. HIF is a member of the basic helix-loop-helix (bHLH) superfamily and is composed of a hypoxia-inducible HIF-α sub-

unit (HIF-1α, HIF-2α, and HIF-3α) and a constitutively expressed HIF-1β subunit. Aside from hypoxia, HIF-1 can be regulated in an oxygen-independent manner, including TGF-β [71, 78, 79]. In retinal pigment epithelial cells, TGF-β_2 increased HIF-1α and as the *ATP1B1* promoter contains a Smad binding domain (SBD) in close proximity to a putative hypoxia-responsive element (HRE), it is likely that both Smad3 and HIF1-α cooperated in regulating the expression of β_1-subunit expression by TGF-β_2 in these cells [71].

In retinal pigment epithelial cells, TGF-β_2 signaling suppressed only β_1-subunit and not α_1-subunit. Nevertheless, TGF-β_1 reduced β_1-subunit as well as α_1-, α_2-, and α_3-subunit mRNA levels in rat thyroid cells [73]. Furthermore, TGF-β_1 prevented the increase in α_1-subunit mRNA level induced by steroid hormones [80]. Thus, Na,K-ATPase subunit regulation by TGF-β may depend on the cellular background. This is consistent with TGF-β signaling playing a complex role in cancer development and progression, where it can function either as a tumor suppressor or a tumor promoter [74]. Whether TGF-β-dependent regulation of Na,K-ATPase contributes to this cell-type specific function of TGF-β remains to be determined.

5 Hypoxia, Na,K-ATPase and EMT

As tumors grow, cancer cells adjust to hypoxia and lack of nutrients by activating angiogenesis-specific and metabolic and glycolytic pathways [81–83] and TGF-β signaling can integrate hypoxia-associated signals [11]. One of the best characterized hypoxia genes is HIF-1α which is frequently upregulated in solid tumors and promotes EMT and tumor progression and facilitates metastasis [84–87]. HIF-1α can upregulate EMT-mediating transcription factors Snail, ZEB1/2 and Twist resulting in repression of E-cadherin levels [88–90] and interacts with other EMT-associated signaling pathways [11]. HIF-1α contains three major domains, a N-terminal DNA-binding domain, an oxygen-dependent degradation domain as HIF-1α is rapidly degraded in normoxia, and a C-terminal domain that recruits transcriptional co-activators [78]. In hypoxic conditions, HIF-1α is stabilized, translocates to the nucleus and forms a dimer with HIF-1β to associate with the HRE in the regulatory regions of target genes. HIF-1α generally functions as a transcriptional activator and transcriptional repression by HIF-1α induction may require additional transcription repressors. In the case of Na,K-ATPase, the *ATP1B1* promoter region contains a Smad binding domain (SBD) and a putative HRE in close proximity. Both HIF-1α and Smad3 bound to *ATP1B1* promoter and likely cooperated in repressing the expression of β_1-subunit [71]. While it is well known that Na,K-ATPase subunit levels are regulated by endocytosis and degradation under hypoxic conditions [91], it is possible that additional transcriptional mechanisms exist in hypoxic tumor cells undergoing EMT to suppress Na,K-ATPase subunit expression.

6 Conclusions

EMT is an important process in cancer development and progression, characterized by induction of mesenchymal genes and repression of epithelial genes. These EMT-associated genes are under tight control at multiple levels, including both, transcriptional and posttranscriptional levels. Notably, many regulators of EMT-related genes also modulate Na,K-ATPase expression. For example, the transcription factor Snail1 induces EMT and also represses the expression of Na,K-ATPase β_1-subunit. miR-192 modulates both ZEB2 and β_1-subunit expression. However, the role of Na,K-ATPase in EMT is not completely clear, yet. Some studies indicate that Na,K-ATPase might facilitate the EMT process. For instance, loss of Na, K-ATPase results in altered epithelial polarity and increased cell motility and invasiveness, which are characteristic events in EMT, β_1-subunit is a target of TGF-β, a major inducer of EMT, and introducing E-cadherin or Na,K-ATPase alone into MSV-MDCK cells cannot induce epithelial polarity and inhibit invasiveness and motility of these cells. However, simultaneous overexpression of E-cadherin and the β_1-subunit rescued the epithelial phenotype. Despite all of this circumstantial evidence, a causal role for Na,K-ATPase in cancer development has not been demonstrated so far. It is likely that only once initial steps in the EMT process are triggered that an additional loss of Na,K-ATPase contributes to EMT progression and tumor formation.

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Chapter 24
Metal Based Compounds, Modulators of Na, K-ATPase with Anticancer Activity

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Abstract Involvement of Na, K-ATPase in different biological processes and its overexpression in pathological states enables its use as a target in anticancer studies. For the past 10 years, a variety of metal-based complexes have been synthesized which offer good tolerance, potent action, selectivity, and less toxicity in cancer treatment. This chapter gives an overview of the interaction of platinum, gold, ruthenium, vanadium, and palladium complexes with Na, K-ATPase and their effect on the enzyme function and activity. The mechanism of Na, K-ATPase activity inhibition with metal based complexes is supported with extensive kinetic analysis. The inhibition can be achieved via the complexes interaction with –SH groups of the enzyme and cleavage of the disulfide bridges, required for the enzyme functionality. Moreover, the inhibitory effect of selected compounds can be prevented and recovered by the addition of –SH donors, L-cysteine and glutathione, the biomolecules usually present in physiological liquids. The conclusion is made that gold, ruthenium, and palladium complexes are expected to overcome platinum complexes toxic side effects.

Keywords Anticancer • Glutathione • Inhibition • L-cysteine • Ligand • Metal complexes • Na, K-ATPase • Prevention • Recovery • Thiol

1 Introduction

A variety of metal-based compounds have been synthesized so far, in an effort to offer better tolerance, more potent action, better selectivity, and less toxicity in cancer treatment [1, 2]. A mechanistic understanding of how metal complexes produce their biological activities is critical for their clinical success. Proteins have been

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proven to be possible targets for antitumor metal complexes as well as importance of their involvement in the overall mechanism of action of anticancer metallo-drugs [3]. The modification of cysteine residues in proteins due to its ability to strongly coordinate complex metal ions is one of the arguments of critical importance for the design of the novel types of pharmacological agents, based on Pt(II), Au(III) and Pd(II) complexes [4–6]. It is well known that sodium pump (Na, K-ATPase) has a critical role in cell survival, proliferation, adhesion, and migration [7–12] as well as its increased expression and elevated activity in some types of cancers [7]. In this chapter, we present an overview of anticancer metal-based complexes which affect and modulate the activity and function of Na, K-ATPase.

2 Na, K-ATPase

2.1 Structure and Function

Na, K-ATPase is a membrane enzyme ubiquitous in animal cells that involve 5′-adenosine triphosphate (ATP) as a substrate for their functioning. It is responsible for establishing and maintaining the electrochemical gradient in animal cells, due to the free energy resulting from the hydrolysis of an intracellular ATP [13–15]. The sodium pump contributes substantially to the maintenance of the ion concentration gradient throughout the membrane, and enables the animal cell to control its volume and actively transport carbohydrates as well as amino acids. It is also required for nerve and muscle excitation [16].

The minimum functional unit of Na, K-ATPase is an oligomer composed of stoichiometric amounts of two major polypeptides, α- and β-subunits. The α-subunit, responsible for the catalytic and transport properties of the enzyme, is a protein with a molecular mass of ~112,000 Da that contains the binding sites for the cations and ATP. It acts as the receptor for specific inhibitors, cardiac glycosides such as ouabain, which are bound to the extracellular side of the protein at very high affinity and lead to the inhibition of enzymatic activity [17–19]. The β-subunit is a polypeptide that crosses the membrane once and has a molecular weight between 40,000 and 60,000 Da, depending on the degree of glycosylation in different tissues. The β-subunit is essential for the normal activity of the enzyme [20], and it appears to be involved in the occlusion of K^+ and the modulation of the K^+ and Na^+ affinity of the enzyme [21]. The enzyme has two major conformation states, E_1 and E_2 which act as "flip-flop" model, in which both α subunits show complementary conformations [22]:

$$E_1E_2 \rightleftharpoons E_2E_1$$

where E is the conformation of each α subunit. According to this model when one of the α subunits is in the E_1 conformation, the other one is necessarily in the E_2. These states have different tertiary structures, different catalytic activities, and different ligand specificities. Biochemical and spectroscopic data show that

long-range E_1–E_2 conformational transitions in the α-subunit mediate interactions between cytoplasmic domains and the cation sites in the intramembrane domain [13, 23, 24]. These transitions couple the scalar processes of ATP binding, phosphorylation, and dephosphorylation to the vectorial extrusion of three Na^+ ions and uptake of two K^+ ions.

The sodium pump is characterized by a complex molecular heterogeneity that results from the expression and differential association of multiple isoforms of both its α- and β-subunits. Individual genes of four α (α1, α2, α3, and α4)-subunit isoforms and at least three β (β1, β2, and β3)-subunit isoforms of Na, K-ATPase have been identified in mammalian cells [25–27]. The distribution of the Na, K-ATPase α and β subunits isoforms is tissue- and developmental-specific, suggesting that they may play specific roles, either during development or coupled to specific physiological processes [27–29].

2.2 Expression of Na, K-ATPase in Pathological States

In some pathological states, like cancer, expression of Na, K-ATPase is changed compared with the normal tissue. Na, K-ATPase is highly expressed in cancer cells including human glioblastoma cells [7, 8, 30, 31]. Human T98G glioblastoma cells that are resistant to the chemotherapy drug temozolomide showed a unique high expression of the Na, K-ATPase α2 and α3 subunits compared to the temozolomide-sensitive cell line LN229 and normal human astrocytes [7]. It was shown that Na, K-ATPase α1 subunit expression is markedly higher in a significant proportion of non-small-cell lung cancer (NSCLC) clinical samples compared to normal lung tissue [32, 33]. In the neuroblastoma cell line the level of β1 subunit mRNA was found to be higher than in neuron primary cell culture. The level of α1 mRNA in investigated tumors was the same as in normal tissues. These results may give evidence of the involvement β2-subunit in the process of tumorigenesis as was shown for some other adhesion molecules [34]. Also, there are the data that some type of cancers showing lower expression of Na, K-ATPase than of normal tissue. In normal human gastric tissues Na, K-ATPase subunits are highly expressed, but in tumorous tissue expression of the enzyme is reduced [35]. The β1-subunit of Na, K-ATPase was isolated and identified as an androgen downregulated gene. Expression was observed at high levels in androgen-independent as compared to androgen-dependent (responsive) human prostate cancer cell lines and xenografts when grown in the presence of androgens [36]. Compared with the benign fields, the mean protein expression for both Na, K-ATPase α- and β-subunits were found to be decreased overall in in situ and invasive bladder tumors, as well as in tumor-adjacent dysplastic fields [37]. It was reported that poorly differentiated human carcinoma cell lines derived from colon, breast, kidney, and pancreas show reduced expression of the Na, K-ATPase β1-subunit [38]. Substantial decrease in the level of Na, K-ATPase β2-subunit mRNA in xenografts of human renal, lung and hepatocellular carcinomas in nude mice as compared with corresponding normal tissues, as well as in the

neuroblastoma cell line as compared with the neuron primary cell culture. The level of β1 mRNA is decreased in kidney and lung tumor cells, but is unchanged in hepatocellular carcinoma. Reduced expression of β-subunit of Na, K-ATPase in human clear-cell renal cell carcinoma (RCC) was found, and at the same time the α-subunit level in RCC lysates was generally near or above the levels relative to normal kidney [39]. Stomach and colon adenocarcinomas showed opposite patterns of β1-isoform glycoprotein expression. Stomach adenocarcinomas showed lower expression levels of Na, K-ATPase β1 subunit than did control tissue, and colon adenocarcinomas showed higher expression of the same isoform than of normal surrounding tissue [40]. In human colorectal cancer upregulation of Na, K-ATPase α3-isoform and downregulation of the α1-isoform was detected [41].

