The many faces of Na\textsuperscript{+},K\textsuperscript{+}-ATPase: understanding the role of human isoforms in health and disease

Karl Weigand
About the cover

Na+,K+-ATPase is an enzyme that facilitates transfer of Na⁺ and K⁺ between the in- and outside of the cell. It accomplishes this by continuously changing its shape, completing its reaction cycle up to several thousand times per minute. Since the shape of the enzyme depends on the chemical composition of its environment, it is possible to slow down or even reverse the reaction cycle, for example by mutations or pharmacological intervention. In the human body, four variants of Na⁺,K⁺-ATPase are present, each variant with its own characteristics. The research described in this thesis aimed to understand, from a biochemical and pharmacological point-of-view, how these four variants behave, either under normal or special circumstances.

The many faces of Na⁺,K⁺-ATPase:
understanding the role of human isoforms in health and disease

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Chapter 1
General introduction
Introduction

In December 1997, Jens Christian Skou was awarded the Nobel Prize in Chemistry for “the first discovery of an ion-transporting enzyme, Na⁺,K⁺-ATPase”. Forty years before, Skou published a paper describing “the influence of some cations on an adenosine triphosphatase from peripheral nerves”. After a suggestion by Robert Post to test for ouabain inhibition of the observed activity, it became clear that the enzyme was involved in active transport across the cellular membrane. Following the first report on an adenosine triphosphatase in neuronal tissue of crab, it was quickly shown that there was an active transporter of Na⁺ and K⁺ present in human red blood cells as well, leading to many more publications describing the presence of Na⁺,K⁺-ATPase in the plasma membrane of all animal tissues tested. Also, other ion transporting pumps were identified, including a Ca²⁺-ATPase in sarcoplasmic reticulum (SERCA), and a H⁺,K⁺-ATPase present in stomach and the gastrointestinal tract, both belonging to the same class II family of P-type ATPases as Na⁺,K⁺-ATPase. In this thesis, the first discovered enzyme of this class, Na⁺,K⁺-ATPase, will be discussed.

The role of Na⁺,K⁺-ATPase

Na⁺,K⁺-ATPase is responsible for a considerable amount of cellular ATP consumption, ranging from 25% of the human body in total up to 50% in brain tissue. An enzyme using these amounts of energy is most likely involved in important cellular processes, which is confirmed by the presence of Na⁺,K⁺-ATPase in a wide variety of animal species. Today, it is known that under physiological conditions the enzyme transports three sodium ions out of the cell in exchange for two potassium ions returning into the cell during each transport cycle, while consuming one molecule of ATP. One of the most prominent features of this transport is the net efflux of cations from the cell. This provides a vital compensatory mechanism for the high intracellular concentration of K⁺ and its induced entrance of water. A second role of Na⁺,K⁺-ATPase is maintenance of the membrane potential. This involves the electrogenic capacity of the enzyme, for example in restoring the membrane potential after impulse-induced depolarization in neurons resulting in entry of Na⁺ and release of K⁺. In addition to its electrogenic property, Na⁺,K⁺-ATPase also generates a steep Na⁺ gradient across the cellular membrane. This gradient provides the driving force for secondary active transporter enzymes, for example glucose and amino acid importers. More recently, a fourth role has been proposed, in which Na⁺,K⁺-ATPase serves as an anchor for enzymes that are active in various signaling pathways after activation by endogenous ligands present in blood. Together, these highly important processes rely on Na⁺,K⁺-ATPase, underlining its importance for cellular function.

The reaction cycle

As mentioned above, Na⁺,K⁺-ATPase exports three sodium ions and imports two potassium ions during each reaction cycle, maintaining high intracellular K⁺ and high extracellular Na⁺
concentrations. The transport of these cations against their chemical gradient requires energy supplied by ATP, which acts as a fuel source. After the discovery of Na+,K+-ATPase, research was devoted to the elucidation of the exact mechanism of transport. This transport cycle is now well characterized by the so-called Post-Albers scheme. This scheme (Fig. 1) depicts how the enzyme exerts its transport function, by going through a cycle of conformational changes induced by binding or release of Na+ and K+, and ATP. Na+,K+-ATPase (and other P-type ATPases) has three characteristic properties used in the transport cycle: presence of two conformational states (E1 and E2) possessing highly different affinities to both Na+ and K+, autophosphorylation of a highly conserved aspartic acid by the γ-phosphate from ATP, and the ability to form cation occluded states trapping either Na+ or K+ by preventing access from either the cytosolic or extracellular sides of the membrane.

The reaction cycle is divided into four steps, the first step involving the E1 conformation of the enzyme. This conformation is characterized by a high affinity for Na+ and ATP, while the ion-binding sites are accessible from the cytosolic side only, allowing Na+ binding to take place. Following high-affinity binding of ATP, the γ-phosphate from ATP is transferred to an aspartic acid present in a highly conserved region of the enzyme, inducing a conformational change to the E1P state resulting in occlusion of the three sodium ions present in the ion-binding sites. Normally, the E1P state spontaneously results in formation of the E2P state, a process that can be inhibited by oligomycin. In the E2P state, the enzyme has a high K+ and low ATP affinity and the ion-binding sites are accessible only from the extracellular side of the cell. Due to the low sodium affinity of the E2P state, the enzyme releases these ions to the outside of the cell, allowing for two potassium ions to bind to the sites that were occupied by Na+ previously. This result is in hydrolysis at the phosphorylated aspartic acid site and subsequent formation of inorganic phosphate (P). Now, the enzyme has reached its fourth enzymatic conformation, the dephosphorylated E2 state in which the two bound potassium ions become occluded, i.e. the extracellular access channel has been shut. Subsequently, ATP binds to the enzyme, greatly increasing the conformational change from the E2P state to the E1 state, opening the intracellular access channel to the ion-binding sites, resulting in release of potassium to the intracellular space and completing the reaction cycle. While under physiological conditions the reaction cycle runs clockwise resulting in consumption of ATP, it is important to mention that each step is an equilibrium reaction. This means that when adjusting the biochemical conditions, it is possible to allow the reaction cycle to run in the opposite (counter-clockwise) direction resulting in formation of ATP.

Isoforms of Na+,K+-ATPase
In the human body, Na+,K+-ATPase is present in every cell. However, there are differences in the types of Na+,K+-ATPase found in specific tissues. In any case, each functional Na+,K+-ATPase consists of at least two different subunits: a 110 kDa α-subunit accompanied by a 50 kDa β-subunit. Many reports have pointed out the presence of a third subunit dubbed γ-subunit, characterized by the FXYD motif present in the extracellular part, although it remains to be established whether this subunit is always present. FXYD proteins play a role in modulating Na+,K+-ATPase activity tissue specifically, reflected by identification of multiple Na+,K+-ATPase associated FXYD proteins in different tissues. The α-subunit is responsible for most of the properties attributed to Na+,K+-ATPase. It contains the ion-binding sites and the ATP-binding and phosphorylation site. In the human body, four different isoforms of the α-subunit have been detected, each with a different tissue expression pattern. For example, the α1-isoform is found in every cell of the body and is therefore the most ubiquitous isoform, whereas the α4-isoform is specifically expressed in testis. The α2- and α3-isoforms possess an intermediate expression pattern evidenced by localization of the α2-isoform in skeletal muscle, heart, and vascular smooth muscle, adipocytes, and brain, whereas the α3-isoform is found mainly in neurons, but also in ovaries, heart, and white blood cells. Interestingly, the α1-, α2-, and α3-isoforms share a high degree of similarity (≈ 90%), whereas the α4-isoform shows less homology to these isoforms (≈ 80%).

As is the case with the α-subunit, multiple isoforms of the β-subunit exist. Usually, three isoforms are reported, although recently a fourth isoform was described, with different functionality. The β-subunit is not only essential for trafficking and insertion into the membrane, but also plays a pivotal role in enzyme activity. Similar to the α-isoforms, the β-isoforms are expressed tissue specifically. The most prominent one, the β1-isoform, is
expressed throughout the body.14,52 The β2-isofom is found in nervous tissue and skeletal muscle33,46, whereas the β3-isofom has been identified in testes, lung, retina, and liver.53,56 Together, the three different β-isofoms combined with four α-isofoms can produce up to 12 different Na+,K+-ATPase enzymes (although α1β1 is by far the most prominent), further diversified upon association with different FXYD proteins, facilitating a vast array of enzyme combinations throughout the body.

**Na’,K’-ATPase related diseases**

Since Na’,K’-ATPase is present in every cell of the human body, loss-of-function mutations in the corresponding genes are likely to have a significant impact on health. Only recently, somatic mutations in the α1-gene have been linked to disease, in this case to aldosterone-producing adenomas.57 Loss-of-function mutations in the germ line of the α1-gene are probably not compatible with life, and this might explain why these have not been reported yet. For the α4-isofom, it is not unlikely that mutations could associate with decreased male fertility, although this has not yet been investigated. Different neurological diseases have been linked to mutations in the α2- and α3-isofoms. The α2-isofom is mutated in familial hemiplegic migraine type 2 (FHM2), a rare yet severe subtype of migraine with aura associated with hemiparesis.58,59 The α2-isofom present in astrocytes normally provides the necessary driving force for reuptake of glutamate from the synaptic cleft following synaptic activation, which is affected when Na’,K’-ATPase is dysfunctional as is the case in FHM2.60,61 Rapid-onset dystonia parkinsonism (RDP), characterized by episodes of bradykinesia and postural instability with rapid onset,52-54,60 as well as alternating hemiplegia of childhood (AHC), featuring recurrent hemiplegic episodes and distinct other neurological symptoms55,56, have been linked to mutations in the α3-gene. How these mutations result in their respective phenotypes remains to be established, but it is worth mentioning that only one overlapping mutation has been reported70,71, indicating relatively specific effects on protein function for both diseases. Also, not all patients suffering from RDP and AHC are shown to have mutations producing adenomas.57 Loss-of-function mutations in the germ line of the α1-gene are likely to have a significant impact on health. Only recently, evidence for a role of DLCs in the pathogenesis of blood volume expansion74-86, even showing an antihypertensive effect of antibodies against digoxin in rats.86 This finding led to a search for endogenously produced DLCs in the human body, and not surprisingly this search revealed that several DLCs are produced in humans as well. These endogenously produced DLCs include ouabain91,92, dihydro-ouabain90, marinobufagenin91, telocinobufagenin,19-norbufalin, and bufalin. 93 However, by the time the first endogenous DLCs were discovered it was clear that these compounds were not involved in blood volume regulation as previously hypothesized. Since increasing numbers of DLCs were being discovered in the human body, a new role emerged for these compounds. The fact that they were found in relatively low amounts in blood (pico- to nanomolar range, unable to inhibit Na’,K’-ATPase activity significantly) led researchers to investigate whether endogenous DLCs could be involved in cellular signaling. In the last ten years, evidence for DLC-mediated cellular signaling has been stacking up.16,25,56 Several signaling pathways have been described, each originating from binding of DLCs to Na’,K’-ATPase. The most studied signaling pathway involves activation of the Ras/Raf/MEK/ERK1,2 cascade via transactivation of EGFR after DLC binding to Na’,K’-ATPase.97-101 One of the proteins in this cascade leading to ERK1,2 activation is Ras, which has also been linked to the production of reactive oxygen species in mitochondria of different cell types.10,100,101 Another route of cellular signaling involves the activation of phospholipase C-γ, leading to subsequent formation of IP3, which can bind to its receptor IP3R that controls calcium release from the endoplasmic reticulum (ER).102,103 Also, a link was found to signaling via the PI3K/PDK/Akt pathway leading to apoptosis104, endocytosis of Na’,K’-ATPase 105, and hypertrophic growth in cardiac myocytes.106 An interesting question regarding all these signaling pathways involves the role of Src, a protein which is implicated in the Na’,K’-ATPase receptor complex. In general, it is thought that Src interacts directly with Na’,K’-ATPase and becomes autophosphorylated and subsequently initiates different signaling cascades upon stabilization of the enzyme in the E2P conformation upon DLC binding.104-106
Na⁺,K⁺-ATPase crystal structures

Ten years after the Nobel prize was awarded for the discovery of Na⁺,K⁺-ATPase, the first crystal structure of the enzyme was published. This publication set the stage for a new era in Na⁺,K⁺-ATPase research: it was now possible to study the enzyme in its real-life appearance. In the following years, more crystal structures were solved, greatly increasing our knowledge on the E₁, E₂, and E₃ conformations (Fig. 2) of the reaction cycle, including the ouabain-bound states. The crystal structures published show clear similarities to other crystallized P-type ATPases.

The α-subunit consists of approximately 1,000 amino acids organized into ten transmembrane domains, with the N- and C-terminus located in the cytosol. The cytosolic domains of the first six transmembrane domains together form three cytosolic domains essential for ATPase activity and are found in all P-type ATPases: the N(ucleotide binding)-domain, the P(hosphorylation)-domain and the A(ctuator)-domain. These domains work together during the reaction cycle: ATP is bound by the N-domain initially in the presence of Mg²⁺, subsequently transferred towards the P-domain allowing for transfer of the γ-phosphoryl to a conserved aspartic acid residue located within the P-domain, aided by the A-domain that protects the phosphorylated aspartic acid and directs dephosphorylation at the appropriate step in the reaction cycle. Two of the ion-binding sites are located within the transmembrane domains 4-6, which are unwound at the center. These two sites (sites I and II) constitute the binding sites for the two K⁺ atoms, reaching these sites via the extracellular DLC binding pocket in the E₂P conformation, and leaving the sites in the E₁ conformation after deocclusion via the cytoplasmic access channel formed by the characteristic kink present in the first transmembrane domain, together with the second and fourth transmembrane domains. The third ion-binding site has been subject of debate for many years, but the recent crystal structures of the E₁ conformation have provided more insight into its exact location. This site (site III, previously site IIIb) has now been located between transmembrane domains 5, 7, and 8. The three Na⁺ atoms access their binding sites via the N-terminal access channel described above, although an alternative access channel leading to the third ion-binding site has been discovered recently, playing a role in transfer of a proton that substitutes for Na⁺ in the E₂ conformation when sites I and II are occluded with K⁺. The sodium ions leave their binding sites in transition to the E₂P conformation via the binding pocket of DLCs, the same channel that is used for entry of K⁺. This access channel is also the binding site for DLCs in the E₂P conformation, a state associated with high affinity binding of DLCs, as opposed to the low affinity E₂ state. A characteristic feature of high affinity DLC binding involves the presence of a magnesium ion in ion-binding site II, as was recently discovered. The β-subunit is shown to consist of one transmembrane domain with a large extracellular domain, consisting of approximately 300 amino acids in total. It interacts with transmembrane domains 7 and 10 of the α-subunit, and the extracellular domain interacts with the extracellular loop between α-isoform transmembrane domains 7 and 8, implicated in correct assembly of the αβ-heteromer. The γ-subunit interacts almost exclusively with transmembrane

Figure 2 Structure of Na⁺,K⁺-ATPase in the E₁ conformation, adapted from Laursen.
domain 9, in close proximity to the third ion-binding site, possibly explaining its impact on the enzyme’s activity.\(^1\) Knowledge of the crystal structures has greatly advanced our understanding of Na\(^+\),K\(^-\)-ATPase structure and the related functions.

**Aim and outline of this thesis**

The research described in this thesis was aimed at increasing our understanding of the role of Na\(^+\),K\(^-\)-ATPase in health and disease, based on various biochemical studies. We have studied all four α-isofoms present in the human body, either related to physiology or disease. In Chapter 2, we have looked at the role of the α1-isoform in signaling events based on DLC binding to Na\(^+\),K\(^-\)-ATPase, using a biochemical assay representing the proposed Na\(^+\),K\(^-\)-ATPase/Src signaling complex. Next, we studied differences in DLC affinity to the α1- and α2-isofoms of Na\(^+\),K\(^-\)-ATPase, with a special focus on the role of the (hydrogenated) lactone ring present in all DLCs, as described in Chapter 3. The effects of FHM mutations on the α2-isoform were studied in Chapters 4 and 5, where we expressed and biochemically characterized mutants in order to understand how these mutations could lead to disease. Also, we have investigated the effect of mutations of the α3-isoform (Chapter 6) implicated in AHC, providing the first biochemical characterization of this unknown disease. Our final experimental studies are presented in Chapter 7, where we studied which amino acids are responsible for the difference in enzyme activity between the highly active α2- and inactive α4-isoforms. Finally, the results are put in a broader context and their significance is discussed in Chapter 8.