3 Metal Complexes Modulating Na, K-ATPase with Anticancer Activity

The activity of metal complexes depends not only on the metal itself but also on its oxidation state, number and types of ligands bound, and the coordination geometry of the metal complex [42]. Even subtle changes in the charge of a metal result usually in a great change in the coordination structure of the metal complex. These changes lead to dramatic alterations of the physicochemical and thus biological properties in the application of the metal complex as drug. Many metal-based drugs act as prodrugs that undergo ligand substitution and redox reactions before they reach their targets [43].

3.1 Platinum Complexes

History of platinum (Pt) based anticancer drugs investigation and application begins with cisplatin which was discovered accidentally by Rosenberg in 1965 [44]. Cisplatin (Fig. 24.1a) was introduced in cancer therapy after the discovery that it inhibits bacterial cell division when platinum-conducting plates are used for cell growth and stops cell proliferation [44]. In 1978, US Food and Drug Administration (FDA) approved cisplatin under the name of Platinol® for treating patients with metastatic testicular or ovarian cancer in combination with other drugs, and for treating bladder cancer as well [45]. Cisplatin is known as an effective anticancer drug for various human solid cancers, such as those of head, neck, lungs, testes, bladder, and ovaries [46–49]. Despite its potent anticancer activity, cisplatin induces nephrotoxicity and gastrointestinal toxicity, which are major dose-limiting factors in its clinical use [50, 51]. In 1979, Bristol-Myers Squibb licensed carboplatin (Fig. 24.1b), a second-generation platinum drug with fewer side effects, which entered the US market as Paraplatin® in 1989 for initial treatment of advanced ovarian cancer in combination with other approved chemotherapeutic agents [52].

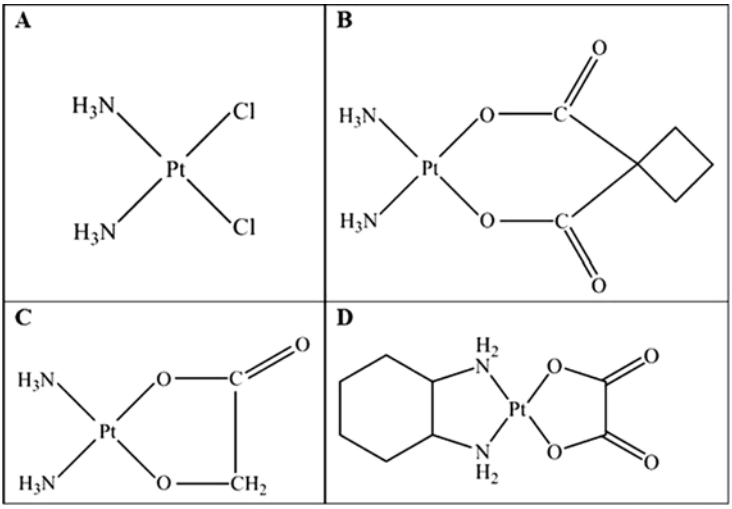

Fig. 24.1 Platinum anticancer agents. Chemical structures of (**a**) cisplatin, (**b**) carboplatin, (**c**) nedaplatin, and (**d**) oxaliplatin

A second cisplatin analog, nedaplatin (*cis*-diammine-glycolatoplatinum) (Fig. 24.1c) was developed in 1983 to provide a treatment with effectiveness similar to that of cisplatin but with decreased renal and gastrointestinal toxicities [53, 54] and approved as a drug (Aqupla®) [55, 56]. Nedaplatin has the same ammine carrier ligands as cisplatin, but has a different leaving group, consisting of a five-membered ring structure in which glycolate is bound to the platinum ion as a bidentate ligand. Similar to cisplatin, nedaplatin reacts with nucleosides forming a nucleoside–platinum complex. It has been confirmed that the types of combined bases in nedaplatin after reaction with DNA are identical to those observed in cisplatin [57]. In 1994, one more platinum anticancer drug was approved—oxaliplatin (Fig. 24.1d) under the trade name of Eloxatin®. It was the first platinum-based drug to be active against metastatic colorectal cancer in combination with fluorouracil and folinic acid [58, 59]. However, the clinical use of platinum anticancer agents is limited by tumor resistance and unwanted normal tissue toxicities [60–62]. The reason for the tumor resistance is the increased platinum accumulation in the tumor tissue due to reduced uptake and/or increased efflux. Cellular platinum accumulation is a key determinant of the sensitivity of a wide range of cancer cells to cisplatin [63], oxaliplatin [64], carboplatin [65], and ZD0473 [66]. Ovarian cancer patients who responded to platinum-containing therapy had higher tumor platinum concentrations than those who did not [67]. Although several toxic side effects induced by platinum anticancer drugs were noticed, nephrotoxicity is the main limiting factor in their clinical use [50, 51]. In recent years, the studies were directed to decreasing tumor tissue resistance induced by interaction of platinum anticancer drugs with thiol-containing molecules. These investigations resulted in the synthesis of picoplatin in which one

of the amines linked to platinum was replaced by a bulky methyl substituted pyridine allowing the drug more time to reach its target, DNA [68, 69]. Development of improved delivery strategies using liposomes and polymers led to discovery of liposomal cisplatin or lipoplatin being under a phase III randomized clinical trial for patients suffering from small cell lung cancer [70, 71]. Besides, polymer-based platinum drug, Prolindac™ has been under investigation for pretreated ovarian cancers in several European countries [72, 73].

3.1.1 Interaction of Platinum Complexes with Na, K-ATPase

Due to many severe toxic side effects coupled to an antitumor activity of platinum complexes, it is important to be aware of its influences on various proteins, such as protein structural alterations and enzymatic changes that are implicated in its mechanism of action. Among all platinum complexes mentioned earlier, cisplatin is the most studied [74]. It is reasonable to assume that the translocation process of ions operated by Na, K-ATPase is influenced by enzyme–ligand interaction, which can induce significant changes in the protein configuration, possibly near the ligand binding sites. Spectroscopic studies have shown that the complexation of the Na, K-ATPase with different metal cations induces significant secondary structural changes, while several other investigations showed that there are minor structural changes [74]. The nature of cisplatin interaction with Na, K-ATPase at physiological pH was characterized with the drug binding constant and the protein secondary structure in the cisplatin–ATPase complexes drug binding mode. The spectroscopic results obtained showed that cisplatin binds Na, K-ATPase through lipid carbonyl group at low concentrations (0.1 μM) and at higher drug concentrations (1 mM) binding extends to polypeptide C=O and C–N groups with the binding constant of $K = 1.93 \times 10^4$ M [74].

The efficacy of complexes is dependent on their accumulation in cells. The uptake of the platinating agents was long thought to be a result of simple passive diffusion. However, years of research have provided better understanding of this important step in the drug's action [75]. The uptake of cisplatin is influenced by factors such as sodium and potassium ion concentrations and pH. Ported and gated channels have been postulated in addition to passive diffusion also [76]. Unfortunately, the exact mechanisms involved have not been completely defined and may differ between different cell types. For instance, the influence of Na, K-ATPase in the uptake of cisplatin in human ovarian carcinoma cells it is reported [77]. This study has shown that cisplatin accumulation in human ovarian carcinoma cells is neither saturable nor competitively inhibited by structural analogs. These data strongly imply that cisplatin transport is not carrier mediated in these cells [77–79]. However, cisplatin accumulation is modulated by cAMP, dependent on the membrane potential, partially ouabain inhibitable, partially energy dependent, and partially sodium dependent [77, 80, 81]. Many of these observations point to a central role for the Na, K-ATPase in cisplatin accumulation [77]. The connection between cisplatin toxicity and Na, K-ATPase has been made also in the kidney and

the inner ear. Cisplatin is particularly damaging to these tissues, not because it inhibits or platinates this key protein, but simply because the presence of high levels of the Na, K-ATPase α1 isoform may cause much higher levels of cisplatin to be brought into these cells. The converse, however, is not true since hepatic tissue is virtually devoid of Na, K-ATPase, yet it accumulates significant amounts of cisplatin [77, 82, 83]. Clearly, the connection between Na, K-ATPase levels and cisplatin toxicity is more complicated than a simple correlation with cisplatin accumulation.

3.1.2 Inhibition of Na, K-ATPase Activity with Platinum Complexes

With the aim to check if the platinum compounds induced nephrotoxicity could be resulted from the inhibition of kidney Na, K-ATPase activity, Kitada et al. investigated in vitro influence of cisplatin, nedaplatin, and carboplatin on the activity of purified renal Na, K-ATPase from pig kidneys and on the viability of human renal proximal tubule epithelial cells (HRPTE cells) [84]. The viability of HRPTE cells which were cultured for 3 days in the presence of various concentrations of the investigated platinum analogs was measured. The obtained results showed concentration-dependent decrease in HRPTE cells, but with varying toxic potencies of the platinum agents. The concentrations to decrease 50 % of the viable cells were 50, 120, and 380 μM for cisplatin, nedaplatin, and carboplatin, respectively [84].

Afterwards, the effects of different concentrations of cisplatin, nedaplatin, and carboplatin on purified renal Na, K-ATPase activity from a pig kidney were tested. Cisplatin and nedaplatin inhibited the enzyme activity in concentration-dependent manner with obtained half maximal inhibition concentrations of 0.6 and 1.5 mM, respectively for cisplatin and nedaplatin, after 3 h preincubation in the presence of these compounds. In these preliminary experiments, it was found that the inhibition was not only concentration dependent but also preincubation time-dependent. The obtained dependence of the enzyme activity on preincubation time shows the preincubation time-dependent inhibition of Na, K-ATPase in the presence of 1 mM platinum analogs. The extent of inhibition after 3 h preincubation was 83, 44, and 29 % ($p < 0.05$) for cisplatin, nedaplatin, and carboplatin, respectively [84].

The inhibitory effect of Na, K-ATPase was rated as cisplatin > nedaplatin > carboplatin, which is in accordance with the rating in decreasing viable HRPTE cells. The observed coincidence indicated that the platinum compounds induced nephrotoxicity could be resulted from the inhibition of kidney Na, K-ATPase activity. This explanation is confirmed by the reported clinic studies about the reduced nephrotoxicity of carboplatin and nedaplatin, second-generation platinum anticancer drugs having equivalent or superior antitumor activity related to cisplatin [85, 86]. The concentrations to decrease the viable cells and to inhibit the Na, K-ATPase activity were different, which may be explained by the difference in the time necessary for the experiments. Three days were necessary to test cell viability, and the change in drugs from an inactive to an active form continued during those 3 days [87]. On the other hand, several hours were necessary to analyze the effects on the ATPase activity, and only a small part of the drugs changed during this period. Preincubation

time-dependent inhibition also might have been caused by the slow change in the drugs from the inactive to the active form [84]. Additionally, the more detailed influence of cisplatin on purified renal Na, K-ATPase activity was tested. The enzyme reaction was done in the presence of various cisplatin concentrations and for various preincubation periods with cisplatin, and then the remaining activity (a percentage of the enzyme activity related to control activity obtained without inhibitor) was measured. The inhibition was evidently both concentration- and preincubation time-dependent [84].