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Chapter 2

Na\(^+\),K\(^+\)-ATPase activity modulates Src activation: a role for ATP/ADP ratio

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Abstract

Digitalis-like compounds (DLCs), specific inhibitors of Na⁺,K⁺-ATPase, are implicated in cellular signaling. Exposure of cell cultures to ouabain, a well-known DLC, leads to up- or down regulation of various processes and involves activation of Src kinase. Since Na⁺,K⁺-ATPase is the only known target for DLC binding an in vitro experimental setup using highly purified Na⁺,K⁺-ATPase from pig kidney and commercially available recombinant Src was used to investigate the mechanism of coupling between Na⁺,K⁺-ATPase and Src. Digoxin was used as a representative DLC for inhibition of Na⁺,K⁺-ATPase. The activation of Src kinase was measured as the degree of its autophosphorylation. It was observed that in addition to digoxin, Src activation was dependent on concentrations of other specific ligands of Na⁺,K⁺-ATPase: Na⁺, K⁺, vanadate, ATP and ADP. The magnitude of the steady-state ATPase activity therefore seemed to affect Src activation. Further experiments with an ATP regenerating system showed that the ATP/ADP ratio determined the extent of Src activation. Thus, our model system which represents the proposed very proximal part of the Na⁺,K⁺-ATPase-Src signaling cascade, shows that Src kinase activity is regulated by both ATP and ADP concentrations and provides no evidence for a direct interaction between Na⁺,K⁺-ATPase and Src.

Introduction

More than 50 years ago, Skou suggested that an adenosine triphosphatase present in crab nerve is involved in the extrusion of sodium.1 Following this hallmark publication, the enzyme involved in this process was shown to transport sodium and potassium ions across the cellular membrane, using ATP as a fuel source. Ouabain, a digitalis-like compound (DLC),2 had already been reported to inhibit active ion transport in red blood cells.3 By combining the findings mentioned above Post et al.4 showed that in human red blood cells, Na⁺ and K⁺ activated transport is fuelled by ATP and inhibited specifically by ouabain. ‘This Na⁺,K⁺-ATPase is now known to be one of the key players in cellular ion homeostasis, present in every human cell, and responsible for consumption of approximately 40% of the cellular ATP reserve.’5

It was suggested that in some cells, two pools of Na⁺,K⁺-ATPase are present on the cell surface: one dedicated to transport of Na⁺ and K⁺ over the cell membrane, whereas another pool resides in caveolae and is implicated in cellular signaling events.6 Several signaling pathways have been linked to this pool; the Src-EGFR-RAS-p42/44 MAPK cascade7, PLC-γ and IP3R microdomains involved in regulating Ca²⁺ homeostasis8, and an AMPK-mediated response to cellular ATP reduction.9 Recent experiments showed, however, that this strict division into ion-pumping and signaling pools of enzyme has to be abandoned. The detailed comparison of the properties of the caveolar and noncaveolar fractions of kidney Na⁺,K⁺-ATPase revealed no difference in either affinities to the specific ligands or molar activities of the enzymes.10 In recent years the Src-EGFR-RAS-MAPK cascade has been studied extensively, resulting in a comprehensive knowledge of the consequences of activation of this cascade.7,11-13 The first player, Src, is a well known non-receptor tyrosine kinase, which can be activated by (auto) phosphorylation of its kinase domain (SH1), more specifically tyrosine 418.14 Phosphorylation of another residue, tyrosine 529, results in a decrease in kinase activity. Src has been implicated in many different signaling pathways within the cell, and may be activated through different receptors including EGFR, PDGFR, ERBB2, and FGFR.14 In addition, Src has been implicated in the Na⁺,K⁺-ATPase mediated cellular response to endogenous ouabain.7,12,13 These studies suggest that Na⁺,K⁺-ATPase serves as an anchor for Src and restrains its kinase activity via two interactions. The first interaction is a constitutive binding (between the Na⁺,K⁺-ATPase CD2 and Src SH2-SH3 domains). The second, between the Na⁺,K⁺-ATPase CD3 and Src kinase/SH1 domain, is ouabain-dependent and exists only in the absence of the ligand. Ouabain binding to Na⁺,K⁺-ATPase revokes the latter domain interaction, releases the kinase domain and thereby causes activation of Src with downstream regulation of proteins within the Src signal transduction cascade.13

In this paper, an in vitro model resembling the proximal part of the Src-mediated signaling cascade is characterized in detail. Recombinant Src protein and purified Na⁺,K⁺-ATPase are used to form a complex. We observed that also in our hands Na⁺,K⁺-ATPase activity prevented Src activation. More importantly, any inhibition of Na⁺,K⁺-ATPase resulted in activation of...
Src: we found no difference between the inhibitors digoxin and vanadate. We found also that both the decrease in ATP level as well as the resulting increase in ADP level caused by Na⁺,K⁺-ATPase activity were unfavorable for Src activation. Since the concentrations of both ATP and ADP, and therefore also the ratio between these two, are heavily dependent on Na⁺,K⁺-ATPase activity, the activating effects of digoxin and vanadate on Src are of functional (inhibition of ATP hydrolysis) but not structural (induction of a certain conformation) origin. This provides an alternative hypothesis concerning the role of Src in DLC-induced signaling.

Materials and methods

Materials

Rabbit polyclonal antibody for detection of activated Src protein (pY418) was purchased from Invitrogen (44660G). Protein was detected using a goat anti-rabbit antibody labeled with HRP obtained from Abcam (AB6721). Protein presence was detected using a Thermo Scientific Supersignal® West Pico Chemiluminescent kit (34080). Pig kidney Na⁺,K⁺-ATPase was purified according to Klodos et al. to a specific activity of 1800 µmol Pᵢ mg⁻¹ h⁻¹. Recombinant full length Src protein obtained from Upstate (14-117) can be phosphorylated both at residues 418 or 529, rendering the active or inactive protein, respectively. Creatine kinase and creatine phosphate were obtained from Roche, and ATP and ADP were purchased from Sigma-Aldrich.

In vitro Src phosphorylation

In a final volume of 40 µL, recombinant Src protein (0.5 µL = 1.5 units) was incubated for 30 min at 37 °C together with 610 ng of Na⁺,K⁺-ATPase in its buffer (250 mM sucrose, 20 mM histidine, 0.9 mM EDTA(II) pH 7.0), in 1 × PBS supplemented with 5.0 mM MgCl₂, or different when indicated. Phosphorylation was started by addition of 2.0 mM MgATP unless described otherwise. An ATP regenerating system, when added, consisted of 100 µg/mL creatine kinase and 10 mM creatine phosphate present with MgATP. After 10 min of incubation at 37 °C, the phosphorylation reaction was stopped by incubation of samples with SDS sample buffer for 10 min at 65 °C, and subsequently the samples were loaded on a 10% SDS-PAGE gel. Proteins were blotted overnight onto a nitrocellulose membrane at 30 V. Then, the membranes were blocked for one hour at room temperature with TBS-T + 5% Elk. After washing with TBS-T, incubation of membranes with (1 : 1000) anti-pSrc-418 antibody was performed in TBS-T + 1% Elk + 1% BSA for two hours at room temperature. After three washes with TBS-T, incubation with (1 : 3000) goat-anti-rabbit HRP conjugate antibody was performed in TBS-T + 1% Elk + 1% BSA for one hour at room temperature. Afterwards, membranes were washed three times with TBS-T and subsequently three times with TBS before imaging was performed. Blots were imaged using a LAS-3000 scanner and images were subsequently analyzed using Image-J 1.42 software.

ATPase activity studies

In order to determine the extent of ATP hydrolysis under different experimental conditions, we tested the conversion of radiolabeled ATP, [γ-³²P]ATP (specific activity 100-500 mCi mmol⁻¹), which upon hydrolysis is converted into ADP and [³²P]Pᵢ. The experimental procedure was identical to the in vitro Src phosphorylation experiments, except for the use of 2.0 mM radiolabeled ATP. After 10 minutes at 37 °C, the reaction was stopped by addition of 500 µL of 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid. After centrifugation of samples, 150 out of 540 µL final volume was subsequently analyzed for radioactivity using a liquid scintillation counter.

Results

Effect of Na⁺,K⁺-ATPase on Src activation

The effect of Na⁺,K⁺-ATPase on Src phosphorylation levels was studied by incubating a fixed amount of Src with increasing amounts of Na⁺,K⁺-ATPase. The level of Src activation was visualized on a Western blot (Fig. 1). In the absence of MgATP pSrc-418 was absent as well. In contrast, when Src was incubated with MgATP phosphorylation of Src was clearly visible.

![Figure 1 Na⁺,K⁺-ATPase inhibits Src phosphorylation.](image-url)
Addition of increasing amounts of Na⁺,K⁺-ATPase resulted in a gradual decrease of the pSrc-418 signal, indicating that Na⁺,K⁺-ATPase prevents Src activation. Presence of 610 ng Na⁺,K⁺-ATPase significantly reduced pSrc-418 levels and therefore was used in further experiments, unless indicated otherwise.

To relate the extent of Src activation to the amount of ATP hydrolysis, we repeated the experiment using radiolabeled ATP. The level of ATP hydrolysis under the different experimental conditions is shown underneath the corresponding lanes of the Western blot image. As expected, the amount of ATP hydrolysed increased with the amount of Na⁺,K⁺-ATPase that was used to inhibit Src activation. In the presence of 610 ng of Na⁺,K⁺-ATPase, approximately 50% of the 2.0 mM ATP present was hydrolyzed during the time of experiment.

**Digoxin-induced Src activation**

The effect of digoxin on Src phosphorylation was tested by incubation of Src with or without Na⁺,K⁺-ATPase in the absence or presence of digoxin. The results of seven independent experiments are shown in figure 2. The signal intensities of Src autophosphorylation are related to the control level reached in the presence of ATP alone (100%). Presence of Na⁺,K⁺-ATPase decreased the average signal intensity to approximately 20%, while simultaneous presence of Na⁺,K⁺-ATPase and digoxin resulted in a signal intensity indistinguishable from that in the control situation.

**Role of sodium and potassium in Src inhibition by Na⁺,K⁺-ATPase**

The effect of varying KCl or NaCl on Na⁺,K⁺-ATPase mediated inhibition of Src phosphorylation is depicted in figure 3. The left part of each panel represents the significant inhibition of Src autophosphorylation by 610 ng of Na⁺,K⁺-ATPase in PBS buffer essentially as described above. This inhibition was reversed by the addition of 10 µM digoxin. The right part illustrates how pSrc-418 levels increase as a result of both decreasing NaCl (150, 50, 15, 5 or 0 mM) (panel A) or KCl concentrations (4.50, 1.50, 0.50, 0.15 or 0 mM) (panel B). This modulation of Src activation by either Na⁺ or K⁺, ligands of Na⁺,K⁺-ATPase, was unexpected.

![Figure 2 Digoxin stimulates Src phosphorylation.](image)

Western blot showing the average signal intensities (± SEM) for seven independent experiments under different experimental conditions. 1.5 units of Src were incubated with or without the presence of (inactive) Na⁺,K⁺-ATPase in PBS for 30 min, subsequently MgATP (final concentration 2.0 mM) was added for 10 min and the amounts of pSrc-418 phosphorylation were quantified and related to pSrc-418 autophosphorylation signal intensities.

![Figure 3 Effects of Na⁺ and K⁺.](image)

Representative Western blot analysis of pSrc-418 formation under different buffer conditions. 1.5 units of Src was incubated with or without 610 ng of purified Na⁺,K⁺-ATPase in PBS or 50 mM Tris-HCl (pH 7.4) with or without NaCl or KCl. Panel A shows the effect of decreasing NaCl concentrations (150, 50, 15, 5 and 0 mM) while maintaining a constant KCl concentration, whereas panel B shows the result of decreasing KCl concentrations (4.5, 1.5, 0.50, 0.15 and 0 mM) under a constant NaCl concentration. After 30 min preincubation, the reaction was started by addition of 2.0 mM MgATP and was allowed to take place for 10 min. For both experiments (panel A and B), the average amount of ATP hydrolysis (mean ± SEM) of three experiments is depicted below the corresponding lane of the Western blot image. Again, the methods were essentially the same as described above, except for the use of radiolabeled ATP to measure its hydrolysis.
The process of Src autophosphorylation itself caused only a limited decrease in the amount of ATP, whereas 55% of ATP was hydrolyzed when Na⁺,K⁺-ATPase was added. Digoxin prevented this drop in ATP concentration. When Src and Na⁺,K⁺-ATPase were incubated in a Tris buffer containing both NaCl and KCl, the ATP hydrolysis increased as expected (up to 75%), due to the absence of inhibitory inorganic phosphate. Lowering the NaCl or KCl concentrations, while maintaining ionic strength (using NMDG-Cl), decreased the level of ATPase consumption in a concentration dependent manner, in agreement with the finding described above showing that Na⁺,K⁺-ATPase activity is responsible for almost all ATP hydrolysis.

Role of ATP concentrations in Src inhibition

Since both Na⁺ and K⁺ are essential for Na⁺,K⁺-ATPase induced Src inhibition, we tested if the state (active vs. inactive) of Na⁺,K⁺-ATPase was important for Src activation. For this purpose the classical P-type ATPase inhibitor vanadate was used. Figure 4 shows that vanadate did not interfere with Src activity (lanes 1 and 2) in the absence of Na⁺,K⁺-ATPase, but did stimulate Src phosphorylation in the presence of Na⁺,K⁺-ATPase. Similar levels of Src activation were obtained by addition of saturating vanadate concentrations or digoxin, i.e. the presence of either inhibitor of Na⁺,K⁺-ATPase restored Src activity.

The data presented in figure 4 reveal that the extent of ATP hydrolysis correlated inversely with the level of Src phosphorylation. This finding suggests that the two enzymes compete for the same substrate: ATP. Increasing amounts of vanadate clearly diminished ATP hydrolysis.

Thus, Na⁺,K⁺-ATPase is responsible for most of the ATP conversion, whereas Src is responsible for <10% of ATP consumption in the absence of Na⁺,K⁺-ATPase (see also Fig. 3). Figure 5 confirms that the inactivation of Src in the presence of Na⁺,K⁺-ATPase under steady-state conditions of enzyme activity is due to ATP depletion. In the presence of a low amount (305 ng) of Na⁺,K⁺-ATPase a significant level of Src activation is observed already at low ATP concentrations (0.50-1.0 mM). Increasing the amount of Na⁺,K⁺-ATPase present (610 and 1220 ng) resulted in higher ATP demands in order to reach the same level of Src phosphorylation. In contrast, only 0.25 mM ATP was required for Src activation when Na⁺,K⁺-ATPase activity was inhibited by addition of 10 µM digoxin.

Effect of ADP in Src activation

ADP, the product of the Na⁺,K⁺-ATPase reaction cycle, is known to inhibit Src activity. Therefore, the effect of increasing ADP concentrations on the level of Src phosphorylation was investigated (Fig. 6). Increasing ADP concentrations attenuated Src phosphorylation. The effect of an ATP regenerating system, which converts ADP and Pi into ATP, points in the same direction. The presence of Na⁺,K⁺-ATPase did not influence these results (Fig. 6B) since the regenerating system maintained high ATP concentrations essentially in the absence of ADP.