Sakakibara et al. studied the effect of cisplatin on partially purified Na, K-ATPase from Ca9-22 cells derived from a human squamous cell carcinoma of the gingiva [88]. The authors started their study from the assumption that Na, K-ATPase is involved in the transport of cisplatin into cells and acts as a modulator of 5-fluorouracil in combination therapy of cisplatin and 5-fluorouracil. Additionally, Na, K-ATPase is expected to have effects on both anticancer therapy and nephrotoxicity induced by cisplatin treatment. As in previously described study, the obtained cisplatin induced inhibition of Na, K-ATPase activity depended on both, the drug concentration and preincubation time of the enzyme with cisplatin. The influence of cisplatin on the partial reactions of the enzyme, Na^+-dependent ATP hydrolysis and K^+-dependent *p*-nitrophenylphosphate hydrolysis activities was also tested, to determine which step in the reaction sequence of Na, K-ATPase was inhibited. Cisplatin inhibited both activities depending on its concentration and the preincubation time, whereas the Na^+-dependent ATP hydrolysis activity was inhibited even at lower concentrations. Formation of a phosphointermediate of Na, K-ATPase was also inhibited by cisplatin, depending on the concentration and preincubation time. These results suggested that the active form of cisplatin inhibits the Na, K-ATPase activity by inhibiting the formation of a phosphointermediate of the enzyme [88].

Uozumi and Litterst studied the effect of cisplatin on renal ATPase activity in in vitro and in vivo conditions to investigate the correlation between nephrotoxicity and the inhibition of ATPase activity induced by cisplatin [89]. In the first set of in vitro experiments, commercially available purified Na, K-ATPase from dog kidney was preincubated during 0–240 min with cisplatin at concentrations of 50–800 μM before the determination of the enzyme activity. The obtained inhibition of the enzyme activity was time- and concentration-dependent. In the experiments performed without preincubation of the enzyme with inhibitor, high concentration of cisplatin of 280 μM was necessary to achieve IC_{50} value (platinum concentration inducing 50 % the enzyme inhibition related to control activity in the absence of inhibitor). In the case of a lower platinum concentration (200 μM), 50 % inhibition of Na, K-ATPase activity was reached at a long period of preincubation (160 min). Another set of in vitro experiments was carried out using kidney homogenate from female Sprague-Dawley rats instead of purified Na, K-ATPase. Na, K-ATPase in rat kidney homogenate was less sensitive toward cisplatin, compared with the purified enzyme. Actually, the activity of the enzyme present in kidney homogenate was inhibited by 50 % after 110 min preincubation with 800 μM cisplatin or 160 min preincubation with 400 μM cisplatin. In in vivo experiments, female Sprague-Dawley rats were treated with 5, 7, or 10 mg/kg of cisplatin. ATPase activity and

platinum concentration in kidney homogenate were evaluated 1 h, 6 h, 1 day, 3 days, and 5 days after cisplatin injection. Normal ATPase activity was preserved after 3 days of the treatment at all administered doses. The highest concentration of platinum determined in kidney tissue was 19.3 μg/g tissue. However, this platinum concentration was not sufficient to significantly inhibit Na, K-ATPase activity determined under the described in vitro conditions. On the basis of this fact, the authors conclude that it seems unlikely that the inhibition of ATPase activity is the cause of nephrotoxicity, although cisplatin can affect ATPase activity [89].

Oppositely, some authors are claiming that cisplatin, however, is an ineffective inhibitor of Na, K-ATPase either in vitro or in vivo, requiring high concentrations to cause an affect if any [75, 77, 90].

3.1.3 Recovery of Cisplatin-Induced Na, K-ATPase Inhibition

Some earlier studies indicated that some drugs containing sulfhydryl reagent can suppress the nephrotoxicity of cisplatin without comprising its antitumor activity [91, 92]. For this reason, Kitada et al. [84] tested the effects of thiol (–SH) containing compounds: 2-mercaptoethanol (2-ME), a reduced form of glutathione (GSH), an oxidized form of glutathione (GSSG), cysteine (Cys), sodium thiosulfate (STS), sodium sulfate, and fosfomycin to find out whether cisplatin-inhibited Na, K-ATPase activity could be recovered by them. Preliminary experiments demonstrated that the tested compounds did not inhibit Na, K-ATPase activity at the concentrations used in the recovery experiments. At first, the enzyme was preincubated with 0.5 mM cisplatin for 120 min, when about 70–80 % of the activity was lost. Then, the solutions containing the tested reagents at the final concentrations were added, and preincubation was continued. The Na, K-ATPase activity was measured at 180 and 240 min after the start of incubation. The activity without any reagent (only cisplatin was present) was continued to decrease until 240 min [84].

The addition of 2-ME gradually recovered cisplatin-inhibited Na, K-ATPase activity, depending on both its concentration and incubation time. Cys, GSH, and STS also recovered the activity, but their extents of recovery were very small compared to that of 2-ME. On the other hand, the recovery of cisplatin-inhibited activity was not observed by the addition of GSSG, sodium sulfate, or fosfomycin, which do not contain thiol groups [84].

Sakakibara et al. investigated possibility of 2-ME to recover Na, K-ATPase activity inhibited by cisplatin [88]. In this purpose, Na, K-ATPase was partially purified from Ca9-22 cells derived from a human squamous cell carcinoma of the gingiva. In recovery experiments, previously inhibited Na, K-ATPase by cisplatin was treated with an appropriate 2-ME concentration. In prevention experiments, cisplatin and 2-ME were added in a ratio before the enzyme reaction. The obtained results demonstrated that 500 μM cisplatin and eightfold higher concentration of 2-ME (4 mM) prevented inactivation of Na, K-ATPase by cisplatin. Furthermore, the Na, K-ATPase activity inhibited by pretreatment with cisplatin was also recovered almost completely by 2-ME addition.

The described ability of the thiol compounds to effectively recover and prevent the inactivated Na, K-ATPase activity suggests that they could be promising candidates to decrease nephrotoxicity during anticancer therapy with platinum containing drugs.

3.2 Gold Complexes

The interests for the application of gold (Au) in the field of medicinal inorganic chemistry as a potential anticancer drug have dramatically increased because of very promising results of its complexes in cancer treatment [93–97]. Great number of gold(III) complexes were synthesized with the aim to overcome side effects of the platinum(II) anticancer drugs, such as gastrointestinal and hematological toxicity, or drug-resistance phenomena [98, 99]. Unlike platinum drugs, it was found that proteins, rather than DNA, are the main target for the biological actions of gold complexes [100]. Thus it was proposed that the molecular basis for the biological action of gold(III) complexes could be the modification of surface protein residues and associate loss of protein function [43, 101]. A number of gold(III) compounds showing significant antiproliferative effects in vitro against a number of cancer cells [102]. It was shown that some bipyridyl gold(III) compounds ($[Au(bipy)(OH)_2][PF_6]$, (bipy = 2,2′-bipyridine) (Aubipy)) exhibit excellent cytotoxic properties towards various human cancer cell lines: ovarian cell lines A2780 and SKOV3, as well as CCRF-CEM leukemic cell line interact with sodium pump [103].

3.2.1 Structure and Chemistry of Gold Complexes

Gold is isoelectronic with platinum(II) and forms tetra-coordinate complexes with the same square-planar geometries as cisplatin [104]. Great number of gold(III) complexes were synthesized in order to reproduce the main features of cisplatin, and were later found to have completely different molecular targets. There is an evidence that borate capped Au nanoparticles interacts with protein molecules due to the chemical bonding between nanoparticles surface and positive charged cysteine moiety [105]. Highly reactive gold(III) complexes have large positive redox potentials but show a relatively poor stability under physiological conditions [99, 106]. Multidentate ligands with nitrogen atoms as donors, such as polyamines, cyclams, terpyridines, phenanthrolines, and bdma (*N*-benzyl-*N*,*N*-dimethylamine), were the most effective in stabilizing the gold(III) center and improving stability of gold(III) complexes [95–97, 101]. In particular, square planar gold(III) complexes that contain functionalized bipyridine ligands of general formula $[Au(N,N)Cl_2][PF_6]$ (where *N*,*N* = 2,2′-bipyridine, 4,4′-dimethyl-2,2′-bipyridine, 4,4′-dimethoxy-2,2′-bipyridine, and 4,4′-diamino-2,2′-bipyridine) (Fig. 24.2) [101, 107] are stable under physiological conditions. These complexes displayed appreciable stability in solution [108]. As an example, $[Au(bipy^c\text{-}H)(OH)][PF_6]$ (where

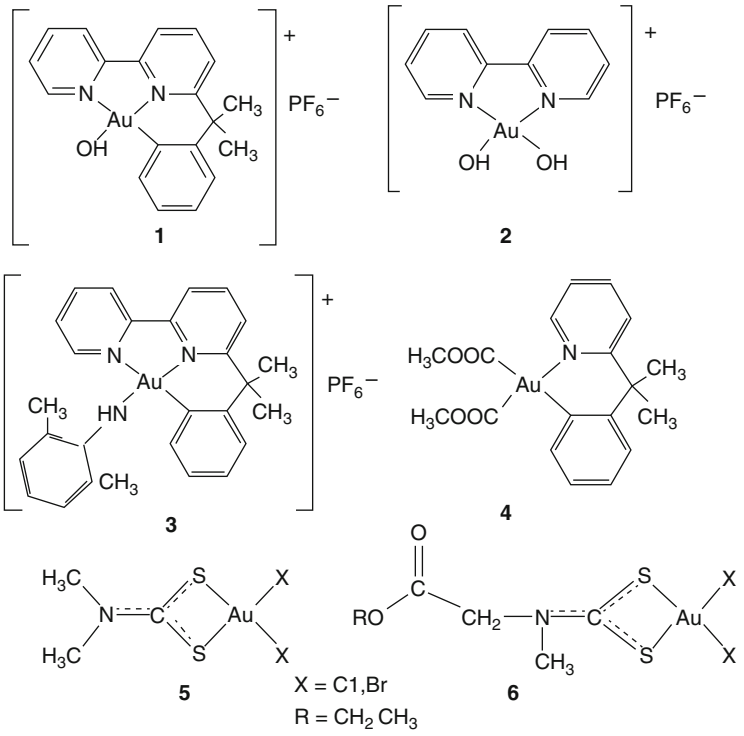

Fig. 24.2 Structures of Au(III) complexes. Schematic drawing of [Au(bipy^{c}-H)(OH)][PF_6] (**1**), [Au(bipy)$(OH)_2$][PF_6] (**2**), [Au(bipy^{dmb}-H)(2.6-xylidine-H)][PF_6] (**3**), [Au(py^{dmb}-H)$(AcO)_2$] (**4**) (where bipy^{dmb} = 6-(1,1-dimethylbenzyl)-2,2′-bipyridine; py^{dmb} = 2-(1,1-dimethylbenzyl)-pyridine) and of the gold(III) dithiocarbamate complexes containing *N*,*N*-dimethyldithiocarbamate (**5**) and ethylsarcosinedithiocarbamate (**6**) ligands

bipy^{c} = 6-(1,1-methylbenzyl)-2,20-bipyridine) (Fig. 24.2, **1**) and [Au(bipy)$(OH)_2$] [PF_6] (bipy = bipyridine) (Fig. 24.2, **2**) exerted only small deviations from ideal square planar geometry characteristic for the classical bipyridyl complexes whereas such deviations are quite large in the case of cyclometallated derivatives. Both mentioned compounds exhibited sufficient solubility in watery solutions. In [Au(bipy) $(OH)_2$][PF_6], the Au(III) center is coordinated by two nitrogens of the bidentate bipyridyl ligand and by two hydroxide groups. On the contrary, [Au(bipy^{c}-H)(OH)] [PF_6] is an organogold(III) complex in which donors to the Au(III) center are two nitrogens from the bipyridyl moiety, the C2 carbon of the phenyl group, and a hydroxide group. Only small deviations from ideal square planar geometry were seen in the classical bipyridyl complexes whereas such deviations are quite large in the case of cyclometallated derivatives [101].

Since Au(III) complexes manifest the strong affinity towards S-donor ligands such as glutathione and L-cysteine, and a limited reactivity against nucleosides and

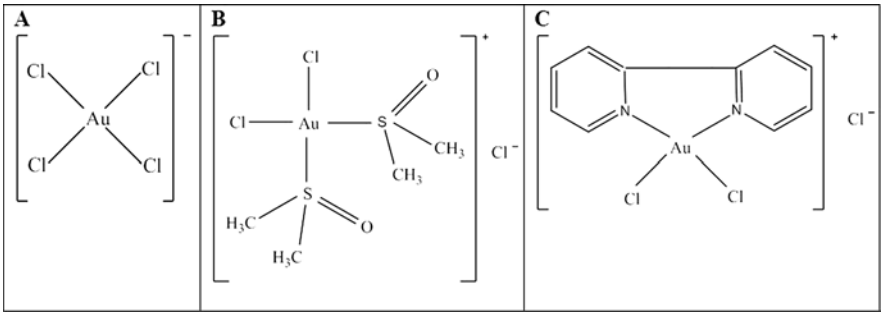

Fig. 24.3 Structural formulas of Au(III) compounds. (**a**) $[AuCl_4]^-$, (**b**) $[Au(DMSO)_2Cl_2]Cl$, and (**c**) $[Au(bipy)Cl_2]Cl$

their bases, exposed cysteine residues of proteins might be their proper targets. They can also cleave the disulfide bond of cystine [109, 110], and oxidize methionine [111–113] and glycine [114], suggesting that amino terminus of peptides and proteins could be deaminated by Au(III).

3.2.2 Inhibition of Na, K-ATPase Activity with Gold(III) Complexes

In vitro inhibition studies of complexes $[Au(bipy)(OH)_2][PF_6]$, $[Au(py^{dmb}\text{-}H)(CH_3COO)_2]$, $[Au(bipy^{dmb}\text{-}H)(OH)][PF_6]$, $[AuCl_4]^-$, $[Au(DMSO)_2Cl_2]Cl$, and $[Au(bipy)Cl_2]Cl$ (Fig. 24.2: **2**, **4**, **1**, respectively and Fig. 24.3) performed with Na, K-ATPase purified from human red blood cells and from porcine cerebral cortex showed that Au(III) complexes dose-dependently inhibit Na, K-ATPase activity (Fig. 24.4) [115, 116]. Inhibitory parameters obtained using Hill analysis of inhibition curves (Fig. 24.4, insets) demonstrate variable potencies of the Au(III) complexes (Table 24.1) [115, 116]. In all cases, the high Hill coefficient ($n>1$) is observed. This suggests a strong positive cooperation of the inhibitor binding to the Na, K-ATPase. The kinetic studies results in curves which obeyed typical Michaelis–Menten kinetics, presented in Fig. 24.5a. Obtained kinetic parameters (V_{max} and K_m) were derived using Lineweaver–Burk transformation of the experimental data (Fig. 24.5b) [116].

Such analysis transformed the hyperbolic Michaelis–Menten function into linear Lineweaver–Burk function with the possibility for much easier determining of V_{max} and K_m values. The obtained results are summarized in Table 24.2 [115, 116]. These data suggest that V_{max} decreased in the presence of the inhibitors, while K_m remained constant, comparing to the control. This kind of enzyme behavior in the presence of Au(III) complexes indicated the noncompetitive reversible type of Na, K-ATPase inhibition. This mode of interaction suggests that the inhibitor and the substrate bind randomly and independently of each other at different enzyme sites. Na, K-ATPase affinity for binding with $[Au(bipy)(OH)_2][PF_6]$, $[Au(py^{dmb}\text{-}H)(CH_3COO)_2]$, and $[Au(bipy^{dmb}\text{-}H)(OH)][PF_6]$ is determined, using the secondary

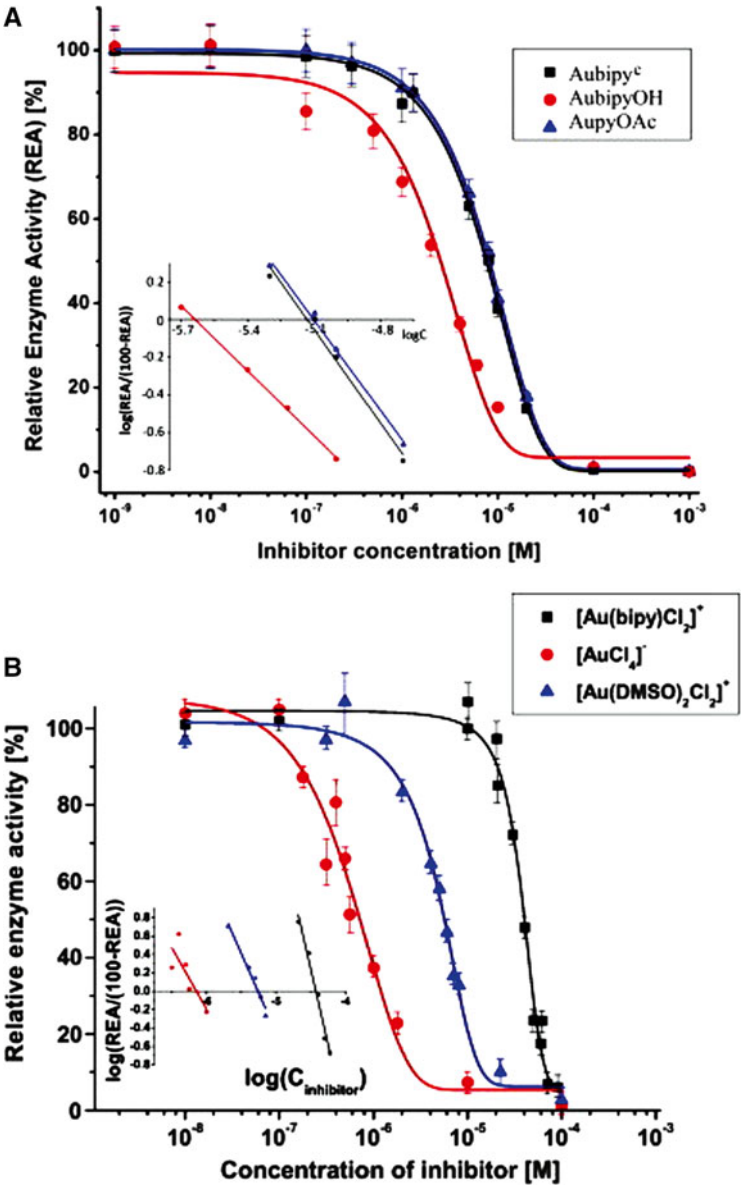

Fig. 24.4 Inhibition of Na, K-ATPase from porcine cerebral cortex activity with Au(III) complexes. (**a**) $[Au(bipy)(OH)_2][PF_6]$, $[Au(py^{dmb}\text{-}H)(CH_3COO)_2]$, $[Au(bipy^{dmb}\text{-}H)(OH)][PF_6]$; and (**b**) $[AuCl_4]^-$, $[Au(DMSO)_2Cl_2]Cl$, and $[Au(bipy)Cl_2]Cl$. The dependencies of relative enzyme activity (REA), expressed as a percentage of the control value (Na, K-ATPase activity obtained without inhibitor on the concentration of Au(III) complexes). *Inset*: Hill analysis of inhibition curves. The values given are the mean of at least three experiments ± S.E.M. [115, 116], respectively

Table 24.1 IC_{50} values and Hill coefficients for the inhibition of Na, K-ATPase, isolated from human blood cells and porcine cerebral cortex (PCC), induced by Au(III) complexes

	$IC_{50} \times 10^6$ (M)		*n*
Complex	Human blood cells	PCC	PCC
AubipyOH [116]	2.5±0.5	3.5±0.1	1.1±0.2
AupyOAc [116]	6.9±0.5	7.6±0.1	1.6±0.4
Aubipy^c [116]	6.4±0.5	7.3±0.1	1.6±0.3
$[AuCl_4]^-$ [115]	Not done	7.24±0.02	5.26±0.01
$[Au(DMSO)_2Cl_2]^+$ [115]	Not done	5.49±0.02	2.25±0.01
$[Au(bipy)Cl_2]_+$ [115]	Not done	3.84±0.02	4.52±0.01

replot of the Lineweaver–Burk graph (Fig. 24.5c), and presented as the inhibitor constant (K_i), i.e., dissociation constant of the enzyme–inhibitor complex in Table 24.2 [116].

3.2.3 Prevention and Recovery of Gold(III) Complexes Induced Na, K-ATPase Inhibition

The inhibitory effect of Au(III) complexes can be prevented and recovered by the addition of –SH donors, L-cysteine and GSH, the biomolecules usually present in physiological liquids. The dose-dependent prevention of enzyme inhibition with $[Au(bipy)(OH)_2][PF_6]$, $[Au(py^{dmb}\text{-}H)(CH_3COO)_2]$, and $[Au(bipy^{dmb}\text{-}H)(OH)][PF_6]$ in the presence of both –SH donors is presented in Fig. 24.6 [115, 116]. For each inhibitor the concentrations were equal to their IC_{20}, IC_{50}, and IC_{100} values, i.e., the concentrations of inhibitor which induce 20 %, 50 % and 100 % enzyme inhibition, respectively. The complete prevention of inhibition in the presence of –SH donors was achieved for inhibitor concentration corresponding to the IC_{20} values of each of the three investigated Au(III) complexes [116]. The recovery effect of –SH donors on the inhibition of Na, K-ATPase induced by the Au(III) complexes was also shown. In the case of GSH, even 1×10^{-4} M showed the significant recovery of the inhibited enzyme activity (about 40 %) in the presence of complexes which induced the complete inhibition [116]. The similar results were obtained for prevention and reactivation of Na, K-ATPase activity with complexes $[AuCl_4]^-$, $[Au(DMSO)_2Cl_2]$ Cl and $[Au(bipy)Cl_2]Cl$ [115].

The likely reason for prevention of inhibition is the fast formation of the inactive $[AuCl(L\text{-}Cys)(DMSO)_2]^+$complex analogous to the $[PtCl(L\text{-}Cys)(DMSO)_2]$ of Bugarčić et al. [117], prior to the formation of the [Au(III)complex (enzyme)]$^+$ complex. Further reduction of Au(III), resulting in the formation of Au(I) and Au colloids in the slower second reaction step, is likely in the prevention and the recovery experiments when GSH and L-Cys were added to the media. This process is common for –SH containing ligands, and the fast replacement of the ligand in coordination sphere of Au(III) complexes leads usually to reduction [118]. This reduction leads also to the formation of colloidal Au particles which are even able to