Discussion

Na⁺,K⁺-ATPase, in parallel to its transport function, serves as a receptor in the ouabain signaling pathway. It was shown that exposure of cell cultures to digitalis-like compounds leads to up- or down-regulation of various processes (e.g. gene activation, motility, cell-cell contact, cell proliferation, apoptosis).
DLC binding to Na⁺,K⁺-ATPase always inhibits enzyme activity in in vitro experiments. On the cellular level, however, the interactions might result in diverse effects. Na⁺,K⁺-ATPase inhibition can cause changes in ionic composition of the cytoplasm or energetic state of the cell. In addition, conformational changes of Na⁺,K⁺-ATPase, acting as a receptor and physically interacting with its downstream partners, might result in activation of signaling pathways. Several studies support the involvement of the non-receptor tyrosine kinase Src in DLC induced changes in cellular homeostasis.\textsuperscript{11-13} The suggested mechanism implied a direct physical interaction between Src and Na⁺,K⁺-ATPase, where signal transduction is mediated via a specific conformational state of Na⁺,K⁺-ATPase. In short, Src is kept in an inactive state by a double intermolecular bond linking the enzyme to Na⁺,K⁺-ATPase.\textsuperscript{13} Binding of ouabain induces the E₂-ouabain bound conformation of Na⁺,K⁺-ATPase, relieving one of the two interactions (Src kinase domain-CD3 domain of Na⁺,K⁺-ATPase) followed by autophosphorylation of tyrosine 418. The phosphorylated Src then initiates diverse signaling pathways, eventually leading to changes in cellular homeostasis.\textsuperscript{11-13,19} The attempts to interpret the stimulating effect of ouabain on Src activity as an indication of direct interactions between Na⁺,K⁺-ATPase and Src were earlier complicated by the fact that vanadate, in contrast to ouabain, did not relieve the inhibitory effect of Na⁺,K⁺-ATPase on Src activity. In a recent publication\textsuperscript{22}, however, it was hypothesized that cellular signaling is activated via the E₂ conformations of Na⁺,K⁺-ATPase, conformations which are stabilized by ouabain binding. Vanadate binding shifts the E₁/E₂ equilibrium towards the E₂ conformation of Na⁺,K⁺-ATPase, and therefore should be expected to stimulate Src activity as well. As the experimental conditions for the in vitro experiments in both publications\textsuperscript{11,12} are identical, as well as the enzymes used are of similar purity and specific activity, it is hard to find an explanation for the lacking vanadate effect observed by Tian et al.\textsuperscript{13} Moreover, the Src-stimulating effect of vanadate was also observed in a study involving intact cells and the viral homolog of Src.\textsuperscript{23} Likewise, recent experiments showed endogenous Src activity in both caveolar and noncaveolar preparations of Na⁺,K⁺-ATPase and no additional Src activation by ouabain.\textsuperscript{20}

Our results confirm that active Na⁺,K⁺-ATPase causes inhibition of Src, while inhibited Na⁺,K⁺-ATPase was unable to inactivate Src kinase. This suggests another explanation for Src activation by binding of DLCs to Na⁺,K⁺-ATPase. Exposure of the presumed Na⁺,K⁺-ATPase-Src complex to different experimental conditions revealed that the magnitude of Na⁺,K⁺-ATPase activity affects the extent of Src phosphorylation. The first observation that evoked this association was the fact that Src phosphorylation was enhanced in response to diminished NaCl and KCl concentrations, both resulting in attenuated Na⁺,K⁺-ATPase activity, as was confirmed by ATPase studies (Fig. 3). Then, the Na⁺,K⁺-ATPase inhibitor vanadate stimulated Src activation in the same way as digoxin did (Fig. 4). Estimation of the amount of ATP present after termination of the reaction showed that 50-60% of ATP was hydrolyzed by Na⁺,K⁺-ATPase in the absence of vanadate, and that the vanadate induced decrease in the amount of ATP hydrolysis correlated with the degree of vanadate-dependent stimulation of Src phosphorylation (Fig. 4). Direct proof that ATP depletion is one of the reasons for Na⁺,K⁺-ATPase induced Src inhibition was obtained in experiments where this effect was counteracted by increasing ATP concentrations (Fig. 5). Finally, we observed that ADP has an inhibitory effect on Src phosphorylation both in the presence and absence of Na⁺,K⁺-ATPase. Src activity significantly decreased when ATP concentrations were below 2.0 mM or in the presence of more than 1.0 mM ADP (Fig. 6). In conclusion, the results of our in vitro experiments show that the hydrolytic activity of Na⁺,K⁺-ATPase determines ATP and ADP concentrations and thereby regulates Src activity.

In terms of whole cell physiology our results suggest that coupling between Src and Na⁺,K⁺-ATPase is based on the control of ATP and ADP concentrations, their common ligands. Inhibition of Na⁺,K⁺-ATPase activity by DLCs will increase the ATP/ADP ratio, activating Src phosphorylation. The effect probably involves local changes in the concentrations of ligands, since DLC plasma concentrations are in the pico- and nanomolar range\textsuperscript{24}, too low to affect the house-keeping function of Na⁺,K⁺-ATPase and bulk concentrations of ATP and ADP. However, not much is known about DLC concentrations in caveolae, the cellular membrane invaginations playing an important role in cell signaling. It is possible that the increase in local DLC concentrations in vivo results in significant Na⁺,K⁺-ATPase inhibition.

The role of Na⁺,K⁺-ATPase as a regulator of Src kinase suggested above is in many aspects similar to its role in maintenance of cellular Ca²⁺ homeostasis. Inhibition of Na⁺,K⁺-ATPase...
activity affects the driving force for the Na⁺/Ca²⁺-exchanger, resulting in gain of [Ca²⁺]_cyt and changes in contractility of heart muscle as well as in cellular signaling. In this study, we report evidence that changes in the energetic status of the cell due to varying Na⁺,K⁺-ATPase activity may be involved in cellular signaling. This hypothesis is supported by the fact that in some tissues, Na⁺,K⁺-ATPase is responsible for almost 50% of ATP conversion. Thus, Soltoff and Hedden showed that ouabain, by inhibition of Na⁺,K⁺-ATPase, increases the phosphorylation of AMPK, which subsequently results in phosphorylation of ERK1/2, converging on the same signaling pathway that has been reported for Src mediated signaling. In conclusion, this study suggests another mode of DLC induced signaling than the proposed direct interaction between Na⁺,K⁺-ATPase and Src. We show that Na⁺,K⁺-ATPase activity regulates Src phosphorylation indirectly, via modulation of ATP and ADP concentrations. Src activation takes place when the ATP concentration is high and ADP concentration is low. The fact that vanadate, acting as an ATPase inhibitor, is also able to activate Src via this mechanism implies that other ligands are capable of stimulating Src activation by interfering with Na⁺,K⁺-ATPase activity.

Disclosure statement
The authors declare that there are no conflicts of interest.

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References
Chapter 3

Na\(^+\),K\(^+\)-ATPase isoform selectivity for digitalis-like compounds is determined by two amino acids in the first extracellular loop

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Abstract

Digitalis-like compounds (DLCs) comprise a diverse group of molecules characterized by a cis-trans-cis ring-fused steroid core linked to a lactone. They have been used in the treatment of different medical problems including heart failure, where their inotropic effect on heart muscle is attributed to potent Na⁺,K⁺-ATPase inhibition. Their application as drugs, however, has declined in recent years due to their small safety margin. Since human Na⁺,K⁺-ATPase is represented by four different isoforms expressed in a tissue-specific manner, one of the possibilities to improve the therapeutic index of DLCs is to exploit and amend their isoform selectivity. Here, we aimed to reveal the determinants of selectivity of the ubiquitously expressed α1-isoform and the more restricted α2-isoform toward several well-known DLCs and their hydrogenated forms. Using baculovirus to express various mutants of the α2-isoform, we were able to link residues Met119 and Ser124 to differences in affinity between the α1- and α2-isoforms to ouabain, dihydro-ouabain, digoxin, and dihydro-digoxin. We speculate that the interactions between these amino acids and DLCs affect the initial binding of these DLCs. Also, we observed isoform selectivity for DLCs containing no sugar groups.

Introduction

In the 18th century, William Withering reported the use of foxglove extracts for various medical conditions, specifically concerning edema, known then as dropsy. Modern science has shown that the most important component of foxglove extract must have been digitoxin, a well-known digitalis-like compound (DLC). More recently, endogenously produced DLCs were discovered in human plasma, implicating these compounds in various physiological processes. However, the most prominent property of DLCs is their ability to bind to Na⁺,K⁺-ATPase with high specificity, inhibiting the transport activity of this enzyme.

Na⁺,K⁺-ATPase is located in the plasma membrane of almost all animal cells. By recurrent transitions between two different conformational states (E₁ and E₂), Na⁺ and K⁺ are transported over the plasma membrane using the energy obtained from hydrolysis of ATP. It is estimated that this transport process consumes approximately 25-50% of cellular energy, highlighting the enzyme’s important role. In return, the enzyme maintains the electrochemical gradients for Na⁺ and K⁺ across the cell membrane, thus providing the driving force for various secondary active transporters. Ion transport as well as ATP hydrolysis are performed by the α-subunit of Na⁺,K⁺-ATPase that is present in four tissue-specifically expressed isoforms. Additionally, a β-subunit is necessary for proper insertion of the heteromeric αβ-complex into the membrane.

Numerous studies have been dedicated to understand exactly how DLCs bind to Na⁺,K⁺-ATPase. Among the first studies was the work by Lingrel and co-workers, who showed that residues Gln118 and Asn129 (human α1-isoform coding) are associated with DLC sensitivity based on mutagenesis studies with the corresponding residues from rat isoforms of Na⁺,K⁺-ATPase. In the meantime, mutagenesis studies complemented by crystal structures of DLC-bound Na⁺,K⁺-ATPase have provided convincing evidence that the first six transmembrane segments of the Na⁺,K⁺-ATPase α-subunit constitute a conserved DLC binding pocket. Following the localization of the binding site of DLCs, Katz and co-workers subsequently investigated the isoform selectivity of DLCs by comparing the binding affinities of various DLCs to the α1-, α2-, and α3-isoforms. Using recombinant Na⁺,K⁺-ATPase, they concluded that isoform selectivity of DLCs is determined by the DLC’s sugar moieties. This affirmed the conclusions from other studies, which had highlighted the role of the sugar moiety in the dissociation rate of ouabain binding.

In this study, we set out to study the differences in binding affinities of 13 well-known DLCs (Fig. 1) for the human α1- and α2-isoforms of Na⁺,K⁺-ATPase, which were expressed in Sf9 insect cells using baculovirus. Focusing on ouabain, dihydro-ouabain, digoxin, and dihydro-digoxin, we aimed to reveal specific amino acids responsible for the observed differences and could successfully identify two crucial residues present in the first extracellular loop of Na⁺,K⁺-ATPase. Interestingly, a previous study identified an important role for these amino acids in the ouabain binding process. Here, we provide evidence that these residues are responsible for the isoform selectivity of dihydro-DLCs as well. Finally, using the crystal structures of...
multiple conformational states of Na⁺,K⁺-ATPase, including the high-affinity ouabain-bound state, we hypothesize how these mutations might affect the affinity of (dihydro-)DLCs.

**Materials and Methods**

**The Gateway system**

Mutant and wild type Na⁺,K⁺-ATPase were produced using the Gateway cloning system (Life Technologies, Breda, The Netherlands). Mutant cDNA was generated by combining two α2-isofrom based PCR products (A and B) obtained by using either a 5'- or 3'-primer in combination with a primer containing the desired mutation(s), resulting in two fragments containing complementary mutated flanks. These two fragments were combined in a subsequent PCR (C), where only the 5'- and 3'-primers were used to obtain full length cDNA containing the desired mutation(s). Subsequently, the cDNA was cloned into an empty entry clone by making use of LR Clonase II enzyme (Life Technologies, Breda, The Netherlands). Amplification of clones was performed by miniprep of colonies visible on a kanamycin selection plate. After successful sequence analysis of entry clones, expression clones containing the (mutated) α-subunit were obtained by combining an expression clone containing the β1-subunit with the entry clone by making use of LR Clonase II enzyme (Life Technologies, Breda, The Netherlands), resulting in a plasmid containing both the (mutated) α-subunit and β1-subunit. Mock transfection using yellow fluorescent protein (YFP) instead of the α-subunit resulted in the generation of a negative control (expressing YFPβ1).

**Generation of recombinant viruses**

Expression clones generated using the Gateway system were transformed into competent DH10Bac *Escherichia coli* cells (Life Technologies, Breda, The Netherlands) harboring the baculovirus genome (bacmid) and a transposition helper plasmid. Upon transposition between the Tn7 sites present in the bacmid and expression clone, recombinant bacmids were selected and isolated. Subsequent transfection of bacmids into Sf9 insect cells using Cellfectin (Life Technologies, Breda, The Netherlands) and incubation for 6 days at 27 °C resulted in generation of virus coding for the desired wild type and mutant Na⁺,K⁺-ATPase isoforms. Using the supernatant of these cells, a fresh batch of Sf9 cells was infected at a multiplicity of infection of 0.1. After another 6 days of infection, the supernatant of these cells was collected and used for further procedures.

**Membrane preparation**

Sf9 cells grown at 27 °C in T175 flasks and later in 1000 mL shaking flasks in Xpress medium (Sigma, Bornem, Belgium) were infected with recombinant virus at a density of 1.5 × 10⁶ cells mL⁻¹ in the presence of 1% (v/v) ethanol, as described before. After 3 days of infection, cells were harvested by centrifugation at 5,000 × g for 5 min, and the pellet was resuspended in ice-cold 250 mM sucrose, 2.0 mM EDTA, and 20 mM HEPES/Tris pH 7.0 buffer. Cell lysis and membrane fraction purification were performed by sonication at 80 W for 30 s, followed by centrifugation at 10,000 × g for 30 min. The pellet was resuspended in ice-cold 250 mM sucrose, 2.0 mM EDTA, and 20 mM HEPES/Tris pH 7.0 buffer. Cell lysis and membrane fraction purification were performed by sonication at 80 W for 30 s, followed by centrifugation at 10,000 × g for 30 min and centrifugation of the resulting supernatant at 100,000 × g for 60 min. The pelleted membrane fraction was resuspended in the above-mentioned resuspension buffer, homogenized, and stored at -20 °C.

**Ouabain competition assay**

Approximately 150-200 µg of protein was incubated in 20 mM histidine, 5.0 mM MgCl₂, 5.0 mM H₂PO₄, 25 nM of radiolabeled ouabain and increasing concentration of nonradiolabeled DCLs at room temperature for 2 h. Then, samples were transferred to ice and filtered through ME27 cellulose filters (GE Healthcare, Little Chalfont, UK) using ice-cold distilled water, retaining only the Na⁺,K⁺-ATPase-bound DCLs. The amount of radioactivity on the filters was determined by addition of 4 mL of OptiFluor (Canberra Packard, Tilburg, The Netherlands) and subsequent liquid scintillation counting.
In house hydrogenation of digoxin to obtain dihydro-digoxin

Digoxin (50 mg) and PtO₂ (10 mg, 6 mol%) were dissolved in 15 mL of ethanol in a Schlenck tube. All air was then removed by vacuum, and the solution cooled to 0 °C. H₂ gas was bubbled through the solution, resulting in a color change from brown to black. The solution was allowed to warm to room temperature, and H₂ bubbling was continued for 16 h. After hydrogenation was completed (which was checked by LCQ) the catalyst was removed by filtration through a 0.45 µm ultra filter. The solvent was removed by rotary evaporation. The product was obtained by recrystallization from EtOH/Et₂O in 46% yield as a white solid.

Data analysis

All data was analyzed using GraphPad Prism 5.02 software. Repeated measures ANOVAs were performed to check for differences in IC₅₀ values among DLCs (α = 0.05). For determination of IC₅₀ values, we performed nonlinear curve fitting using variable slope on averaged (n ≥ 3), log-transformed data with a bottom restraint ≥ 0.

Molecular docking

The molecular docking calculations were based on the crystal structure of the Na⁺,K⁺-ATPase E₂P-ouabain complex (PDB code 4HYT) and performed in Schrödinger Suite 2011 (Schrödinger LLC, 2011). The receptor was prepared in Protein Preparation Wizard according to standard procedures, including addition of hydrogens and assignment of bond orders and protonation states. Energy minimization of the receptor was performed with the OPLS 2005 force field. Ouabain and the two dihydro-ouabain isoforms were build in Maestro and minimized with MacroModel v9.5, using the MMFF94s force field with 10,000 steps of conjugate gradient iterations, or until convergence was reached, according to default settings. For the molecular docking calculations, the induced-fit docking (IFD) protocol was used, allowing side chains and, to some extent, the backbone of the binding site to be flexible during the docking calculations. The IFD calculations were performed with extra precision Glide and the center of the enclosing box was defined by the centroid of the co-crystallized ouabain molecule.

Results

Screen for differences in DLC binding affinities between α1 and α2 wild type enzymes

We expressed the α1β1- and α2β1-isoforms of Na⁺,K⁺-ATPase together with a negative control (expressing YFPβ1) using the baculovirus expression system. Purified membrane fractions of these enzymes were used to screen for differences in IC₅₀ values among α1 and α2 isoforms (e.g. ouabain), (ii) lower affinity for the α2-isoform (e.g. dihydro-ouabain), and (iii) higher affinity for the α2-isoform (e.g. digoxin). Next, we focused on four DLCs: ouabain, dihydro-ouabain, digoxin, and dihydro-digoxin, since hydrogenation of the lactone ring in both cases worsened their affinities to Na⁺,K⁺-ATPase, which was especially present for the α2-isoform.