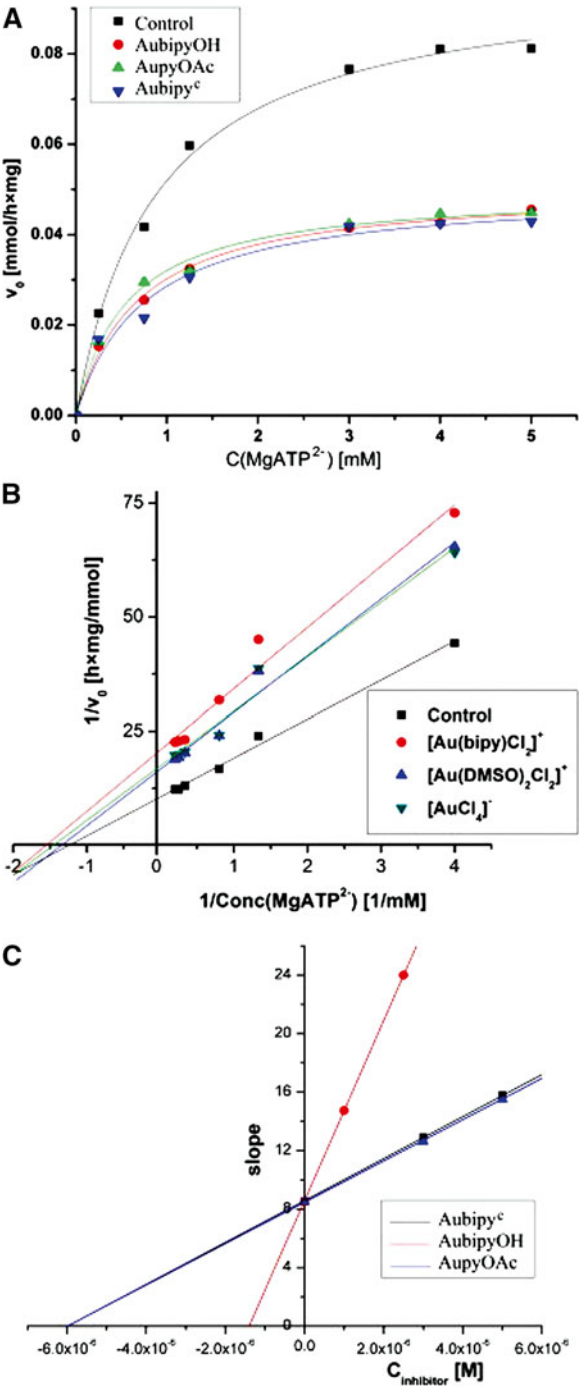

Fig. 24.5 Kinetic analysis of Na, K-ATPase porcine cerebral cortex showed that interaction with [Au(bipy)$(OH)_2$][PF_6], [Au(py^{dmb}-H)$(CH_3COO)_2$], and [Au($bipy^{dmb}$-H)(OH)][PF_6]. (**a**) Initial reaction rate (v_0) vs. $MgATP^{2-}$ concentration in the absence (control) and presence of 2.5×10^{-6} M AubipyOH, 5×10^{-6} M AupyOAc and 5×10^{-6} M Aubipyc. The values given are the mean of at least three experiments; (**b**) Lineweaver–Burk linearization of the obtained results. (**c**) Secondary replots of Lineweaver–Burk graphs: slope vs. the inhibitor concentrations for Au(III) complexes [116]

Table 24.2 The values of kinetic parameters and inhibitory constants for Au(III) complexes determined from Lineweaver–Burk linearization of Michaelis–Menten hyperbola

Sample	K_m (mM)	V_{max} (mmol/h/mg)	K_i (M)
Control [116]	0.69 ± 0.07	0.094 ± 0.005	–
AubipyOH [116]	0.68 ± 0.10	0.052 ± 0.005	1.42×10^{-6}
AupyOAc [116]	0.68 ± 0.12	0.050 ± 0.006	5.97×10^{-6}
Aubipyc [116]	0.67 ± 0.09	0.051 ± 0.004	6.02×10^{-6}
Control [115]	0.79 ± 0.07	0.094 ± 0.005	–
$[AuCl_4]^-$ [115]	0.63 ± 0.10	0.047 ± 0.004	–
$[Au(DMSO)_2Cl_2]^+$ [115]	0.73 ± 0.11	0.059 ± 0.005	–
$[Au(bipy)Cl_2]^+$ [115]	0.67 ± 0.11	0.057 ± 0.009	–

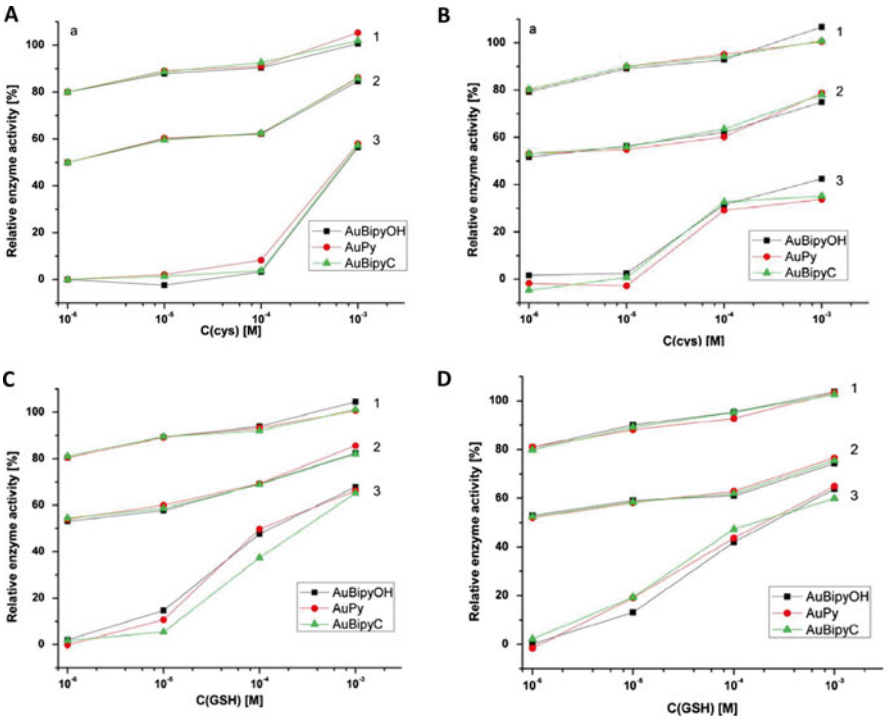

Fig. 24.6 Prevention and recovery of Au(III) complexes inhibition of Na, K-ATPase with –SH donors. Prevention of Na, K-ATPase inhibition (**a**) and recovery (**b**) by L-cysteine; and prevention (**c**) and recovery (**d**) by GSH, in the presence of gold(III) complexes at concentrations which induce 20 % (*line group 1*), 50 % (*line group 2*) and 100 % (*line group 3*) enzyme inhibition. These characteristic concentrations were: $IC_{20} = 6 \times 10^{-6}$ M, $IC_{50} = 2.5 \times 10^{-6}$ M, and $IC_{80} = 6 \times 10^{-7}$ M, for AubipyOH; $IC_{20} = 1.5 \times 10^{-5}$ M, $IC_{50} = 7 \times 10^{-6}$ M, and $IC_{80} = 2 \times 10^{-6}$ M for AupyOAc and $IC_{20} = 1.5 \times 10^{-5}$ M, $IC_{50} = 7 \times 10^{-6}$ M, and $IC_{80} = 2 \times 10^{-6}$ M for Aubipyc [115, 116]

enhance the enzyme activity [105]. The recovery of the activity was achieved when the concentration of thiols was equal or higher than the concentration of the complexes. These results can be explained by enzyme extruding from [Au(III) complex(enzyme)] complex and its substitution with smaller and more reactive –SH donor. A similar effect was earlier demonstrated on Pd(II) complexes [119]. In addition, Au(III) compounds can oxidatively cleave disulfide bonds of β unit, and this may contribute to the lost non-recoverable enzyme activity [109, 110, 120].

For all studied Au(III) complexes the action of gold is more complex than simple interaction with the –SH groups of the enzyme, and the redox reactions with L-Cys residues and disulfide bonds of Na, K-ATPase must be also taken into account. The disulfide bridges in the Na, K-ATPase are required for enzyme functionality. The removal of disulfide bonds from the β subunit due to the redox reactions of gold complexes can lead to the significant functional alterations [120].

3.3 Ruthenium Complexes

Ruthenium (Ru) arene anticancer complexes are being widely studied as potential alternatives to platinum chemotherapeutics especially because resistance to platinum-based drugs represents a major clinical drawback for compounds such as cisplatin and oxaliplatin [121, 122]. Compared to Pt drugs Ru complexes cause less side effects and resistances against the drug are less likely [123, 124].

Ruthenium coordination compound KP1019 indazolium *trans*-[tetrachlorobis (1*H*-indazole)ruthenate (III)] (FFC14A) (Fig. 24.7) is a ruthenium complex with promising anticancer activity [123]. It was one of the two first of its kind transferred into clinical trials. Ruthenium complex KP1019 entered phase I clinical trials in 2003 as an anticancer drug which is among others very active against colon carcinomas

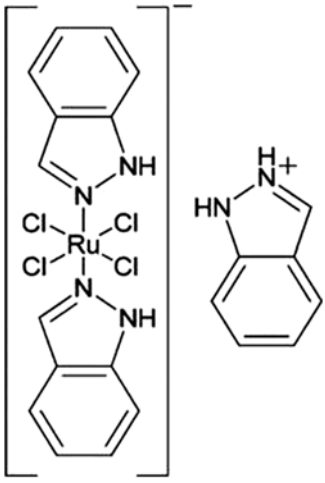

Fig. 24.7 Chemical structure of KP1019

and their metastases [125]. KP1019 more recently showed activity in vitro on colorectal cancer cell lines [126, 127] and in vivo on the chemoresistant MAC15A colon carcinoma—main tumor type effected colorectal human and animal [128]. KP1019 has been shown to act as a cytostatic and cytotoxic drug on colorectal tumor cells in vivo and in vitro [129, 130]. Resistance of tumor cells against this compound during chemotherapy is very low. This feature distinguishes this ruthenium compound from many other anticancer drugs. It was shown that KP1019 acts as a substrate and as an inhibitor of P-glycoproteins including Na, K-ATPase, Ca-ATPase and mitochondrial ATPase. KP1019 dose-dependently inhibited P-glycoproteins activity, with an IC_{50} value of approximately 31 μM. A week intrinsic resistance against KP1019 in highly P-glycoprotein-overexpressing tumors has to be taken in consideration [123].

Iminopyridine complexes of general formula [Ru(η6-*p*-cymene)(*N*,*N-dimethyl-N′*-[(*E*)-pyridine-2-ylmethylidene]benzene-1,4-diamine)X]PF_6 bearing two different halido ligands X = Cl (Complex 1) or X = I (Complex 2) (Fig. 24.8) possess antiproliferative effect [121]. The structural features of these Ru^{II} "piano-stool" complexes allow fine-tuning of their physical and chemical properties and optimization of their biological activity [2, 131–133]. These complexes contain three basic building blocks as shown in Fig. 24.11: an arene ligand (the "seat" of the "stool"), used to control hydrophobicity and to stabilize the oxidation state of the metal center, a monodentate ligand, X, initially included as an activation site, and a bidentate ligand which provides additional stability [134–136].

Antiproliferative effect for the complexes in A2780 ovarian, A549 lung, HCT116 colon, and MCF7 breast carcinoma cells are presented in Table 24.3 [136]. Changes in the monodentate ligand can modify the cellular uptake and accumulation pathways involved in the first stages of drug action. This leads to variations in cellular distribution of the drug and, in turn, to different apoptotic pathways being triggered as a consequence of cellular compartmentalization, hence determining differences in IC_{50} values [137]. Role of Na, K-ATPase in cellular metal accumulation as a facilitated diffusion endocytosis pathway was shown. A2780 human ovarian carcinoma cells were co-incubated with the complexes and various concentrations of ouabain, during 24 h at 37 °C. The amount of Ru taken up by the cells was determined by ICP-MS. Co-administration of complexes 1 and 2 with cardiac glycoside ouabain impaired cellular Ru accumulation. Ruthenium accumulation

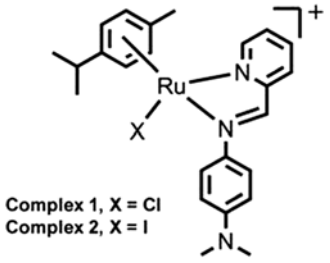

Fig. 24.8 Structure of Ru complexes 1 and 2

Table 24.3 Antiproliferative activity of complexes 1 and 2, in A2780, A549, HCT116, and MCF7 cell lines and cellular accumulation of Ru in A2780 cells after 24 h of the drug exposure at 37 °C*

	IC_{50} (μM)				Cell accumulation
Complexes	A2780	A549	HCT116	MCF7	A2780
1	16.2±0.9	105±0.8	3.4±0.4	12.1±0.3	7.8±0.5
2	3.0±0.2	15.3±0.9	8.6±0.8	4.4±0.3	11.5±0.8

*Cell accumulation experiments did not include recovery time in drug free media. Results are expressed as ng Ru per 10^6 cells, and the concentrations used were equipotent, in all cases $IC_{50}/3$

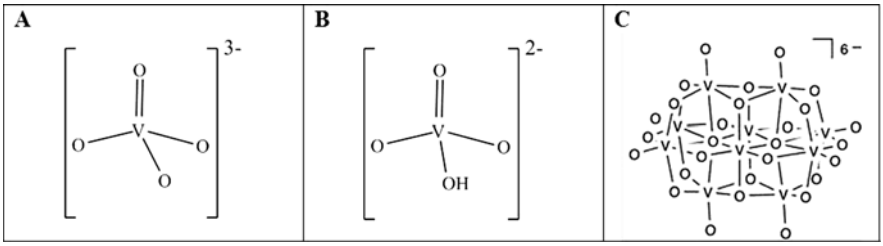

Fig. 24.9 Schematic structures of vanadium complexes. (**a**) Orthovanadate, (**b**) oxovanadium, and (**c**) decavanadate anions. Va, Vb, and Vc represent the three different types of vanadium atoms

from complex 1 is almost halved when co-administered with 200 mM of ouabain. Similarly, for complex 2, Ru accumulation decreases by ca. 40 % when co-administered with the same concentration of ouabain. These results suggest that the cellular uptake of these ruthenium complexes relies at least in part on facilitated diffusion, and in particular is dependent on the membrane potential [121].

3.4 Vanadium Complexes

Vanadium (V), is a metal possessing a well-defined geometry of its inner coordination sphere, regarding to the usual oxidation states of the metal [(III), (IV) and (V)], while its outer sphere (moieties) is not subject to such limitations. There is, thus, a donation of an electron pair from ligands of the inner sphere to the metal, conferring a high degree of covalent character to the resulting bond. Structural information on the oligomeric species in aqueous solution is difficult to obtain [138]. At high pH V(V) (Fig. 24.9a) is the most stable form of vanadium in aqueous solution, whereas at low pH the V(IV) (Fig. 24.9b) species is more favored. From pH 2 to 6, the major V(V) species is the decamer, $[V_{10}O_{28}]^{6-}$ (Fig. 24.9c) and its various protonated forms. The biological effects of aqueous solutions containing mixtures of oxovanadates reflect that some structural analogy to the corresponding phosphate derivatives does exist [138]. Vanadate (V) and oxovanadium (IV) complexes are, both inhibitors of protein phosphotyrosines (PTPs) in many cells, acting with different

mechanisms [139–141]. The inhibitory studies assume that vanadate acts as a phosphate analog and presumably inhibits the enzymes as a transition state analog for the phosphoryl group transfer [142–144].

It is suggested that vanadium complexes might be used in chemotherapy due to their antiproliferative activity, cytostatic/cytotoxic activity (in apoptotic or necrotic way) and antimetastatic activity [145, 146]. It was reported that vanadium complexes prevent animal carcinogens by inhibition of mouse and rat cells proliferation and differentiation [147] and that early protective effect of vanadium in chemically induced rat colon carcinogenesis may be mediated by a reduction of carcinogen-induced DNA damage [148]. It was reported that CoV_{10} in vitro inhibited the proliferation in human SMMC-7721 cells (liver cancer) and human SK-OV-3 cells (ovary cancer), and also the tumor growth was inhibited with the administration of CoV_{10} in vivo, which showed higher antitumoral activity both in vitro and in vivo. Although the mechanism of antitumoral activity of CoV_{10} had not been clarified yet the data suggest that CoV_{10} specifically induced cell apoptosis [149]. Antitumor activity of two ammonium decavanadate compounds $(H_{2tmen})_3V_{10}O_{28} \times 6H_2O$ and $(H_{2en})_3V_{10}O_{28} \times 2H_2O$ against human A549 (lung carcinoma) and mouse P388 (leukemia) cells lines was shown using MTT-based assay. Both compounds inhibit proliferation of these two kinds of tumor cells [150]. It was shown that intracellular vanadium blocks several events essential for terminal differentiation, one of which is a decrease in Na, K pump activity [12].

3.4.1 Inhibition of Na, K-ATPase Activity with Vanadate Complexes

Vanadate is a specific and potent Na, K-ATPase inhibitor in vitro. The dog kidney Na, K-ATPase is inhibited 50 % by 40 nM Na_3VO_4 under optimal conditions (28 mM Mg^{2+}) and the inhibition is 100 % reversible by millimolar concentrations of norepinephrine [151]. Vanadate interactions with the Na, K-ATPase were characterized. Vanadate binds to one high affinity site ($K_1 = 4$ nM) and one low affinity site ($K_2 = 0.5$ μM) per enzyme molecule (i.e., per ouabain binding site) under optimal conditions. Saturation of the high affinity site is sufficient to cause greater than 95 % inhibition of Na, K-ATPase activity. Competitive inhibition experiments indicate that the high affinity vanadate site is identical with a low affinity ATP site ($K_m = 3$ mM) on the enzyme. The low affinity vanadate site is responsible for inhibition of Na^+-stimulated adenosine triphosphatase activity and is probably the high affinity ATP site associated with sodium-dependent protein phosphorylation [152]. Using fluorescein-labeled Na, K-ATPase Karlish et al. showed that vanadate inhibits Na, K-ATPase by blocking a conformational change of the unphosphorylated form [153].

Cytoplasmic reduction of vanadate by glutathione has been recognized since 1980 [154]. During vanadate uptake and reduction by red cells, Na, K-ATPase was inhibited 60 %. Cytoplasmic reduction of vanadium (V) to vanadium (IV) can explain why the Na, K-ATPase in vivo seemed resistant to inhibition by vanadate [154]. Vanadium (IV) is a less potent inhibitor of the Na, K ATPase and when vanadium (V)

is reduced to vanadium (IV), it cannot inhibit this enzyme at attainable in vivo concentrations of vanadium (IV) [154, 155]. Tetravalent vanadium was a relatively potent inhibitor of membrane bound kidney Na, K-ATPase producing nearly complete inhibition at a concentration of less than 5 μM in some highly purified preparations of the enzyme. It was not possible to define an equilibrium constant for the inhibition since the degree of inhibition varied greatly between different enzyme preparations and vanadyl ion stability over time [156].

It was reported that decavanadate (V_{10}) is the major protein-bound species of vanadium and have a stronger effect on various enzymes, when compared to other vanadate oligomers [157]. Inhibitions of several adenosine triphosphatases, such as P-type ATPases have been shown [158]. The effect of V_{10} on synaptic plasma membrane (SPM) and commercial porcine cerebral cortex Na, K-ATPase activity was investigated by in vitro exposure to the enzymes [159]. The results show that V_{10} induce inhibition of enzymatic activity in a concentration-dependent manner in both cases (Fig. 24.10a). The IC_{50} values of the investigated compound for both samples

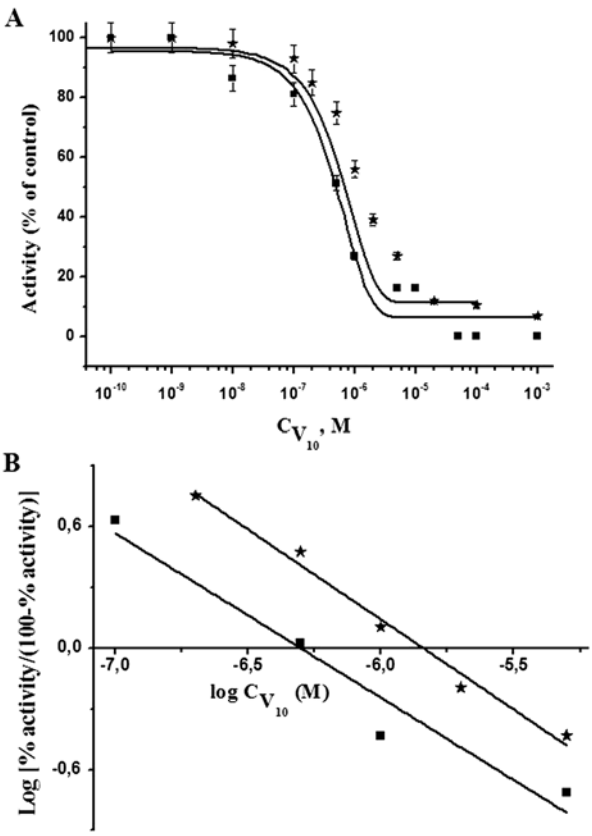

Fig. 24.10 Inhibition of Na, K-ATPase with V_{10}. (**a**) The concentration-dependent inhibition, and (**b**) Hill analysis of SPM Na, K-ATPase (*square*) and commercial porcine cerebral cortex Na, K-ATPase (*asterisk*) by V_{10} [159]

Table 24.4 The inhibition parameters of the SPM and commercial Na, K-ATPase inhibition by V_{10} obtained by fitting the experimental points using sigmoidal function and Hill analysis. Kinetic analysis of commercial porcine cerebral cortex Na, K-ATPase activity in the absence (control) and presence of V_{10} (1.30×10^{-6} M) [159]

	Sigmoidal fitting	Hill analysis	
	IC_{50} (M)	n	IC_{50} (M)
SPM Na, K-ATPase	$(4.74 \pm 1.15) \times 10^{-7}$	0.81 ± 0.13	4.79×10^{-7}
Commercial Na, K^+-ATPase	$(1.30 \pm 0.10) \times 10^{-6}$	0.88 ± 0.03	1.71×10^{-6}
Michaelis–Menten kinetics			
	Control	V_{10}	
K_m (mM)	1.68 ± 0.14[a] 1.76 ± 0.02[b]	2.38 ± 0.20[a] 2.69 ± 0.09[b]	
V_{max} (μmol P_i/h/mg)	59.93 ± 2.02[a] 61.13 ± 1.75[b]	39.65 ± 1.72[a] 41.87 ± 1.35[b]	

[a]Values obtained by Lineweaver–Burk transformation
[b]Values obtained by nonlinear regression fitting-hyperbola function; n—Hill's coefficient; K_m—Michaelis constant; V_{max}—maximum enzymatic velocity; P_i—released inorganic phosphate

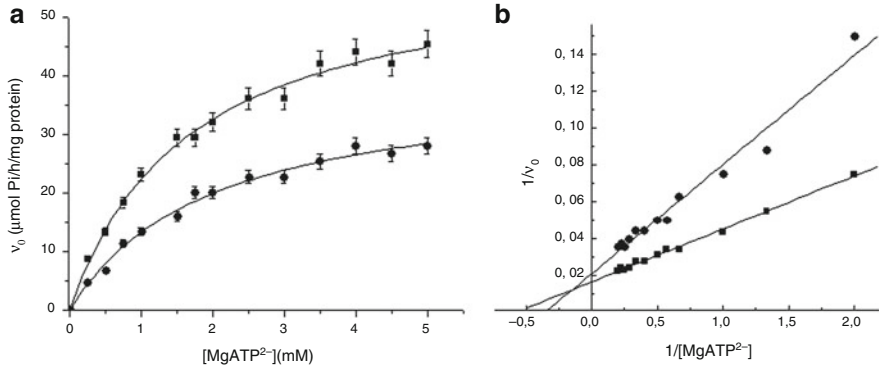

Fig. 24.11 Kinetics of Na, K-ATPase inhibition with V_{10}. (**a**) Na, K-ATPase activity dependence on ($MgATP^{2-}$) in the absence (*square*) and presence of 1×10^{-6} M (*circle*) V_{10}, and (**b**) Lineweaver–Burk transformation of the data. The values given are the mean of at least three experiments ± SEM, conducted in duplicate [159]

were determined by sigmoidal fitting the experimental results (Fig. 24.10b) as well as by Hill analysis and are summarized in Table 24.4 [159]. The nature of commercial porcine cerebral cortex Na, K-ATPase inhibition by V_{10} was characterized as mixed type of inhibition. V_{10} induced significant decrease of enzyme maximal velocity (V_{max}), as well as decrease of its apparent affinity for the substrate (ATP) (increased K_m value) (Table 24.4 and Fig. 24.11) [159].