Mutagenesis studies focused on the DLC binding pocket

On the basis of differences in the amino acid sequence of the α1- and α2-isoforms in the vicinity of the DLC binding pocket, six α2-isoform mutants were designed with one or more α2-isoform residues substituted by their α1-isoform counterparts. These mutants included α2-G114S-M119T-D121E-S124Q-β1 (mutant A), α2-V306L-G312E-S314T-β1 (mutant B), α2-I883L-L885V-T891W-M892I-β1 (mutant C), α2-A135S-β1 (mutant D), α2-L789I-β1 (mutant E), and α2-Q291H-L292I-β1 (mutant F). As expected, the affinities for ouabain were similar to those of the wild type α1- and α2-isoforms for all six mutants (Fig. 2A). The binding properties of mutants B-F were comparable to those of the α2-isoform of the wild type enzyme for dihydro-ouabain, digoxin, and dihydro-digoxin (Fig. 2B-D), whereas mutant A showed the highest degree of similarity to the α1-isoform for these three DLCs, a trend that was even statistically significant for dihydro-ouabain. Together, these results show that mutant A possesses one or more mutations responsible for the isoform-specific differences in affinity toward dihydro-ouabain and, to a lesser extent, also for digoxin and dihydro-digoxin.
In essence, radiolabeled ouabain was incubated for 2 h with increasing concentrations of ouabain, digoxin, and dihydro-digoxin. The mutated residues were all located in the loop between transmembrane helices αM1 and αM2, highlighting this area for further studies.

### Single mutations G114S, M119T, D121E and S124Q

The individual effects of the amino acid residues substituted in mutant A were tested by investigating four single mutants: α2-G114S-β1, α2-M119T-β1, α2-D121E-β1, and α2-S124Q-β1. None of the single mutations significantly changed the affinity for ouabain (Fig. 3A) compared to that of the α2-isoform of the wild type enzyme. However, experiments with the other three DLCs revealed that mutations M119T and S124Q (dihydro-ouabain), M119T (digoxin), and S124Q (dihydro-digoxin) significantly changed the affinities compared with those for the α2-isoform of the wild type enzyme, although the effect was not as strong as that observed with mutant A. Thus, Met119 and Ser124 seemed to affect DLC binding for dihydro-ouabain and dihydro-digoxin as well as digoxin itself.

#### Met119 and Ser124 mutations

On the basis of the change in affinity from α2-isoform to α1-isoform levels upon replacing either Met119 or Ser124 with their corresponding α1-isoform residues, the final round of mutagenesis studies was aimed at investigating the role of the amino acid side chains. To this end, we generated the following mutants: α2-M119A-β1, α2-M119D-β1, α2-M119F-β1, α2-M119K-β1, α2-M119L-β1, α2-M119N-β1, α2-M119S-β1, α2-S124A-β1, α2-S124E-β1, α2-S124K-β1, α2-S124L-β1, α2-S124N-β1, and α2-S124T-β1. Again, the apparent affinities of ouabain, dihydro-ouabain, digoxin, and dihydro-digoxin were tested.

As expected, none of the Met119 mutations improved the affinity for ouabain (Fig. 4A). In contrast, replacing methionine with aspartic acid (M119D) decreased the ouabain affinity for this mutant significantly. For dihydro-ouabain (Fig. 4B), only introduction of serine (M119S) significantly increased the affinity, reflecting its structural resemblance to threonine, which is present in the α1-isoform. Digoxin (Fig. 4C) showed a decreased affinity for several mutations: M119D, M119K, M119N, and M119S. A significant decrease in dihydro-digoxin affinity was induced by the M119D mutation (Fig. 4D).

Finally, the apparent DLC affinities upon substitution of Ser124 were tested. As expected, no significant changes in affinity for ouabain (Fig. 5A) and digoxin (Fig. 5C) were observed, except for a minor decrease in affinity of S124K for ouabain. However, for dihydro-ouabain (Fig. 5B), replacing Ser124 with alanine (S124A), glutamic acid (S124E), lysine (S124K), or leucine (S124L) significantly increased the affinity, whereas replacing serine with threonine (S124T) did not. Finally, alanine (S124A), lysine (S124K) and leucine (S124L) showed nonsignificant increases in affinity for dihydro-digoxin (Fig. 5D).

**Figure 2** Overview of competition assays and relative IC₅₀ values for mutants A–F.

In essence, radiolabeled ouabain was incubated for 2 h with increasing concentrations of ouabain, digoxin, and dihydro-digoxin. The amount of radioactivity retained on the filters following filtration, reflecting the amount of enzyme-bound ouabain, was determined using liquid scintillation analysis. Values represent mean ± SEM of at least three experiments performed on at least two independently produced protein batches. * P < 0.05 versus α2 wild type, ** P < 0.01 versus α2 wild type, *** P < 0.001 versus α2 wild type.
Radiolabeled ouabain was incubated for 2 h with increasing concentrations of ouabain (A), dihydro-ouabain (B), digoxin (C), and dihydro-digoxin (D). The amount of radioactivity retained on the filters following filtration, reflecting the amount of enzyme-bound ouabain, was determined using liquid scintillation analysis. Values represent mean ± SEM of three experiments performed on independently produced protein batches. * P < 0.05 versus a2 wild type, ** P < 0.01 versus a2 wild type, *** P < 0.001 versus a2 wild type.

Figure 3 Overview of competition assays and relative IC\textsubscript{50} values for single mutants G114S, M119T, D121E and S124Q.

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Radiolabeled ouabain was incubated for 2 h with increasing concentrations of ouabain (A), dihydro-ouabain (B), digoxin (C), and dihydro-digoxin (D). The amount of radioactivity retained on the filters following filtration, reflecting the amount of enzyme-bound ouabain, was determined using liquid scintillation analysis. Values represent mean ± SEM of three experiments performed on independently produced protein batches. * P < 0.05 versus a2 wild type, ** P < 0.01 versus a2 wild type, *** P < 0.001 versus a2 wild type.

Figure 4 Overview of competition assays and relative IC\textsubscript{50} values for various mutations of Met\textsuperscript{119}.
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Computational prediction of the binding mode of dihydro-ouabain

As described above, ouabain and dihydro-ouabain showed a significant difference in isoform selectivity. This is in spite of the fact that (i) the structural differences between the two DLCs are confined to the degree of saturation of the lactone ring and (ii) the lactone moiety of ouabain is located more than 15 Å away from Met119 and Ser124 in the crystal structure of

Figure 5 Overview of competition assays and relative IC₅₀ values for various mutations of Ser124.

Radiolabeled ouabain was incubated for 2 h with increasing concentrations of ouabain (A), dihydro-ouabain (B), digoxin (C), and dihydro-digoxin (D). The amount of radioactivity retained on the filters following filtration, reflecting the amount of enzyme-bound ouabain, was determined using liquid scintillation analysis. Values represent mean ± SEM of three experiments performed on independently produced protein batches. * P < 0.05 versus a2 wild type, ** P < 0.01 versus a2 wild type, *** P < 0.001 versus a2 wild type.

Figure 6 DLC binding site, showing the computational predicted binding mode of both dihydro-ouabain isoforms (depicted in blue and magenta sticks, respectively).

The crystal structure of the high affinity E₆P-ouabain complex (7) is depicted by a green cartoon, with the co-crystallized ouabain molecule represented by green sticks. To demonstrate the large conformational rearrangements that αM1 and αM2 are undergoing during the catalytic cycle, crystal structures of the [K₂E₂·Pi] and [Na₃E₁P·ADP] conformational states have been superimposed onto the E₆P-ouabain complex. Residues identified via mutagenesis are shown in sticks, and are distant from the DLCs upon high affinity binding. Arg₈₈₃ is predicted to form a hydrogen-bond with Gln₁₂₄ in the [K₂E₂·Pi] state.
the high affinity E$_P$-ouabain complex. To gain insight into whether hydrogenation of the lactone prevents dihydro-ouabain from obtaining a binding mode similar to that previously described for ouabain, an induced-fit docking approach was applied to computationally predict the binding mode of dihydro-ouabain (Fig. 6). Initial redocking of ouabain into the binding site of the high affinity E$_P$-ouabain complex confirmed that the docking approach applied was able to predict the binding mode of ouabain as being similar to the one obtained in the crystal structure. Moreover, the molecular docking calculations predicted the binding mode of dihydro-ouabain to be similar to that of ouabain, i.e. the steroid core was superimposable with that of ouabain, with the concave apolar α-surface interacting with bulky hydrophobic side chains of αM4-αM6 and the hydrophilic groups on the β-surface taking part in an extensive hydrogen bonding network with polar side chains of the αM1-αM2 loop (flexible in the IFD calculations): the lactone is located in a hydrophobic funnel formed by αM4-αM6, with a putative Mg$^{2+}$ bound in cation binding site II, whereas the sugar moiety is located in a wide hydrophilic cavity exposed to the extracellular environment. No spatial clashes were found between the lactone of dihydro-ouabain and the receptor. However, the docking scores obtained for ouabain were slightly better than those obtained for the two dihydro-ouabain isoforms.

**Discussion**

This study was designed to elucidate the mechanism of Na$_+_K$-ATPase isoform-specific selectivity toward DLCs. We have determined the apparent affinities of the wild type α1- and α2-isoforms and various mutants of the α2-isoform for a selection of DLCs and conclude that residues Met$_{119}$ and Ser$_{124}$, present in the first extracellular loop of Na$_+_K$-ATPase, are responsible for the low affinity of hydrogenated DLCs for the α2-isoform.

The screen of Na$_+_K$-ATPase isoform affinities toward different DLCs (Table 1) revealed three groups of ligands: (i) ouabain, dihydro-digoxin, convallatoxin, peruvoside and proscillaridin A were bound with similar affinities by both isoforms (0.5 < ratio α2/α1 < 2.0), whereas (ii) digoxin and digitoxin showed a preference for the α2-isoform (0.5 ≥ ratio α2/α1), and (iii) dihydro-ouabain, ouabagenin, digoxigenin, digitoxigenin, strophanthidin, and strophanthidin bound to the α1-isoform preferably (ratio α2/α1 > 2). On the basis of the absence or presence of differences in affinities of closely related DLCs, it is possible to rule out the effect of various hydroxyl groups: for example, the hydroxyl group at position 12 did not affect the affinity for either isoform (digoxin and digoxigenin versus digitoxin and digitoxigenin, respectively). Also, substitution of the hydroxyl group at position 19 by a ketone did not affect the affinities as well (strophanthidin versus strophanthidin). In addition, the hydroxyl groups at positions 1 and 11 did not influence DLC affinity (ouabagenin versus strophanthidin). With respect to the α1-isoform, the lowest affinities were detected for digoxin, dihydro-digoxin, and digitoxin, implying that the presence of three sugar groups decreases the affinity for this isoform. Although different from recent studies, these findings are in line with the earlier work of Forbush and co-workers, who showed that digitoxigenin bound to the α1-isoform of Na$_+_K$-ATPase to a lower extent than did digitoxin and ouabain. In contrast to the α1-isoform, the presence of three sugar moieties (digoxigenin and digitoxigenin versus digoxin and digitoxin) did not result in a decreased affinity for the α2-isoform. In fact, the α2-isoform possessed the lowest affinities for DLCs containing a hydrogenated lactone moiety (dihydro-ouabain and dihydro-digoxin). Lastly, the isoform selectivity was decreased in the presence of sugar moieties: ouabain, digoxin, digitoxin, and convallatoxin possessed 3-14 times lower isoform selectivity than their aglycones ouabagenin, digoxigenin, digitoxigenin, and strophanthidin, respectively. The most recent study concerning isoform preference of DLCs was performed with human Na$_+_K$-ATPase isoforms expressed in Pichia pastoris, and the authors concluded that this selectivity was mediated via the sugar group because aglycones showed no preference for either of the isoforms. Although the results obtained in this study confirm the preference of digoxin and digitoxin for the α2-isoform, we also observed significant selectivity for several aglycones, including digoxigenin and digitoxigenin. This inconsistency might be explained by different experimental conditions used in the present study, including the baculovirus expression system, the degree of β-subunit glycosylation, the enzymatic conformations induced in the competition experiments (vanadate-induced versus Mg$^{2+}$ and P$_i$-induced phosphorylated enzyme), and the time and temperature used for incubation of membranes.

On the basis of the isoform selectivity screen described above, four DLCs were selected for further studies: ouabain, digoxin, and their hydrogenated forms, dihydro-ouabain and dihydro-digoxin. Hydrogenation of the lactone ring in these ligands has different consequences. Whereas the α1-isoform bound dihydro-ouabain and ouabain with comparable affinities, the dihydro-digoxin affinity was 2.5-fold lower compared with that of digoxin. The affinities observed for the α2-isoform were decreased significantly by hydrogenation in both cases. From a series of mutagenesis studies (Figs. 2 and 3), two residues located in the first extracellular loop of the α2-isoform were shown to influence isoform selectivity of dihydro-ouabain, dihydro-digoxin, and digoxin: it appears that Met$_{119}$ is responsible for the decreased affinity for dihydro-ouabain as well as the higher digoxin affinity observed for the α2-isoform compared with that for the α1-isoform, whereas Ser$_{124}$ is responsible for the decreased affinity of the α2-isoform for dihydro-ouabain and dihydro-digoxin.

Met$_{119}$ and Ser$_{124}$ are located in the first extracellular loop, which is highly important for DLC binding as evidenced by mutagenesis studies as well as crystal structures, although the recent high-affinity ouabain bound crystal structure predicts that neither residue interacts directly with ouabain. Instead, the residue corresponding to Met$_{119}$ (Thr$_{119}$) is located at the entry site of the DLC binding pocket, lining the sugar binding cavity, whereas the amino acid corresponding to Ser$_{124}$ (Gln$_{124}$) faces the lipid bilayer. However, careful analysis of various available crystal structures representing the [Na$_3$][E], P$_i$-ADP, E$_i$-P-ouabain, and [K$_3$] E$_i$-P states shows that the αM1-αM2 and αM3-αM4 extracellular segments undergo large conformational changes during the Na$_+_K$-ATPase reaction cycle (Fig. 6). Located
within the first extracellular loop. Met\textsuperscript{119} and Ser\textsuperscript{124} are involved in various hydrogen bonding networks involving the extracellular loops αM7-αM8 and αM9-αM10. Also, both residues are located close to Glu\textsuperscript{122}, which forms a hydrogen bond to the steroid core of ouabain in the high affinity structure.

Met\textsuperscript{119} and Ser\textsuperscript{124} have been shown to play an important role in the ouabain binding kinetics of the α1- and α2-isoforms in Xenopus laevis.\textsuperscript{15} The authors showed that the ouabain association and dissociation rates of the α2-isoform were decreased in parallel upon substitution of Met\textsuperscript{119} and Ser\textsuperscript{124} with their α1-isoform corresponding amino acids. On the basis of the crystal structure, Met\textsuperscript{119} and Ser\textsuperscript{124} are not predicted to interact directly with ouabain in the high affinity E\textsubscript{P,}P-ouabain complex, yet docking of dihydro-ouabain and ouabain in the DLC binding site is highly similar as evidenced by molecular docking studies (Fig. 6). Therefore, we hypothesize that the mutations described here mainly affect the ligand–receptor interaction during the association state of ligand binding. Still, it is worth mentioning that electrophysiological studies have provided evidence that dihydro-ouabain has an increased dissociation rate when compared with that of ouabain, implicating the residues identified here in the dissociation process.\textsuperscript{31, 32}

Met\textsuperscript{119} plays a two-sided role in DLC selectivity: the presence of an amino acid containing a hydrophobic side chain is shown here to benefit digoxin affinity, whereas the presence of a hydroxyl group decreased the dihydro-ouabain and dihydro-digoxin affinities (opposite to a previous study which, based on lanthanide-based resonance energy transfer (LRET), hypothesized an alternative docking sequence with the lactone ring facing the extracellular side, which has been addressed in the high-affinity crystal structure paper.\textsuperscript{8, 33} Also, no spatial clashes between the lactone ring of dihydro-ouabain and the cavity are predicted. On the basis of previous studies pointing toward a Mg\textsuperscript{2+} bound in cation binding site II as a key determinant for high affinity ouabain binding, a hypothesis for the general loss of affinity following hydrogenation of the lactone ring is that the polarization of the lactone leads to a reduced ability to form long-range electrostatic interactions. Also, the saturation of the ring leads to loss of planarity around C20 and loss of the ability to form electrostatic π-interactions. Our docking calculations predict that the orientation of dihydro-ouabain is similar to that of ouabain, with its modified lactone moiety pointing toward the cation binding site, an apparent contrast to a previous study which, based on lanthanide-based resonance energy transfer (LRET), hydrogenation of the lactone ring.\textsuperscript{34}

In conclusion, we performed a screen for isoform selectivity of various DLCs and identified two amino acids in the first extracellular loop of Na\textsuperscript{+},K\textsuperscript{-}-ATPase that determine the selectivity of hydrogenated DLCs for the α1- and α2-isoforms. It is most likely that this interaction affects the initial binding steps in the ligand-receptor interaction.

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**References**

Chapter 3

Na⁺,K⁺-ATPase isoform selectivity for DLCs is caused by two amino acids


Chapter 4

Biochemical characterization of sporadic/familial hemiplegic migraine mutations

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Abstract
Sporadic hemiplegic migraine type 2 (SHM2) and familial hemiplegic migraine type 2 (FHM2) are rare forms of hemiplegic migraine caused by mutations in the Na⁺,K⁺-ATPase α2-encoding gene. Today, more than 70 different mutations have been linked to SHM2/FHM2, randomly dispersed over the gene. For many of these mutations, functional studies have not been performed. Here, we report the functional characterization of nine SHM2/FHM2 linked mutants that were produced in Spodoptera frugiperda (Sf)9 insect cells. We determined ouabain binding characteristics, apparent Na⁺ and K⁺ affinities, and maximum ATPase activity. Whereas membranes containing T345A, R834Q or R879W possessed ATPase activity significantly higher than control membranes, P796S, M829R, R834X, del 935-940 ins Ile, R937P and D999H membranes showed significant loss of ATPase activity compared to wild type enzyme. Further analysis revealed that T345A and R879W showed no changes for any of the parameters tested, whereas mutant R834Q possessed significantly decreased Na⁺ and increased K⁺ apparent affinities as well as decreased ATPase activity and ouabain binding. We hypothesize that the majority of the mutations studied here influence interdomain interactions by affecting formation of hydrogen bond networks or interference with the C-terminal ion pathway necessary for catalytic activity of Na⁺,K⁺-ATPase, resulting in decreased functionality of astrocytes at the synaptic cleft expressing these mutants.

Introduction
Na⁺,K⁺-ATPase is a cation-transporting membrane protein involved in active transport of sodium and potassium ions against their concentration gradients across the cell membrane. For every molecule of ATP consumed, three sodium ions are exported from the cell in exchange for import of two potassium ions. Na⁺,K⁺-ATPase serves many roles, including cell-volume homeostasis as well as maintenance of the membrane potential. In addition, the generated Na⁺ and K⁺ gradients drive various secondary active transporters. Since transport of Na⁺ and K⁺ across the cell membrane takes place against such steep concentration gradients, the enzyme consumes high amounts of energy: approximately 25-50% of cellular ATP is consumed by Na⁺,K⁺-ATPase.1,2 Na⁺,K⁺-ATPase consists of at least two different subunits: an α-subunit (110 kDa), containing ten transmembrane α-helices (αM1-αM10) and two large cytosolic loops (αM2-αM3 and αM4-αM5), together with a highly glycosylated single transmembrane domain spanning β-subunit (50 kDa). In some tissues, a third subunit containing a characteristic FXYD motif is present as well (γ-subunit).3 In the human genome, four α-subunit encoding genes have been described. The proteins encoded by these genes share a sequence homology of approximately 80-90% and are expressed tissue specifically.4 For example, the α1-isoform is expressed ubiquitously5, whereas the α4-isoform is detected only in the testis.6 The α3-isoform is present in both the central and peripheral nervous systems7,8, whereas the α2-isoform is expressed in different tissues including heart, skeletal, and vascular smooth muscle, bone, adipocytes, and astrocytes.9 Genetic studies have linked over 70 mutations in the ATP1A2 gene (chromosome 1q23) encoding the α2-isoform of Na⁺,K⁺-ATPase to sporadic/familial hemiplegic migraine type 2 (SHM2/FHM2), shown in figure 1.10 Sporadic (only one affected family member) and familial (two or more affected family members) hemiplegic migraine are autosomal-dominant monogenic subtypes of migraine with aura (MA), characterized by neurologically aura symptoms preceding the migraine attack. In addition to the mutations present in the α2-isoform of Na⁺,K⁺-ATPase, two other genes have been linked to FHM: CACNA1A has been linked to SHM1/FHM1 whereas SCN1A has been associated with FHM3 (no sporadic mutations reported).11 Together, SHM and FHM show a prevalence of approximately 0.005%.12,13 The exact pathophysiology of SHM2/FHM2 remains to be elucidated, but it is assumed that α2-isoform Na⁺,K⁺-ATPase mutations result in altered enzyme functionality at the synaptic cleft, where astrocytes expressing the α2-isoform are present as supporting cells. The Na⁺,K⁺-ATPase present at the plasma membrane of these cells presumably plays a role in uptake of K⁺ from the synaptic cleft following nerve excitation, resulting in a favorable driving force for the re-uptake of glutamate by the co-localized glutamate transporter.14 Changes in the enzymatic properties of Na⁺,K⁺-ATPase might therefore have an indirect effect on signal transmission via the synaptic cleft, leading to hemiplegic migraine attacks.
Over 70 SHM2/FHM2 causing mutations in the ATP1A2 gene have been reported, randomly dispersed over the gene. For many of these mutations the effects on enzyme functionality have not been reported yet, whereas investigation of the biochemical effects of each of these mutations on protein functionality is an essential step towards understanding the pathogenesis of SHM2/FHM2 mutations. Using Spodoptera frugiperda (Sf)9 insect cells lacking endogenous Na+,K+-ATPase, we set out to characterize the enzymatic effects of nine SHM2/FHM2 mutations (all except T345A) located within the carboxy-terminal transmembrane segment: T345A, P796S, M829R, R834Q, R834X (truncated protein), R879W, del 935-940 ins Ile, R937P and D999H. The carboxy-terminal segment is present only in PII- and PIIIA-type ATPases of the P-type ATPase family. It plays a vital role in the catalytic activity of Na+,K+-ATPase as was illustrated by the discovery of an essential C-terminal half-channel forming a gateway to the third ion-binding site, allowing protons to access the third ion-binding site as required for K+ occlusion. Interestingly, various SHM2/FHM2 mutations have been mapped to this half-channel, leading us to investigate extensively the biochemical effects of mutations located within this region of Na+,K+-ATPase.

Figure 1 Mutant localization.

Homology model of the α2-isoform of Na+,K+-ATPase (based on PDB file 2ZXE*) showing the residues that are associated with SHM2/FHM2 mutations in literature highlighted in green, whereas the mutations described in the present study are highlighted in yellow. The α-subunit is shown in blue, whereas red represents the β-subunit: these two subunits together constitute a functional Na+,K+-ATPase.

Materials and methods

The Gateway system

The cDNA for the α2-isofrom was obtained by purchasing a vector (Thermo Scientific MHS1011-9199751) containing the cDNA obtained from a human teratocarcinoma cell line (cDNA: NM_000702). The desired mutations were obtained by performing two PCRs (A and B) on human Na+,K+-ATPase α2-encoding wild type cDNA using either a 5'- or 3'-primer together with a mutagenesis primer (Biolegio, Nijmegen, the Netherlands) containing the desired mutation. This resulted in generation of two fragments that were subsequently combined in a PCR (C) using only the 5'-and 3'-primers, allowing for annealing of the two fragments. Using Gateway cloning (Life Technologies, Breda, the Netherlands), the mutated Na+,K+-ATPase α2-encoding gene was then transferred to an entry vector containing a kanamycin resistance cassette using BP Clonase II enzyme according to the manufacturer’s instructions. After successful transformation of entry clones into Escherichia coli DH5α cells and overnight selection on kanamycin LB-agar plates, colonies were inoculated in LB medium containing kanamycin, grown overnight and subsequently isolated using the Genelute™ mini-prep isolation kit (Sigma-Aldrich). Then, full-length sequencing of the different constructs was performed to check for successful mutagenesis. Subsequently, expression clones were obtained by inserting the α2-encoding gene carrying the mutation in the Gateway Reading Frame Cassette B behind a polyhedrin promoter in an ampicillin resistant pFastBac Dual transfer vector containing the β1-subunit behind the p10 promoter using LR Clonase II enzyme (Life Technologies, Breda, the Netherlands). After successful transformation of expression clones into E. coli DH5α cells and overnight selection on ampicillin LB-agar plates, colonies were inoculated in LB medium containing ampicillin, grown overnight and subsequently isolated using the Genelute™ mini-prep isolation kit (Sigma-Aldrich). Throughout the cloning procedures, YFP was used as a negative control, resulting in the generation of an expression clone encoding YFPβ1.

Generation of recombinant baculoviruses

The expression clones generated using the Gateway system were transformed to competent DH10Bac E. coli cells (Life Technologies, Breda, the Netherlands) harboring the baculovirus genome (bacmid) and a transposition helper plasmid, conferring resistance against kanamycin, gentamycin, and tetracycline. Upon transposition between the Tn7 sites present in both the bacmid and expression clones, recombinant bacmids were selected on LB-agar plates containing kanamycin, gentamycin, and tetracycline. Subsequently, resistance-selected bacmids were transfected to S. frugiperda (Sf)9 insect cells grown in Grace medium (Sigma, Bornem, Belgium) at 27 °C in 175 cm² monolayers using Cellfectin reagent (Life Technologies, Breda, the Netherlands). After a 6-day period, recombinant baculoviruses were harvested and used to infect fresh Sf9 cells at a multiplicity of infection of 0.1. After another 6 days of culture of infected Sf9 cells, amplified baculoviruses were harvested.
Protein production using recombinant virus

Sf9 cells grown at 27 °C in 175 cm² monolayers and later in 500 mL shaking flasks in Xpress medium (Sigma, Bornem, Belgium) were infected at a density of 1.5 ·10⁶ cells·mL⁻¹ in the presence of 1% (v/v) ethanol as described before. After three days of infection, the cells were harvested by centrifugation at 2,000 × g for 5 min. Then, the pelleted cells were resuspended at 0 °C in a 0.25 M sucrose, 2 mM EDTA and 20 mM Heps/Tris (pH 7.0) buffer. Sonication was performed for 30 s at 80 W prior to centrifugation at 10,000 × g at 4 °C for 30 min, followed by centrifugation of the resulting supernatant at 100,000 × g at 4 °C for 60 min. The pelleted membranes were resuspended using the buffer mentioned above, homogenized and stored at -20 °C and the protein concentration was determined using the modified Lowry method.

Western blotting

Approximately 10 µg of membranes was treated with SDS-PAGE solubilization buffer overnight at room temperature before loading on a 10% polyacrylamide gel, as described previously. After separation, the proteins were transferred overnight to a nitrocellulose membrane at 30 V. The alpha- and beta-subunits were detected using either the C356-M09 (α-subunit) or the C385-M77 (β-subunit) antibodies, respectively.

Ouabain binding experiments

Ouabain binding was determined by incubation of approximately 150-200 µg of membranes in the presence of 20 mM histidine, 5 mM MgCl₂, 5 mM H₃PO₄, pH 7.0 and 25 nM of radiolabeled ouabain (Perkin-Elmer, Waltham, MA, USA) in a final volume of 60 µL at room temperature for 2 h. After incubation for 15 min on ice, the amount of bound ouabain was determined by washing the samples over a 0.8 µm ME27 filter using distilled H₂O, retaining the enzyme-bound ouabain to the filters. The amount of radioactivity on the filters was then determined in a liquid scintillation counter after addition of 4 mL OptiFluor (Canberra Packard, Tilburg, the Netherlands). Correction for endogenous ouabain binding was performed by subtracting the amount of ouabain bound in YFPβ1 samples.

ATPase activity studies

ATPase activity was determined in the Na⁺ or K⁺ affinity studies by incubating 20 µL of membranes in a final volume of 100 µL containing 50 mM Tris-Ac pH 7.0, 0.1 mM EGTA, 1.2 mM MgCl₂, 1.0 mM Tris-Ν₂, and 100 µM of radiolabeled ATP in the presence of 50 mM NaCl or 5.0 mM KCl and increasing concentrations of KCl or NaCl, respectively. After incubation of the samples for 30 min at 37 °C, the reaction was stopped by addition of 500 µL of 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid and, after 10 min at 0 °C, the mixture was centrifuged for 30 s (10,000 × g). Subsequently, 150 µL of the clear supernatant was mixed with 4 mL OptiFluor before analysis using a liquid scintillation counter to determine the amount of formed P₃ (inorganic phosphate), a measure of ATPase activity. Na⁺,K⁺-ATPase specific activity was determined by subtracting the amount of P₃ formed in the presence of 1.0 mM ouabain. [γ-³²P]ATP (3000 Ci mmol⁻¹) was obtained from Perkin-Elmer (Waltham, MA, USA).

Data analysis

All data were analyzed using Graphpad Prism 5.02. Repeated measures ANOVA was used to test for differences in apparent Na⁺ and K⁺ affinities and maximum ATPase activity levels compared with α2-wild type enzyme, with α=0.05. Ouabain binding characteristics (maximum ouabain binding (Eₙₙₒₘₚₐₓ)) and Kd) were tested for differences compared to wild type enzyme using Students t-test. With regard to the Na⁺ and K⁺ affinities the Origin 6.1 (Microcal, Northampton, MA) software was used to perform non-linear regression analysis. The Na⁺ and K⁺ values (defined as the concentrations of Na⁺ and K⁺ resulting in half-maximal activation) were calculated via the Hill equation using the averaged data and SEM values, and the Hill coefficients (nH) obtained for wild type Na⁺,K⁺-ATPase were also used for calculation of the mutant half-maximum Na⁺ and K⁺ concentrations. Maximum ATPase activity was determined in the presence of 50 mM NaCl, 5.0 mM KCl, 1.2 mM MgCl₂ and 0.1 mM ATP at pH 7.0.

Structural analysis

Homology models based on different crystal structures of the α1β1 Na⁺,K⁺-ATPase (enzyme obtained from pig and shark) and sarcoendoplasmic reticulum Ca²⁺-ATPase (SERCA) were used as templates to investigate the possible interactions between residues of interest using the YASARA software. The following PDB entries were used (corresponding enzyme conformation): 2C9M (E1Na⁺), 1T5S (E1Na⁺-ATP), 1T5T (E1Na⁺-P-ADP), 3B9R (E1Na⁺-P), 3B9B (E2K⁺-P-ATP), 2ZXE (E2K⁺-P), and 2C88 (E2K⁺ ATP). The homology models were built using the YASARA software and the WHAT IF Twinset using default parameters. YASARA-calculated Z-scores were used for validation and selection of the best model and ranged from -1.990 (3B9R), -1.788 (3B9B), -1.762 (2C9M), -1.699 (1T5T), -1.644 (3B9A), -1.557 (2C88), -1.548 (1T5S) to -0.454 (2ZXE). Minimization was performed using an automatic YASARA protocol and default settings.

Results

General characterization of ATP1A2 migraine mutants

We selected 9 ATP1A2 SHM2/FHM2 causing mutations identified in the C-terminal part of the α2-subunit of Na⁺,K⁺-ATPase to investigate the effects of these mutations on the enzyme's apparent Na⁺, K⁺, and ouabain affinities, as well as maximum ATPase activity and ouabain binding. Baculovirus infected Sf9 insect cells were used to obtain membrane fragments containing the desired mutants. Figure 2 shows that the amount of protein detected was
equal for all mutants compared to wild type enzyme, indicating similar expression levels. As expected, the α-subunit could not be detected in membranes from cells infected with YFP (neg. control).

Subsequently, we investigated protein functionality as reflected by ouabain binding. Ouabain is a well-known and highly specific ligand of Na⁺,K⁺-ATPase that binds in a pocket constituted by the first six transmembrane spanning domains and specific residues present in several extracellular loops connecting these transmembrane domains. Therefore, binding of ouabain implies that the first six transmembrane domains of Na⁺,K⁺-ATPase are properly folded. Figure 3 shows the amount of ouabain binding under equilibrium after incubation with 25 nM radiolabeled ouabain. Wild type, T345A, R879W, R834Q, and R937P membranes showed significantly higher ouabain binding levels compared with control membranes expressing YFP.

Next, we tested the ATPase activity of all mutants by incubation of membranes in the presence of 50 mM NaCl, 5.0 mM KCl, 100 µM ATP and 1.2 mM MgCl₂ for 30 min. Table 1 shows that mutants T345A, R834Q and R879W possessed significantly higher ATPase activity than YFP control membranes. We observed that two mutants (R879W and R834Q) showed decreased levels of maximum ATP conversion compared with wild type enzyme: R834Q showed a statistically significant decrease of 78% (P = 0.03) and mutant R879W showed a near-significant decrease in ATPase activity of 49%.

Based on the presence of ATPase activity as described above, we tested mutants T345A, R879W and R834Q with regard to their ouabain binding characteristics (Fig. 4, Table 2) and determined the maximum ouabain binding (E_{Omax}) as well as ouabain affinities for the different mutants. E_{Omax} values ranged from 4.8 pmol/mg for wild type enzyme to 6.3 pmol/mg for T345A, whereas mutants R879W and R834Q showed lower maximum ouabain levels of 2.7 and 0.5 pmol/mg, the latter being significantly decreased when compared

Figure 2 Expression of mutants.