3.5 *Palladium Complexes*

Palladium (Pd) complexes are closely related to their platinum analogs, due to their structural similarities and significant overlap of coordination chemistry for the two metals. Due to the steric effect that results from the bulk on the donor atoms, these ligands could minimize any possible *cis–trans* isomerism and ensure the direct separation of the desired *trans*-Pd isomers [160]. The importance of the *trans*-geometry around the palladium center has been attributed to the comparatively higher cytotoxicity values as those for *cis*-isomers [161]. Palladium complexes exhibit promising activity against different human tumor cell lines: HCT-15 (colon cancer), MCF-7 and MDA-MB-231 [162] (breast cancer), K-562 CML (leukemia), U-251 Glio (central nervous system), PC 3 and DU145 [163] (prostate cancer) cell lines [164], G361 (malignant melanoma) [165], HeLa (cervical cancer), and HCT 116 (colon adenocarcinoma) [166], $A2780^{cisR}$ (ovarian cancer) [167].

3.5.1 Mechanism of $[Pd(dien)Cl]^+$ Interaction with Na, K-ATPase

It was confirmed that Pd(II) complexes interact with Na, K-ATPase preferably through bonding to the enzymatic –SH groups. The mechanism of Pd(II) complex—(μ^3-diethylentriamino)-chloro-palladium(II)-chloride ([PdCl(dien)]Cl) with Na, K-ATPase from porcine cerebral cortex was investigated in vitro [4]. The complex formation between $[PdCl(dien)]^+$ and –SH containing ligands L-cysteine or GSH was monitored spectrophotometrically in the standard reaction medium for proper functioning of the enzyme, at pH 7.4 (Fig. 24.12) [4]. The change of the absorption spectrum of $[PdCl(dien)]^+$ in the presence of the enzyme is analogous to its changes in presence of –SH containing ligands, cysteine or GSH. Such similarity suggested that the complex ion interacts with sulfhydryl groups of Na, K-ATPase. The enzyme–inhibitor interaction was verified by ^{1}H NMR spectra indicating that the intensity of the peak of the free enzyme (δ 3.823 ppm) decreased with time while intensity of the new peak that can be ascribed to the product of this interaction (δ 3.044 ppm) increased (Fig. 24.13) [4]. The time course of the reaction between $[PdCl(dien)]^+$ and the enzyme is shown in Fig. 24.14 [4]. The consequence of this interaction was the concentration-dependent inhibition of the enzyme activity.

3.5.2 Inhibition of Na, K-ATPase Activity with Palladium Complexes

In vitro studies showed that Pd(II) complexes $[PdCl_4]^{2-}$, $[PdCl(dien)]^+$ and $[PdCl(Me_4dien)]^+$ inhibit dose-dependently Na, K-ATPase from porcine cerebral cortex [119]. Sigmoid-shaped inhibition curves were obtained in all cases (Fig. 24.15). IC_{50} values were determined by Hill analysis of the experimental curves (Fig. 24.15, inset) and presented in Table 24.5 together with Hill coefficients [119]. Results indicated that inhibition power of complexes is depended on the

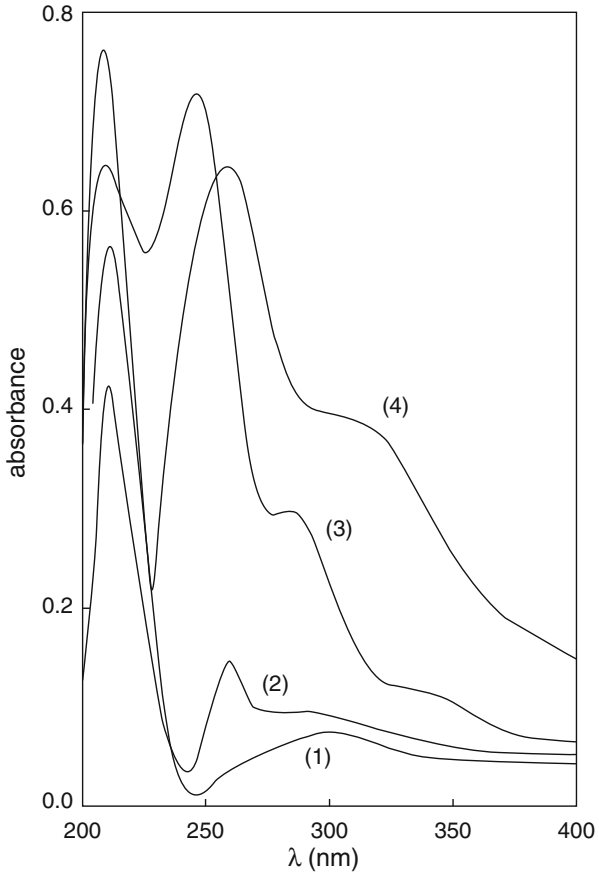

Fig. 24.12 Interaction of $[PdCl(dien)]^+$ and –SH containing ligands. Absorption spectra of 0.1 mM $[PdCl(dien)]^+$ (*curve 1*) in the presence of 2 mg/ml Na, K-ATPase (*curve 2*), 5 mM L-cysteine (*curve 3*), and 5 mM GSH (*curve 4*) at pH 7.4 [4]

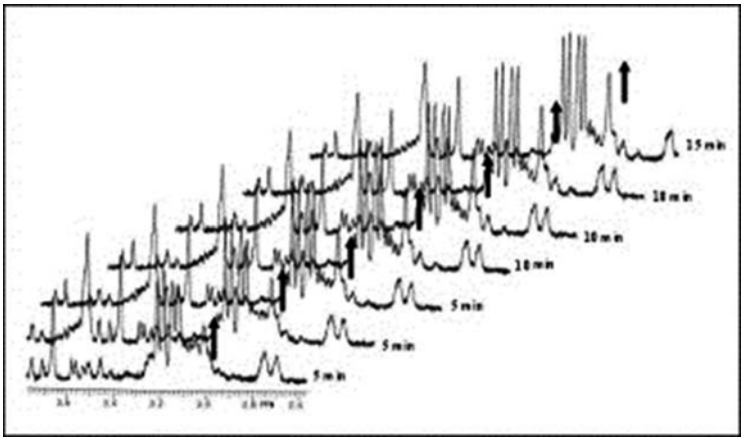

Fig. 24.13 ^1H NMR spectra of the reaction of $[PdCl(dien)]^+$ (5 mM) with Na, K-ATPase as a function of time [4]

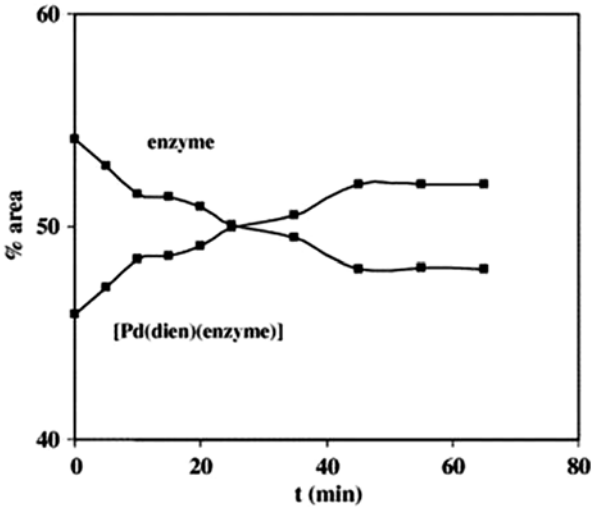

Fig. 24.14 Product formation during the reaction of $[PdCl(dien)]^+$ with Na, K-ATPase. Calculations were performed by relative integration (estimated error 5 %) of suitable proton signals of reaction product and starting materials during reaction [4]

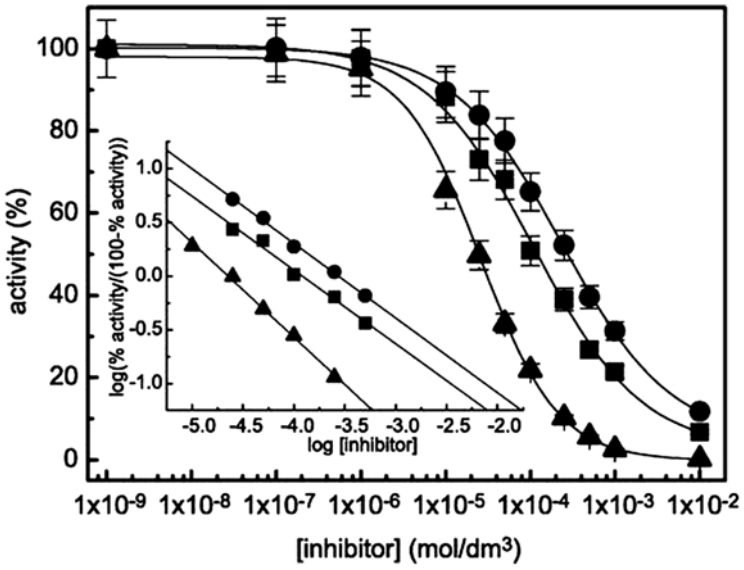

Fig. 24.15 Inhibition of Na, K-ATPase activity by Pd(II) complexes. Dependence of Na, K-ATPase activity on $[PdCl_4]^{2-}$ (*up triangles*), $[PdCl(dien)]^+$ (*squares*) and $[PdCl(Me_4dien)]^+$ (*circles*) concentration. The values given are the mean of at least three experiments ± S.E.M. The Hill plots constructed from the data obtained by inhibition experiments is shown in the *inset*. Regression lines were calculated by means of the least square method [119]

Table 24.5 IC_{50} values and Hill coefficients (n) for Pd(II) complexes-induced inhibition of Na, K-ATPase in the absence and the presence of 1 mM L-cysteine and GSH [119]

Complex	Thiol	IC_{50} (M)	n
$[PdCl_4]^{2-}$	/	[a]$2.25 \pm 0.21 \times 10^{-5}$	[a]0.88 ± 0.03
	L-cys	$2.5 \pm 0.6 \times 10^{-4}$	
	GSH	$3.1 \pm 0.3 \times 10^{-4}$	
$[PdCl(dien)]^+$	/	[a]$1.21 \pm 0.13 \times 10^{-4}$	[a]0.70 ± 0.05
	L-cys	$7.4 \pm 0.8 \times 10^{-4}$	
	GSH	$8.0 \pm 0.5 \times 10^{-4}$	
$[PdCl(Me_4dien)]^+$	/	[a]$2.36 \pm 0.30 \times 10^{-4}$	[a]0.69 ± 0.02
	L-cys	$8.2 \pm 0.9 \times 10^{-4}$	
	GSH	$1.4 \pm 0.2 \times 10^{-3}$	

[a]Data obtained by Hill analysis

structure of ligands. Complexes with massive tridentate ligands and high steric hindrance, such as $[PdCl(dien)]^+$ and $[PdCl(Me_4dien)]^+$ showed lower potency to inhibit Na, K-ATPase activity. Hill coefficients for the investigated Pd(II) complexes were below 1 ($n < 1$) and indicated negative cooperation for binding of these inhibitors to Na, K-ATPase. Steric hindrance seems to be responsible for a decrease in n value below 1 and the loss of the cooperativity. The kinetic properties of the enzyme were determined in the presence of inhibitor concentrations as the concentrations that inhibited 40–60 % of the enzyme activity and using Eadie–Hofstee transformation of the experimental data (Fig. 24.16) [119]. Pd(II) complexes induced significant decrease of enzyme V_{max}, while K_m values remained the same as in the control sample (Table 24.6 and Fig. 24.16), i.e., substrate ($MgATP^{2-}$) and inhibitor were bonded to different sites on enzyme, and the binding of the inhibitor did not affect binding of the substrate. According to these results, the nature of Na, K-ATPase inhibition by Pd(II) complexes was characterized as noncompetitive type of inhibition. Affinities of Na, K-ATPase for binding with Pd(II) complexes were determined and characterized with inhibitor constants (K_i) (Fig. 24.17 and Table 24.6) [119]. Pd(II) complex affinity for binding to Na, K-ATPase deducted from K_i values is: $[PdCl_4]^{2-} > [PdCl(dien)]^+ > [PdCl(Me_4dien)]^+$. Most likely, the main reason for low enzyme–inhibitor affinity of $[PdCl(dien)]^+$ and $[PdCl(Me_4dien)]^+$ was steric bulkiness that hindered contact of these complexes with enzyme. The values of stability constants of enzyme–inhibitor complex, generated as reciprocal inhibitor constants ($K_s = 1/K_i \approx 10^4$), are close to the value of overall binding constants that have been reported for the interaction of Na, K-ATPase with Pt(II) complexes [74].