Western blot showing equal protein expression for all mutants tested in this study. For each mutant, 10 µg of Sf9 membranes was incubated overnight in solubilization buffer and used for SDS-PAGE using 10% acrylamide gels followed by transfer to a nitrocellulose membrane and subsequent detection with antibodies against the α-subunit (C356-M09) and β-subunit (C385-M77). Note that the lower band for mutant R834X (insertion stop codon) reflects a truncated protein, as expected.

Figure 3 Ouabain binding screen.

Binding was determined using scintillation counting after two hours of incubation with 25 nM ouabain at room temperature. Compared to the negative control membranes expressing YFPβ1, wild type and mutants T345A, R834Q, R879W, and R937P showed significantly increased levels of ouabain binding at equilibrium. * = p < 0.05 versus neg. control.

Table 1 Maximum ATPase activity.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Max. ATPase (µmol P, mg⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1.05 ± 0.27</td>
</tr>
<tr>
<td>T345A</td>
<td>1.34 ± 0.38</td>
</tr>
<tr>
<td>P796S</td>
<td>-0.02 ± 0.03*</td>
</tr>
<tr>
<td>M829R</td>
<td>0.00 ± 0.01*</td>
</tr>
<tr>
<td>R834Q</td>
<td>0.23 ± 0.05*</td>
</tr>
<tr>
<td>R834X</td>
<td>-0.03 ± 0.02*</td>
</tr>
<tr>
<td>R879W</td>
<td>0.54 ± 0.08</td>
</tr>
<tr>
<td>del 935-940 ins le</td>
<td>-0.05 ± 0.01*</td>
</tr>
<tr>
<td>R937P</td>
<td>-0.04 ± 0.01*</td>
</tr>
<tr>
<td>D999H</td>
<td>-0.02 ± 0.01*</td>
</tr>
</tbody>
</table>

Results were obtained by averaging the amount of ATP consumed at 37 °C for 30 min in a medium consisting of 50 mM NaCl, 5.0 mM KCl and 100 µM ATP, corrected for non-specific ATPase activity by incubation of membranes with 1.0 mM ouabain and subsequently background-corrected by subtracting the activity observed for YFPβ1 expressing control membranes. * = p < 0.05 versus wild type.
with wild type enzyme. The ouabain affinities were identical for wild type and T345A ($K_d = 21 \pm 11$ and $21 \pm 6$ nM), whereas R879W and R834Q showed non-significant increases in ouabain affinities compared with wild type enzyme ($K_d = 12 \pm 3$ and $12 \pm 4$ nM).

**Apparent Na⁺ and K⁺ affinities and turnover numbers**

6 out of 9 mutants possessed no significant ATPase activity (table 1), providing a hypothesis for their pathogenicity: loss of catalytic activity. However, the lack of ATPase activity also excluded these mutants from further biochemical characterization using ATPase related studies. As a result, we could only determine the Na⁺ and K⁺ affinities for the remaining three mutants: T345A, R834Q, and R879W (Fig. 5, Table 2).

The apparent Na⁺ affinity was studied by investigating the amounts of radioactive ATP hydrolyzed under increasing concentrations of NaCl in the presence of 5.0 mM KCl, 100 µM ATP and 1.2 mM MgCl₂. The results of these experiments can be observed in figure 5A and table 2. We observed no significant changes for mutants T345A and R879W compared with wild type, whereas mutant R834Q showed a significant two-fold decrease in apparent Na⁺ affinity compared with wild type (P = 0.0007).

The apparent K⁺ affinity was determined from the amounts of radioactive ATP hydrolyzed under increasing KCl concentrations in the presence of 50 mM NaCl, 100 µM ATP and 1.2 mM MgCl₂. The results of these experiments are shown in figure 5B and table 2. There were no significant changes in apparent affinities for T345A and R879W compared with wild type enzyme, whereas mutant R834Q showed a significant two-fold increase in apparent K⁺ affinity (P = 0.013). This finding is a mirror image of the result observed for the Na⁺ affinity of the same mutant, which showed a two-fold decrease in affinity.

**In silico studies based on homology models**

Finally, we performed in silico mutagenesis studies using homology models based on crystal structures of the α1β1-isoform of Na⁺,K⁺-ATPase as well as the highly similar sarcoplasmic reticulum Ca²⁺-ATPase (SERCA). For the three mutants showing ATPase activity, no interactions involving Tyr345 and Arg879 or their substitutes (alanine and tryptophan, respectively) were predicted, however a hydrogen bond network was predicted between residue Arg834 and residues Glu285 and Glu363 in the E₂K⁺·Pi conformation, Asp767 in the E₂P conformation, and Asp839 in the E₂K⁺ ATP conformation (Fig. 6). Regarding the six

**Figure 4 Ouabain binding characteristics.**

Membranes were incubated with different concentrations of radiolabeled ouabain at room temperature for 2 h, followed by filtration over ME-27 filters. Radioactivity retained on the filters, containing the enzyme-ouabain complex, was subsequently quantified using liquid scintillation analysis.

**Figure 5 Affinities for Na⁺ and K⁺.**

Average (n ≥ 3) background corrected Na⁺ (A) and K⁺ (B) ATPase activities for wild type, T345A, R834Q and R879W enzymes. ATPase activities were determined by measuring the amount of radioactive ATP hydrolyzed after incubation of membranes for 30 min at 37 °C under different concentrations of either NaCl (A) or KCl (B).

**Table 2 Overview of results.**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Max. ATPase activity (µmol P/mg⁻¹ hour⁻¹)</th>
<th>Max. binding (µmol mg⁻¹)</th>
<th>$K_{d,ATP}$ (nM)</th>
<th>$K_{d,ouabain}$ (nM)</th>
<th>Affinity (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1.00 ± 0.3</td>
<td>1.10 ± 0.1</td>
<td>5.9 ± 0.2</td>
<td>4.80 ± 1.2</td>
<td>21.0 ± 10.4</td>
</tr>
<tr>
<td>T345A</td>
<td>1.34 ± 0.4</td>
<td>1.30 ± 0.1</td>
<td>6.2 ± 0.2</td>
<td>6.30 ± 2.1</td>
<td>21.0 ± 6.3</td>
</tr>
<tr>
<td>R834Q</td>
<td>0.23 ± 0.1*</td>
<td>0.50 ± 0.1*</td>
<td>11.6 ± 0.4*</td>
<td>0.50 ± 0.1*</td>
<td>11.8 ± 4.0</td>
</tr>
<tr>
<td>R879W</td>
<td>0.54 ± 0.1</td>
<td>1.00 ± 0.0</td>
<td>6.5 ± 0.3</td>
<td>1.00 ± 0.0</td>
<td>11.0 ± 3.0</td>
</tr>
</tbody>
</table>

Characteristics of ATPase activity and ouabain binding for wild type, T345A, R834Q and R879W enzymes. ATPase experiments using radiolabeled ATP were performed to determine apparent Na⁺ and K⁺ affinities and maximum ATPase values, whereas radiolabeled ouabain was used to determine apparent ouabain affinity and maximum binding level. $nH = Hill coefficient$, $^* = p < 0.05$ versus wild type.
mutants showing a complete loss of ATPase activity: a hydrogen bond was predicted between serine when introduced at position 796 (P796S) and Arg484, a residue predicted to interact with Gln907 as well. Also, introduction of arginine at residue 829 (M829R) led to formation of two hydrogen bonds with Val756.

Discussion

We investigated the biochemical characteristics of nine mutations/deletions in the α2-isoform of Na+,K+-ATPase linked to sporadic/familial hemiplegic migraine type 2 (SHM2/FHM2) in order to increase our knowledge on the pathogenesis of each of these mutations. All mutants could be expressed in Sf9 insect cells. However, further experiments revealed that six mutants could not be studied in detail due to significant loss of ATPase activity. Three mutants (T345A, R834Q, and R879W) were characterized biochemically, showing significant effects on apparent Na+- and K+-affinities for mutant R834Q. Below, we will describe the findings for each mutant and provide hypotheses on how each mutation might lead to the observed effects.

T345A was linked to FHM2 in a study describing a Finnish family of which 10 individuals were experiencing FHM attacks, whereas two individuals carried the mutation but most likely did not yet reach the age of onset-of-disease. We did not detect significant changes for any of the parameters tested, although we observed a slightly higher maximum ATPase activity, which was accompanied by a parallel increase in EQmax. Although we were not able to identify any interactions of the highly conserved Tyr345, Van der Waals' interactions with Val362 were hypothesized before. Previously, a decreased K’ affinity and unchanged turnover number, which were linked to a reduced maximum phosphorylation rate. In contrast to these studies, we used a non-mammalian expression system with two important benefits over the two expression systems mentioned above: the absence of background Na+,K+-ATPase as well as equimolar expression of the α- and β-subunits. The differences between these expression systems might provide explanations for the differences between these studies and our results. The R834Q mutation was discovered in a French family containing eleven individuals suffering from FHM. We identified a significantly decreased apparent Na’ affinity, increased apparent K’ affinity, and decreased maximum ATPase activity and maximum ouabain binding compared to wild type enzyme. An increase in apparent K’ affinity has been reported before, but we are the first to report a decrease in apparent Na’ affinity for this mutation. Based on the lower maximum ATPase activity, re-uptake of K’ from the synaptic cleft might be decreased, although an increased apparent K’ affinity could diminish this effect. As predicted by the homology models used in this study, Arg484 is involved in interactions with other residues present in the cytosolic domains of Na+,K’-ATPase, serving an important role in interactions between different domains during the catalytic cycle as pointed out before. Being located in the cytosolic loop between the sixth and seventh transmembrane domains (αM6-αM7), Arg484 is also part of the C-terminal half-channel, serving as a gateway to the third ion-binding site by allowing protons to access this site as required for K’ occlusion. We hypothesize that substitution of Arg484 with a glutamine (resulting in removal of a positive charge at this residue) disturbs hydrogen bond interaction networks shown in figure 6, thereby affecting the structure and/or accessibility of the C-terminal half-channel, interfering indirectly with apparent Na’ and K’ affinities and the transitions between different conformations.

Mutation P796S was linked to FHM2 in a Portuguese family by a cell assay that showed no survival of HeLa cells transfected with the mutated Na+,K’-ATPase, inferring pathogenicity for this mutation. Mutation R879W was found in a screen for mutations in patients suffering from sporadic hemiplegic migraine (SHM) and was identified together with another mutation of the same non-conserved residue, R879Q. Although no mutations were detected in 92 controls, both ATP1A2 mutations were also found in non-affected family members, questioning the dominance in SHM. Our data seem to confirm this lack of causality, as they show a non-significant increase in ouabain affinity as well as a non-significant decrease in apparent maximum ATPase activity compared to wild type enzyme. Based on analysis of different homology models, we were not able to identify any hydrogen bond interacting residues for Arg484, in line with the low conservation of this residue among different isoforms. Previously, we reported on another mutation at the same position (R879Q), which showed only minor changes in the enzyme’s biochemical properties, leading us to conclude that this mutation might not be pathogenic. Here, we conclude the same for R879W, strengthening our hypothesis that there is no link to SHM for mutations involving Arg484.

Mutation P796S was linked to FHM2 in a Portuguese family by a cell assay that showed no survival of HeLa cells transfected with the mutated Na+,K’-ATPase, inferring pathogenicity for this mutation. Introduction of serine resulted in prediction of a hydrogen bond with Arg484, which in turn is involved in a hydrogen bond with Gln907, located in the C-terminal half of the extracellular loop between αM7 and αM8, implicated in interactions between the α- and β-subunits. Possibly, substitution of proline with serine results in affected interactions between different domains during the catalytic cycle as pointed out before. Being located in the cytosolic loop between the sixth and seventh transmembrane domains (αM6-αM7), Arg484 is also part of the C-terminal half-channel, serving as a gateway to the third ion-binding site by allowing protons to access this site as required for K’ occlusion. We hypothesize that substitution of Arg484 with a glutamine (resulting in removal of a positive charge at this residue) disturbs hydrogen bond interaction networks shown in figure 6, thereby affecting the structure and/or accessibility of the C-terminal half-channel, interfering indirectly with apparent Na’ and K’ affinities and the transitions between different conformations.

Figure 6 Interactions involving Arg484.

Conformation-dependent hydrogen bond interactions between Arg484 and various other amino acids that take place during the Na’-K’-ATPase reaction cycle. Arg484 is predicted to interact with Glu500 and Glu504 in the E,K’-Pi conformation (homology model based on 2ZXE), Arg484 in the E,P conformation (homology model based on 3B98), and Arg484 in the E,K’-ATP conformation (homology model based on 2C88).
between both subunits leading to a complete loss of ATPase activity as observed here, or might result in improper insertion of the enzyme into the membrane due to loss of these interactions.

M829R was identified in a study describing multiple new mutations linked to FHM2 and subsequently reported to result in a minor increase in apparent K⁺ affinity in oocytes. Our study shows a complete loss of ATPase activity for this mutant, implying an even more severe effect of this mutation. Again, the difference in experimental results might be related to the insect cells used here, which are cultured at 27 °C instead of 18 °C used for oocytes. Based on analysis of the first crystal structure of Na⁺,K⁺-ATPase, the highly conserved Met has been implicated in important interactions stabilizing the interdomain interface region between αM6 and αM7 during the catalytic cycle. Therefore, it is likely that replacement of methionine with arginine results in disturbance of these essential hydrogen bond networks, leading to loss of ATPase activity as shown here.

The mutations R834X, del 935-940 ins Ile, R937P and D999H, all showing complete loss of ATPase activity, affect one or more residues involved in the structure of the C-terminal pathway. This half-channel controls the access of protons from the cytosol to the third proposed ion-binding site and plugs this channel upon occlusion of the third ion binding site (IIIb) by either Na⁺ (E-state) or H⁺ (E-state). Disruption of this half-channel could therefore easily lead to loss of ATPase activity resulting from the absence of Na⁺ and K⁺ occlusion, in particular for the mutations affecting multiple residues of the C-terminal segment including R834X (insertion stop codon) and del 935-940 ins Ile. Arg was found to play a role in controlling the C-terminal tyrosine residues essential for ATPase activity by forming hydrogen bonds and cation-π interactions. The same study also described Asp as one of the residues facing intowards into the proposed channel, highlighting the importance of this residue for ATPase activity as well. We hypothesize that mutation of this residue into a positively charged histidine disturbs C-terminal function, interfering with the affinity and occlusion of Na⁺ and/or H⁺, thereby indirectly affecting ATPase function.

In conclusion, we found a statistically significant decrease in maximum ATPase activity and apparent K⁺ affinity together with an increase in apparent Na⁺ affinity for mutation R834Q. Mutants T345A and R879W did not show any significant changes for the parameters studied here. Mutants P796S, M829R, R834X, del 935-940 ins Ile, R937P and D999H could not be tested due to a lack of ATPase activity, but this might explain how these mutations, as well as R834Q, lead to development of SHM2/FHM2 by affecting transport of K⁺ and Na⁺ between the synaptic cleft and supporting cells expressing the α2-isozyme of Na⁺,K⁺-ATPase. Lack of transport of Na⁺ and K⁺ is believed to affect the secondary active transport of glutamate eventually leading to decreased recovery from neural excitation associated with development of SHM2/FHM2 attacks.

Disclosure statement
The authors declare that there are no conflicts of interest.

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References


Chapter 5

Familial hemiplegic migraine mutations affect Na$^+$,K$^+$-ATPase domain interactions

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Abstract

Familial hemiplegic migraine (FHM) is a monogenic variant of migraine with aura. One of the three known causative genes, \textit{ATP1A2}, which encodes the α2-isoform of Na\textsuperscript{+},K\textsuperscript{-}-ATPase, causes FHM type 2 (FHM2). Over 50 FHM2 mutations have been reported, but most have not been characterized functionally. Here we study the molecular mechanism of Na\textsuperscript{+},K\textsuperscript{-}-ATPase α2-subunit missense mutations. Mutants E700K and P786L inactivate or strongly reduce enzyme activity. Glutamic acid 700 is located in the phosphorylation (P)-domain and the mutation most likely disrupts the salt bridge with lysine 35, thereby destabilizing the interaction with the actuator (A)-domain. Mutants G900R and E902K are present in the extracellular loop at the interface of the α- and β-subunits. Both mutants likely hamper the interaction between these subunits and thereby decrease enzyme activity. Mutants E174K, R548C and R548H reduce the Na\textsuperscript{+} and increase the K\textsuperscript{-} affinity. Glutamic acid 174 is present in the A-domain and might form a salt bridge with lysine 432 in the nucleotide binding (N)-domain, whereas arginine 548, which is located in the N-domain, forms a salt bridge with glutamine 219 in the A-domain. In the catalytic cycle, the interactions of the A- and N-domains affect the K\textsuperscript{-} and Na\textsuperscript{+} affinities, as observed with these mutants. Functional consequences were not observed for \textit{ATP1A2} mutations found in two sporadic hemiplegic migraine cases (Y9N and R879Q) and in migraine without aura (R51H and C702Y).

Introduction

Migraine is a disabling common brain disorder typically characterized by attacks of severe headache and associated with autonomic and neurological symptoms.\textsuperscript{1} Auras that consist of transient mainly visual and sensory symptoms can precede headaches in one-third of patients (hence migraine with aura). Auras are likely caused by cortical spreading depression (CSD) events, which are slowly propagating cortical waves of neuronal and glial depolarization.\textsuperscript{2} Familial hemiplegic migraine (FHM) is a rare monogenic variant of migraine with aura with some degree of hemiparesis during the aura.\textsuperscript{1} Of the three known FHM genes, two encode α1-subunits of voltage-gated Ca\textsubscript{2+} channels (\textit{CACNA1A}; FHM1) or voltage-gated Na\textsubscript{+} channels (\textit{SCN1A}; FHM3).\textsuperscript{3-5} \textit{ATP1A2} (FHM2) encodes the catalytic α2-subunit of the sodium potassium pump.\textsuperscript{6} This pump transfers Na\textsuperscript{+} out and K\textsuperscript{-} into the cell, using ATP as energy source. The sodium pump is present in all human cells, but the α2-isoform is mainly restricted to brain and muscle. In the adult brain the α2-isoform is located in glial cells where it generates the Na\textsuperscript{+} gradient that is essential for the re-uptake of the excitatory transmitter glutamate. Moreover, it also removes excess K\textsuperscript{-} from the intracellular space.\textsuperscript{7}

Since the discovery of the first FHM2 mutation in 2003,\textsuperscript{6} over 50 \textit{ATP1A2} mutations have been published, but only a minority have been functionally studied in detail.\textsuperscript{7} \textit{ATP1A2} mutations have been reported to be associated also with non-FHM phenotypes such as basilar migraine, alternating hemiplegia of childhood, and common migraine with or without aura.\textsuperscript{7} For most of the mutations, however, functional evidence supporting that these mutations are causal is lacking. Here we set out to investigate \textit{ATP1A2} mutations associated with FHM and non-FHM phenotypes in an attempt to find functional evidence for an association with a broader spectrum of migraine-related phenotypes. In addition, we investigated whether some of the mutations may be involved in specific domain interactions within the sodium potassium pump. Our functional analyses reinforce the association between \textit{ATP1A2} mutations and sporadic and familial hemiplegic migraine, but do not provide robust functional evidence for a relationship with other types of migraine.

Materials and methods

The Gateway system

The wild type and mutants of the Na\textsuperscript{+},K\textsuperscript{-}-ATPase α2-isoform were cloned by Gateway-adapted PCR procedures according to the manufacturer’s instructions (Invitrogen, Breda, The Netherlands). A destination vector was generated by subcloning the cDNA of the human Na\textsuperscript{+},K\textsuperscript{-}-ATPase β1-subunit in the pFastbacdual vector (Life Technologies, Breda, The Netherlands) after the p10 promoter and the α2-subunit in the Gateway Reading Frame Cassette B (Invitrogen) that was introduced after the polyhedrin promoter. The (mutated) entry vectors were recombined with this vector by using Gateway LR Clonase II Enzyme
Mix (Invitrogen). Site-directed mutagenesis was performed using the Single Base mutation system, DpnI method (Stratagene, La Jolla, CA). The mutagenic primer (Biolegio, Nijmegen, The Netherlands) introduced the desired mutation in the α-subunit. After selection the mutants were checked by Sanger direct sequence analysis.

**Generation of recombinant viruses**

The pFastbacduall transfer vector containing the different (mutant) cDNAs was transformed to competent DH10bac *Escherichia coli* cells (Life Technologies) harboring the baculovirus genome (bacmid) and a transposition helper vector. Upon transposition between the Tn7 sites in the transfer vector and the bacmid, recombinant bacmids were selected and isolated. Subsequently, insect Sf9 cells were transfected with recombinant bacmids using Cellfectin reagent (Life Technologies). After a three-day incubation period, recombinant baculoviruses were isolated and used to infect Sf9 cells at a multiplicity of infection of 0.1. Four days after infection, the amplified viruses were harvested.

**Preparation of Sf9 membranes**

Sf9 cells were grown at 27 °C in 175 cm² monolayers and later in 500 mL shaking flasks cultures. For production of Na⁺,K⁺-ATPase, 1.5 ·10⁶ cells mL⁻¹ were infected at a multiplicity of infection of 1-3 in the presence of 1% (v/v) ethanol, and 0.1% (w/v) Pluronic F-68 (Sigma, Bornem, Belgium) in Xpress medium (Biowittaker, Walkersville, MD) as described before. After 3 days, the Sf9 cells were harvested by centrifugation at 2,000 × g for 5 min. The cells were resuspended at 0 °C in 0.25 M sucrose, 2 mM EDTA, and 25 mM Hepes/Tris (pH 7.0), and sonicated for 30 s at 60W (Branson Power Company, Denbury, CT). After centrifugation for 30 min at 10,000 × g the supernatant was collected and centrifuged again for 60 min at 100,000 × g at 4 °C. The pelleted membranes were resuspended in the above-mentioned buffer and stored at -20 °C and the protein concentration was determined with the modified Lowry method.

**Western blotting**

Protein samples from the membrane fraction were solubilized in SDS-PAGE sample buffer and separated on SDS-gels containing 10% acrylamide as described previously. For immunoblotting, the separated proteins were transferred to Immobilon polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA). The α-subunit of Na⁺,K⁺-ATPase was detected with the polyclonal antibody C356-M09.10

**Na⁺,K⁺-ATPase assay**

The ouabain sensitive ATPase activity was determined using a radiochemical method. For this purpose, 0.6-5 µg of Sf9 membranes were added to 100 µL of medium, which contained 10-200 µM [γ-32P]-ATP (specific activity 20-100 mCi mmol⁻¹), 1.2 mM MgCl₂, 0.2 mM EGTA, 0.1 mM EDTA, 1.0 mM Tris-Na⁺, 25 mM Tris-HCl (pH 7.0) and various concentrations of KCl and NaCl in the presence and absence of 0.1 mM ouabain. After incubation for 30 min at 37 °C, the reaction was stopped by adding 500 µL 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid and after 10 min at 0 °C the mixture was centrifuged for 10 s (10,000 × g), to 0.15 mL of the cleared supernatant, containing the liberated inorganic phosphate (32P), 3 mL OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added and the mixture was analyzed by liquid scintillation analysis. In general, blanks were prepared by incubating in the absence of membranes. The Na⁺,K⁺-ATPase activity is presented as the difference of the activity in the absence and presence of ouabain.

**Ouabain binding capacity**

Ouabain binding was determined as described before. Sf9 membranes (100 µg) were incubated at 21 °C in 20 mM histidine (pH 7.0), 5.0 mM MgCl₂, and 5.0 mM P₃ in a volume of 50 µL. 10 µL of [3H]-ouabain (specific activity 30 Ci mmol⁻¹, Perkin Elmer) was added and the mixture was incubated for 2 h at 21 °C. The protein was collected by filtration over a 0.8 µm membrane filter (Schleicher and Schuell, Dassel, Germany). After washing twice with 2 mL water (4 °C), the filters were analyzed by liquid scintillation analysis. Data are corrected for the levels of nonspecific ouabain binding obtained with mock-infected membranes.

**Chemicals**

Cellfectin, competent DH10bac *Escherichia coli* cells and all enzymes used for DNA cloning were purchased from Invitrogen (Breda, The Netherlands). [γ-32P]ATP (3000 Ci mmol⁻¹ was obtained from Perkin-Elmer (Waltham, MA, USA).

**Analysis of data**

All data are presented as mean values for three individual enzyme preparations with standard error of the mean. Differences were tested for significance by means of the Student’s t-test. IC₅₀ and K₅₀ values were determined by analyzing the plots using the Non-Linear Curve Fitting program (Hill equation function) of Origin 6.1 (Microcal, Northampton, MA). The Na⁺₀ and K⁺₀ values were calculated via the Hill equation in Origin, on the averaged data with standard error of the mean. The Hill coefficient value obtained for the wild type Na⁺,K⁺-ATPase (1.73 for Na⁺ and 1.3 for K⁺) was also used for the mutants studied. The maximal ATPase activity (V max) is the activity in the presence of 50 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 0.1 mM ATP at pH 7.0.

**Molecular modeling**

The consequences of the mutations studied can be interpreted by studying the location and interactions of the specific amino acid in the sodium pump structures. The role of the amino acids can be understood better when its role during the catalytic cycle is visualized. Therefore,
we made homology models of the human sodium pump in different conformations using SERCA and pig and shark Na+,K+-ATPase as a template. E, Na+ (2C9M), E, Na+-ATP (1T5S), E, Na+·P·ADP (1T5T), E, Na+·P·ATP (3BA6), E, K+·P·ATP (3B9R), E, K+·P (2ZXE), E, K+·ATP (2C88). The homology model was built with the YASARA and WHAT IF Twinset using default parameters. YASARA calculated “Z-scores” where used for validation of the Na+,K+-ATPase enzyme. All mutations had been identified in clinical genetic studies.

Results

Functional expression of ATP1A2 migraine mutants in insect cells

We selected 11 ATP1A2 mutations that were located in different domains of the α2-subunit of the Na+,K+-ATPase enzyme. All mutations had been identified in clinical genetic studies and postulated as disease-causing. A PCR-based mutagenesis method was used to introduce the respective mutations in the human ATP1A2 cDNA. The (mutant) Na+,K+-ATPase α2- and β1-subunits were cloned into recombinant baculoviruses and expressed in Sf9 insect cells. The membrane fractions of cells expressing the recombinant ATPase proteins were isolated. Western blot analysis revealed similar expression levels for mutant and wild type Na+,K+-ATPase protein (Fig. 1).

The immunological detection of proteins on Western blots does, however, not indicate whether the expressed proteins are functionally active. To investigate functionality of the mutant proteins, we performed several functional assays. For instance, binding of the Na+,K+-ATPase inhibitor ouabain in a pocket of the enzyme close to the cation binding sites indicates that the protein is able to adopt a conformation that binds ouabain.9,11,14 The ouabain equilibrium saturation was analyzed and the affinity for ouabain was similar for all mutants. However, the maximum binding (EO_{max}) varied between the different mutants (Table 1). The maximum binding of the wild type and Y9N, R51H, E174K, R548C, and R548H varied between 2.5 and 3.6 pmol/mg and the binding of C702Y and R879Q varied between 1.6 and 1.8 pmol/mg. In contrast, the maximum binding of E700K, G900R, and E902K was greatly reduced (approximately 0.8 pmol/mg), whereas P786L was not able to bind ouabain at all. This indicates that although none of the mutations affected the expression levels, four mutations (i.e. E700K, P786L, G900R, and E902K) seriously influence functional expression.

Kinetic alterations

One of the most important properties of Na+,K+-ATPase that could be influenced by mutations is substrate affinity. In previous studies by Segall et al.15,16 three mutations (T345A, R689Q, and M731T) showed an affected K+ and vanadate affinity. In addition, Tavraz et al.17,18 showed that some mutants possessed altered K+ affinities. To investigate whether the 11 mutants exhibit kinetic alterations, we determined Na+, K+, and ATP affinities for the wild type and mutant enzymes. The Na+,K+-ATPase activity was determined in the presence of 100 μM ATP, 5 mM KCl and varying concentrations of NaCl. The apparent affinities for Na+ are depicted in Table 1 and were not significantly different for Y9N, R51H, C702Y, R879Q, G900R and E902K when compared to wild type enzyme. The ATPase activity of E700K and P786L was not sufficient to obtain reliable ATPase activities under different circumstances. E174K, R548C, and R548H, however, had a reduced apparent Na+ affinity (15.7, 17.0, and 13.8 mM, respectively) compared to wild type enzyme (7.1 mM).

Summary of the catalytic properties of wild type and mutant Na+,K+-ATPase. The values presented are the mean ± SEM of 3–4 enzyme preparations. The values of the mutant enzyme preparations are compared to those of the wild type. n.d. = not detected, * = p < 0.05 versus wild type, ** = p < 0.01 versus wild type, *** = p < 0.001 versus wild type.

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<th>K+ affinity</th>
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</tr>
<tr>
<td>G900R</td>
<td>0.75 ±0.15 ***</td>
<td>11.8 ±1.1</td>
<td>0.16 ±0.04</td>
</tr>
<tr>
<td>E902K</td>
<td>0.76 ±0.08 **</td>
<td>12.6 ±2.5</td>
<td>0.18 ±0.04</td>
</tr>
</tbody>
</table>

Figure 1 Expression of wild type and mutant Na+,K+-ATPase.

A: Western blot of the different Na+,K+-ATPase membrane preparations (10 μg) isolated from infected Sf9 cells. The presence of Na+,K+-ATPase α-subunit was detected with antibody C356-M09. B: Ouabain binding to wild type and mutant Na+,K+-ATPase. The assay was performed at 21 °C in 20 mM histidine buffer (pH 7.0), 5.0 mM MgCl₂, 5 mM P, and 12 nM ³H-ouabain. Average with SEM for 3 different expression experiments.
Familial hemiplegic migraine mutations affect \(Na^+\),\(K^+\)-ATPase domain interactions

In figure 2 the apparent affinities for \(Na^+\) of the R548C mutant and wild type are shown. In the presence of 100 \(\mu\)M ATP, 50 mM NaCl and varying concentrations of KCl, the activity of \(Na^+\),\(K^+\)-ATPase was determined. The apparent \(K^+\) affinity of Y9N, R51H, E174K, C702Y, R879Q, G900R and E902K was not significantly changed compared to the wild type enzyme (Table 1). R548C and R548H, however, had an increased apparent \(K^+\) affinity (0.81 and 0.45 mM, respectively) compared to wild type enzyme (1.35 mM).

The apparent ATP affinity in the presence of 50 mM NaCl and 5 mM KCl was similar for all mutants and the wild type enzyme (Table 1). We determined the maximal ATPase activity at 0.1 mM ATP, 50 mM NaCl and 5 mM KCl (Table 1). Mutants Y9N and R51H had a similar \(V_{\text{max}}\) as wild type enzyme. E174K, C702Y, and R879Q had a somewhat reduced \(V_{\text{max}}\) (47-64%), whereas the activities of R548C, R548H, E900R, and E902K were greatly reduced (22-33%). The P786L was completely inactive and the activity of E700K was only 11% of that of wild type enzyme.

The catalytic turnover determined as the ratio of \(V_{\text{max}}/E_{\text{0.5}}\) is plotted in figure 3 and the calculated values are shown in Table 1. Most mutants had a similar turnover number as wild type enzyme. The catalytic turnovers of E174K and E700K seem to be lower, but were not significantly decreased when compared to wild type. In contrast, the turnover numbers of R548C and R548H were significantly reduced. Although their ouabain binding level was comparable to that of wild type, their \(Na^+\),\(K^+\)-ATPase activity was largely reduced.

**Discussion**

Familial hemiplegic migraine type 2 (FHM2) is caused by mutations in \(ATP1A2\) that encodes the \(\alpha2\)-subunit of \(Na^+\),\(K^+\)-ATPase. Here we studied the catalytic function of 11 \(Na^+\),\(K^+\)-ATPase mutants that were not functionally characterized (in detail) before. For several of these mutants, we predicted that the domain interactions of the \(\alpha2\)-subunit were disrupted, which suggested a novel pathogenic mechanism for \(Na^+\),\(K^+\)-ATPase dysfunction in relation to migraine. The identified functional consequences of the \(ATP1A2\) mutants will be discussed below grouped by domain.

Within the A-domain we investigated mutant E174K. The negative charge at residue 174 is highly conserved in PIIc-type ATPases. In a previous study the E174K mutation had been characterized to some extent, and showed no difference from the wild type \(\alpha2\)-subunit, suggesting perhaps a more subtle effect on protein function than revealed by the activity assay used.\(^{19}\) Our functional studies show a slightly reduced catalytic turnover, a clearly reduced apparent \(Na^+\) affinity, and an increased apparent \(K^+\) affinity compared to that of wild type enzyme. In the A-domain Glu\(^{174}\) is predicted to be exposed to the cytosol in most conformations. However, in the E\(_p\)P conformation Glu\(^{174}\) is predicted to be in close proximity.
to Lys432 in the N-domain (Fig. 4). In this conformation a salt bridge between both residues might stabilize the E$_1$P sodium binding conformation. Substitution of the negatively charged Glu with the positively charged Lys will likely disrupt this stabilizing interaction and could thereby be responsible for the reduced apparent Na$^+$ affinity by decreasing its association rate or increasing its dissociation rate.