3.5.3 Prevention and Recovery of Palladium(II) Complexes-Induced Na, K-ATPase Inhibition

The inhibitory effect of Pd(II) complexes can be prevented and recovered by the addition of L-cysteine or GSH. Pd(II) complexes have a great affinity for substitution of the Cl^- ligand by SH-donor ligands, GSH, and L-cysteine [168, 169].

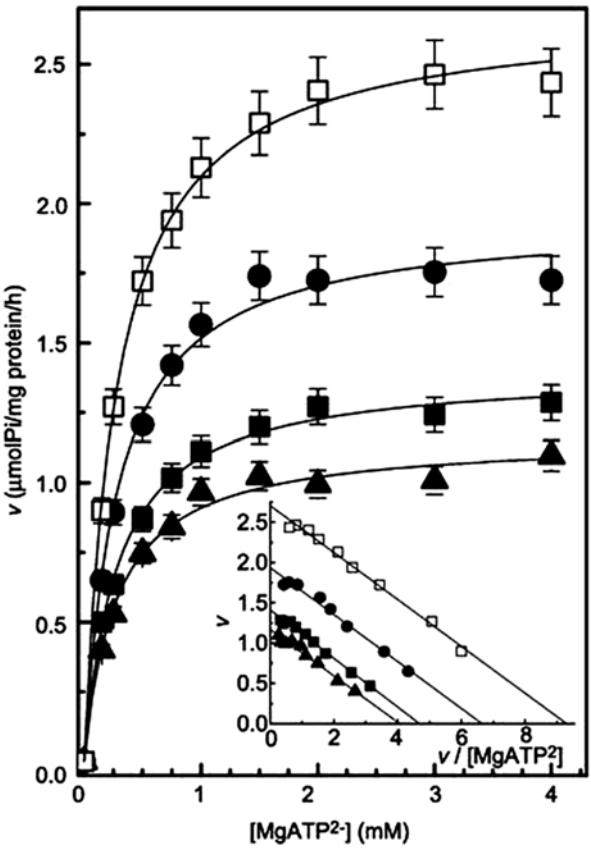

Fig. 24.16 Kinetic analysis of Na, K-ATPase interaction with Pd(II) complexes. Initial reaction rate (v) vs. $MgATP^{2-}$ concentration in the absence (*open squares*) and presence of 5×10^{-5} M $[PdCl_4]^{2-}$ (*solid up triangles*), 1×10^{-4} M $[PdCl(dien)]^{+}$ (*solid squares*), and 1×10^{-4} M $[PdCl(Me_4dien)]^{+}$ (*solid circles*). The values given are the mean of at least three experiments ± S.E.M. *Inset*: The Eadie–Hofstee transformation of the data [119]

Table 24.6 Kinetic analysis of Na, K-ATPase in the absence (control) and presence of Pd(II) complexes [119]

Inhibitor	Conc. (M)	K_m (mM)	V_{max} (μM P_i/h/mg)	K_i (M)
Control	0	0.29 ± 0.01	2.71 ± 0.03	–
$[PdCl_4]^{2-}$	5×10^{-5}	0.29 ± 0.02	1.17 ± 0.03	3.97×10^{-5}
$[PdCl(dien)]^{+}$	1×10^{-4}	0.29 ± 0.01	1.39 ± 0.02	1.04×10^{-4}
$[PdCl(Me_4dien)]^{+}$	1×10^{-4}	0.29 ± 0.01	1.94 ± 0.03	4.20×10^{-4}

Na, K-ATPase can be observed as a SH-donor ligand since the enzyme has 36 –SH groups which are held responsible for interactions of this enzyme with various metal ions [170]. It is generally considered that the nonspecific bonding of metal ions to enzyme sulfhydryl groups is accompanied by the inhibition of enzymatic activity [170, 171]. The inhibitor constants for Pd(II) complexes, obtained by kinetic analysis, indicated low stability of inhibitor–enzyme complex compared to the stability of $[PdCl_{3-n}L_n(GSH)]^{(2-(3-n))}$ or $[PdCl_{3-n}L_n(L\text{-}Cys)]^{(2-(3-n))}$ [117]. The prevention

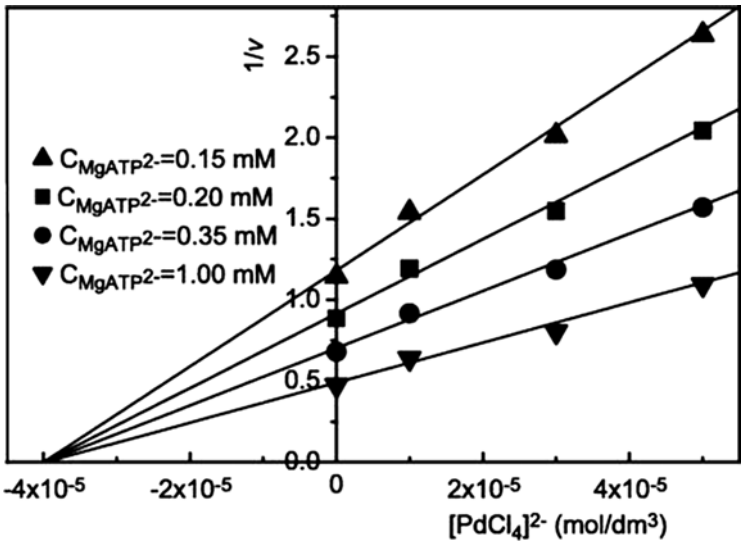

Fig. 24.17 Dixon transformation of the kinetic analysis data. Reciprocal value of initial reaction rate ($1/v$) vs. $[PdCl_4]^{2-}$ concentration in the presence of several $MgATP^{2-}$ concentrations. The *symbols* representing $MgATP^{2-}$ concentrations are shown in the *inset* [119]

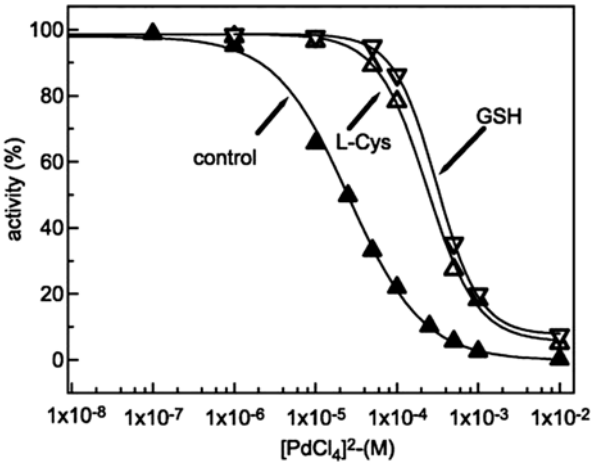

Fig. 24.18 Prevention of $[PdCl_4]^{2-}$ induced inhibition of Na, K-ATPase activity with. 1 mM L-Cys (*open up triangles*) and 1 mM GSH (*open down triangles*). The activity of control sample without SH-donor ligands is given as *solid up triangles* [119]

of Na, K-ATPase inhibition with $[PdCl_4]^{2-}$ has been achieved using L-Cys and GSH (Fig. 24.18) [119]. Analogous inhibition curves in the presence of thiols were observed for the other two Pd(II) complexes [4]. The IC_{50} values determined from inhibition curves in the absence and presence of 1 mM L-cysteine and GSH for all investigated Pd(II) complexes are presented in Table 24.7. Obviously, the sensitivity of Na, K-ATPase to Pd(II) complexes decreased in the presence of SH-containing

Table 24.7 The IC_{50} values for $[PdCl(dien)]^+$-induced inhibition of Na, K-ATPase in absence and presence of 1×10^{-3} M L-cysteine and 1×10^{-3} M GSH determined by sigmoid fit of experimental data [4]

Complex	IC_{50} (M)
$[PdCl(dien)]^+$	$(1.1 \pm 0.1) \times 10^{-4}$
$[PdCl(dien)]^+ + $L-cysteine	$(6.9 \pm 0.8) \times 10^{-4}$
$[PdCl(dien)]^+ + $GSH	$(7.7 \pm 0.5) \times 10^{-4}$

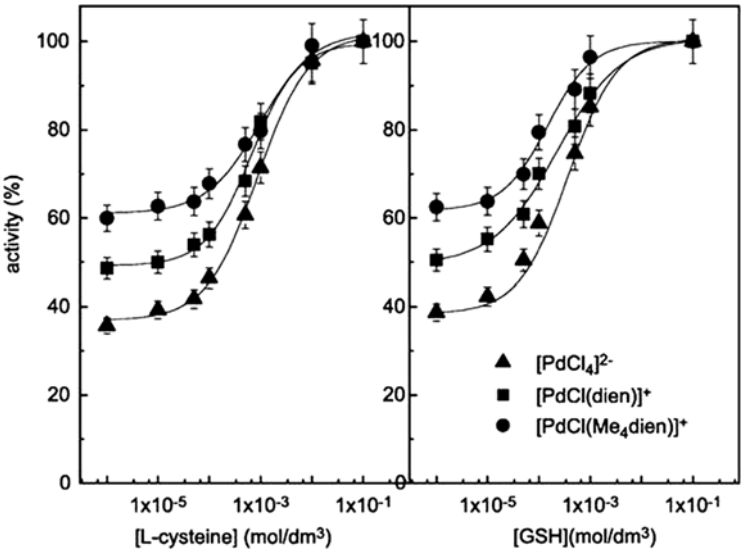

Fig. 24.19 Recovery effect of L-cysteine and GSH on the Na, K-ATPase activity inhibited in the presence of 5×10^{-5} M $[PdCl_4]^{2-}$ (*up triangles*), 1×10^{-4} M $[Pd(dien)Cl]^+$ (*squares*) and 1×10^{-4} M $[Pd(Me_4dien)Cl]^+$ (*circles*) [119]

ligands. It was shown that that L-cysteine and GSH have a dose-dependent recovery effect on Na, K-ATPase activity (Fig. 24.19). Full recovery was achieved when the concentration of SH-containing ligands was equal or higher than the Pd(II) complex concentration [4].

These Pd(II) complexes could be employed for investigation of their ability to detoxify after chemotherapy, since the intracellular concentration of GSH is up to 8 mM [172], and is usually much greater than those of cysteine.

4 Conclusions

Involvement of Na, K-ATPase in different biological processes and its overexpression in pathological states enables its use as a target in anticancer studies. Metal based complexes inhibit Na, K-ATPase activity via the –SH groups of the enzyme.

This inhibitory effect of complexes can be prevented and recovered by the addition of –SH donors, L-cysteine and GSH, the biomolecules usually present in physiological liquids. These interactions of –SH donor molecules with Na, K-ATPase inhibited with the complexes could be used for the development of approaches for prevention of toxic side effects of chemotherapy by metal-based drugs.

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