Several of the studied ATP1A2 mutations are located in the N-domain. For this study we focused on mutants R548C and R548H. Arginine 548 is strongly conserved in P-type ATPases. It most likely forms a salt bridge with Glu221 that stabilizes the interaction between the A- and N-domains in the E$_2$P conformation (Fig. 5). However, when ATP is bound, Arg$_{548}$ probably forms a salt bridge to the β-phosphate of ATP. Indeed, the substitution of Arg$_{548}$ with Gln was shown to abolish high-affinity ATP binding and Na$^+$.K$^+$-ATPase activity, whereas substitution with Lys was shown not to affect ATP binding, but reduced ATPase activity to 30% of the wild type enzyme. Nucleotides might disrupt the Arg$_{445}$-Glu$_{221}$ salt bridge, aiding dissociation of the A- and N-domains and accelerating K$^+$ release. In the E$_1$ conformation the A- and N-domains are far apart and the salt bridge between Arg$_{548}$ and Glu$_{221}$ is absent (Fig. 5). In our functional studies we observed that both R548C and R548H had a reduced catalytic turnover number, a slightly reduced Na$^+$ affinity and an increased K$^+$ affinity. Interestingly, the replacement of arginine by cysteine removes the salt bridge between the N- and A-domains, due to which binding of K$^+$ might be facilitated. In R548H similar effects were observed, suggesting that also in this case a salt bridge cannot be formed.

Within the Na$^+$.K$^+$-ATPase P-domain we analyzed mutant E700K. Our functional studies showed no detectable changes in apparent Na$^+$, K$^+$, or ATP affinities. However, the number of active E700K transporter molecules is significantly decreased compared to wild type, supporting a causal relation with disease. Glu$_{700}$ is highly conserved in P-type IIc ATPases and located at the surface of the P-domain, where it might be involved in the formation of a salt bridge with Lys$_{35}$ of the A-domain in the E$_2$K$_1$P$_0$ conformation (Fig. 6).
Recently, it was shown that E700K exhibits a reduced rate of E2P dephosphorylation and a reduced vanadate affinity. These observations are in agreement with our observed reduced activity. Moreover, the disruption of the salt bridge might destabilize the E2P conformation and thereby inhibit the E1P to E2P transition. In contrast to Schack et al., we did not observe an increased ouabain affinity. The P786L mutation is located within the transmembrane (αM) domain, more specifically αM5. P786L previously did not show survival of cells in our specialized assay. Our present functional studies showed no ouabain binding or ATPase activity for this mutant. In the sarcoplasmic reticulum Ca2+-ATPase substitution of the corresponding amino acid Cys774 with alanine caused inhibition of Ca2+ transport and ATPase activity. This cysteine has been proposed to play a central role in proton transfer. Homology modeling studies suggested that Pro786 in αM5 might be necessary for the Na+ ions to reach their binding site. This might be the reason why αM5 possesses the sequence PEITP, similar to αM4 (PEGLP). The prolines and polar amino acids in αM5 are also responsible for the very inefficient signal anchor sequence. Its correct membrane insertion is probably mediated by posttranslational hairpin formation with αM6, which is favored by a proline pair in the connecting loop. The proline mutant had decreased apparent Na+ and K+ affinity and a reduced turnover, whereas we did not observe any ATPase activity. The discrepancy is most likely due to the expression of Xenopus laevis Na+,K+-ATPase in Xenopus laevis oocytes at 18 °C, whereas we expressed human Na+,K+-ATPase in insect cells at 27 °C. Previously, it was demonstrated that folding defects of mutant proteins can be revealed in a temperature-dependent fashion.

Several studied ATP1A2 mutations are located in the extracellular loop between transmembrane segments 7 and 8: R879Q, R879W, W887R, G900R, E902K, and R908Q. This part of the Na+,K+-ATPase α-subunit has been implicated in binding of the Na+,K+-ATPase β-subunit. Glycine does not carry a side chain and therefore is important for the position of neighboring amino acids. The crystal structures of Na+,K+-ATPase indeed show that in this region several hydrogen bonds between both subunits are likely. For instance, Tyr899 binds to Lys250 and Gln901 binds to the backbone atoms of Ile185 (Fig. 7). Glutamic acid 902 is also well conserved (Asn or Glu) and forms a hydrogen bond with Arg183 of the β-subunit. Replacement of Gly900 or Glu902 will likely disrupt hydrogen bridges and affect...
the interaction between the α- and β-subunits of the Na+/K+-ATPase enzyme. Due to these mutations, the formation of an α-β enzyme complex is predicted to be hampered. However, if the α- and β-subunits were able to form a functional complex, its functional properties were not affected.

The R879Q mutant showed Na+, K+, ATP, and ouabain affinities that are similar to that of the wild type enzyme. Moreover, the number of active R879Q transporter molecules was not significantly decreased compared to the wild type. This non-conserved arginine is located in the first half of the extracellular αM7-αM8 loop, that most likely is not involved in direct binding of the β-subunit.31 Y9N and R51H are both located in the Na+-,K+-ATPase N-terminus and did not show any functional differences. Functional studies also showed no difference in apparent Na+, K+, or ATP affinities of C702Y compared to wild type enzyme. Moreover, the number of active C702Y transporter molecules was, at best, only slightly decreased compared to wild type enzyme. A cysteine-less Na+,K+-ATPase α1-subunit in which all 23 cysteines were replaced by serine residues still possessed the same catalytic turnover number and only minor changes in Na+ and K+ affinities were observed.34 The number of active cysteine-less Na+,K+-ATPase molecules was still 30% of that of the wild type. Thus, the Na+,K+-ATPase α1-subunit seems to contain no cysteine residues that are essential for its function which is in line with our finding that the single C702Y mutation did not affect Na+,K+-ATPase α2β1 function. This observation, therefore, casts considerable doubt on whether this mutant caused disease in the patient.

Our functional studies provide important experimental evidence as to which ATP1A2 mutations are likely disease-causing or not. For the three typical FHM2 families with mutations R548C, E700K and E902K that were absent in healthy relatives and large subsets of control chromosomes30,35,36, we could clearly show catalytic dysfunction of the Na+,K+-ATPase. This is true for mutation G900R, that was observed in a family with patients having FHM, epileptic seizures or both35, indicating that the mutation may underlie both phenotypes. We showed that the functional consequences of G900R are comparable with those of E902K. Another substitution of Arg548 (R548H) was found in an Italian family that was reported suffering from basal migraine and absent from a large group of controls.37

Both Arg548 mutations resulted in similar functional effects and are likely disease-causing. The difference between FHM and basal migraine could be due to the minor difference in apparent K+ affinities, yet undiscovered differences in genetic background, or perhaps diagnosing an atypical case of FHM as basal migraine.

The ATP1A2 P786L variant was identified in a family with sporadic hemiplegic migraine (SHM) without additional associated neurologic symptoms like cerebellar ataxia or epilepsy.23 The P786L mutation had occurred de novo as it was not present in the proband’s parents and false paternity was excluded. P786L completely disrupted the catalytic activity of Na+,K+-ATPase.

E174K was found in a family in which several members suffered from migraine with aura, whereas the mutation was not observed in 520 control chromosomes.19 The high degree of conservation of this negative charge and the finding that the kinetic properties of Na+,K+-ATPase are affected provide some evidence that E174K may be involved in causing the migraine phenotype and is not merely a rare non-causal polymorphism. Still, as the mutation was also present in the unaffected maternal grandfather, the functional effects of E174K appear not sufficient to cause the migraine phenotype.

In contrast to the disease-causing ATP1A2 mutants described above, we observed no or only very minimal catalytic effects for mutants R879Q, C702Y, Y9N and R51H. Of them, R879Q was identified in a Danish nationwide search of SHM patients.26 It was not detected in the 92 controls and was found in several unaffected family members.26 C702Y occurred in a family with occipitotemporal epilepsy and migraine without aura and was present in four unaffected individuals and absent in 170 control individuals.25 Y9N was identified in two SHM patients and the healthy mother of one of the probands.26 Moreover, Y9N has been observed in the unaffected mother of a carrier with the R583Q mutation.25 Finally, a Portuguese three-generation family with migraine without aura harbored the R51H variant.25 R51H was not present in 346 control chromosomes and seemed to co-segregate reasonably well with migraine without aura in the proband’s nuclear family. Cell survival assays39 and the present study, however, fail to provide any evidence for a functional consequence of this variant. The absence of functional abnormalities in this study suggests that R879Q, C702Y, Y9N and R51H for the moment should be regarded rare missense variants without a clear pathogenic effect.

In conclusion, in this study, we analyzed 11 mutations in the Na+,K+-ATPase α2-subunit that have been reported in patients with various migraine phenotypes. Four FHM mutations (R548C, E700K, G900R, E902K) and one SHM mutation (P786L) clearly affected Na+,K+-ATPase pump function and must be regarded disease-causing. Most of them probably hamper the interactions between Na+,K+-ATPase domains during the catalytic cycle. Of the non-hemiplegic migraine mutations, E174K (migraine with aura) and R548H (basilar migraine) affected Na+,K+-ATPase pump function as well. No functional effects were, however, observed for SHM mutations Y9N, P786L, and R879Q and for R51H and C702Y that were found in patients with migraine with or without aura. Our current results strengthen the role of ATP1A2 mutations in FHM2 and SHM.

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Chapter 5

Familial hemiplegic migraine mutations affect Na⁺/K⁺-ATPase domain interactions

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Chapter 6
Alternating hemiplegia of childhood mutations have a differential effect on Na⁺,K⁺-ATPase activity and ouabain binding

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Abstract

De novo mutations in ATP1A3, the gene encoding the α3-subunit of Na⁺,K⁺-ATPase, are associated with the neurodevelopmental disorder Alternating Hemiplegia of Childhood (AHC). The aim of this study was to determine the functional consequences of six ATP1A3 mutations (S137Y, D220N, I274N, D801N, E815K, and G947R) associated with AHC. Wild type and mutant Na⁺,K⁺-ATPases were expressed in Sf9 insect cells using the baculovirus expression system. Ouabain binding, ATPase activity, and phosphorylation were absent in mutants I274N, E815K and G947R. Mutants S137Y and D801N were able to bind ouabain, although these mutants lacked ATPase activity, phosphorylation, and the K⁺/ouabain antagonism indicative of modifications in the cation binding site. Mutant D220N showed similar ouabain binding, ATPase activity, and phosphorylation to wild type Na⁺,K⁺-ATPase. Functional impairment of Na⁺,K⁺-ATPase in mutants S137Y, I274N, D801N, E815K, and G947R might explain why patients having these mutations suffer from AHC. Moreover, mutant D801N is able to bind ouabain, whereas mutant E815K shows a complete loss of function, possibly explaining the different phenotypes for these mutations.

Introduction

Alternating hemiplegia of childhood (AHC) is a rare, severe neurodevelopmental disorder, reported for the first time in 1971 by Verret and Steele. The incidence of AHC has been estimated at 1 in 1,000,000 births, with disease usually ensuing within the first six months. AHC is characterized by episodes of hemiplegia on alternating sides of the body, which can last from a few minutes up to several days. Other symptoms that can occur during AHC episodes include pallor, abnormal eye movements, movement disorders, dystonia and severe cognitive impairment. Patient studies have reported different provoking factors: water exposure, extreme temperatures, physical activity, bright light, and stress. Falling asleep leads to disappearance of all symptoms, although they may return after waking up. The exact mechanism of disease is unknown, although treatment with Flunarizine (a Ca²⁺ influx inhibitor specific for vascular smooth muscle and neurons) has been reported to reduce symptoms. However, the effectiveness and long-term effects of this treatment are unknown. AHC was recently linked to de novo mutations in ATP1A3, the gene encoding the Na⁺,K⁺-ATPase α3-subunit. Most mutations are located in or near the ten transmembrane domains of Na⁺,K⁺-ATPase. Interestingly, two mutations (D801N and E815K) found in 66% of the AHC patients show differences with regard to disease severity, where E815K has been associated with a more severe phenotype. Previously, ATP1A3 mutations were identified in patients suffering from rapid-onset dystonia parkinsonism (RDP). Until now, there has been only one mutation that is reported in AHC and RDP cases (D923N), indicating minor overlap between both diseases.

Na⁺,K⁺-ATPase plays a major role in maintaining the electrochemical gradient across the plasma membrane. The α-subunit is the catalytic component of this transport protein, which together with the β-subunit forms a functional transporter enzyme, in some tissues accompanied by a third or gamma-subunit. In humans, four different isoforms of the α-subunit exist. Three of these isoforms are expressed in the human brain: α1 in multiple cell types due to its ubiquitous expression, including neurons and glial cells, α2 predominantly in astrocytes, and α3 in peripheral and central nervous system neurons. When catalytically active, Na⁺,K⁺-ATPase transports three sodium ions out of the cell and two potassium ions into the cell, a process fueled by hydrolysis of one molecule ATP. During this catalytic cycle, Na⁺,K⁺-ATPase is present in the E₁ or the E₂ conformation, depending on the association of either Na⁺ or K⁺ to the ion binding sites (Fig. 1A). The presence of both ATP and Na⁺ is necessary for phosphorylation of the protein in the E₁ state, which leads to a conformational change into the Na⁺-bound phosphorylated conformation (E₁P-ADP). During transition to the E₂P state, Na⁺ is released out of the cell, followed by binding of K⁺, which initiates dephosphorylation of the protein and transition into the K⁺-bound E₂ state. Binding of ATP drives the protein back into the E₁ state, completing the catalytic cycle. The effect of ATP1A3 mutations on protein functionality is hardly studied. Moreover, whether different phenotypes can be linked to specific mutations is not known. In this study, we
Alternating hemiplegia of childhood mutations affect Na+,K+-ATPase differentially

Chapter 6

Materials and methods

The Gateway system

The desired mutations were obtained by first performing two PCRs (A and B) on human Na+,K+-ATPase alpha 3 wild type cDNA using either a 5'- or 3'-primer in combination with a primer containing the desired mutation, resulting in two different fragments that were subsequently combined for a final PCR (C) using only the 5'- and 3'-primers, allowing annealing of the two fragments (A and B) containing the desired mutation. Next, the obtained mutant Na+,K+-ATPase α3-isoform was cloned into an entry vector using BP Clonase II enzyme according to the manufacturer's instructions (Invitrogen). After successful transformation of entry clones into DH5α cells and overnight selection, colonies were grown overnight in liquid medium and subsequently isolated using the Genelute™ mini prep isolation kit (Sigma-Aldrich). Following a restriction analysis, full length sequencing of the different constructs was performed to check for successful mutagenesis. Subsequently, the entry clones were combined with an empty destination vector already containing the β1-encoding gene (ATP1B1) using LR Clonase II (Invitrogen), resulting in an expression clone containing the desired mutation. As a negative control, an expression clone containing YFP in combination with the β1-subunit was used for mock transfection.

Generation of recombinant viruses

The expression clones, generated using the Gateway system, were transformed to competent DH10Bac Escherichia coli cells (Life Technologies, Breda, the Netherlands) harboring the baculovirus genome (bacmid) and a transposition helper plasmid. Upon transposition between the Tn7 sites present in both the bacmid and expression clone, recombinant bacmids were selected and isolated. Subsequently, the obtained bacmids were transfected to Spodoptera frugiperda (Sf)9 insect cells using Cellfectin reagent (Life Technologies, Breda, the Netherlands). After a 6-day period, recombinant baculoviruses were harvested and used to infect fresh Sf9 cells at a multiplicity of infection of 0.1. After another 6 days of culture of infected Sf9 cells, amplified baculoviruses were harvested.

Protein production

Sf9 cells grown at 27 °C in T175 and later in 500 mL shaking flasks in Xpress medium (Sigma, Bornem, Belgium) were infected at a density of 1.5 ·10^6 cells mL⁻¹ in the presence of 1% (v/v) ethanol, as described before. After three days of infection, cells were harvested by centrifugation at 2,000 x g for 5 min. Then, the pelleted cells were resuspended at 0 °C in a

Figure 1 Na+,K+-ATPase ion-transport scheme and structure.

(A) Albert-Post scheme of the reaction cycle of Na+,K+-ATPase: transport of Na⁺ and K⁺ across the cell membrane is accomplished by a series of conformational changes of Na+,K+-ATPase based on binding of either Na⁺ or K⁺, leading to phosphorylation or dephosphorylation, changing the affinity of the enzyme for its ligands. During each reaction cycle, three Na⁺ ions are transported out of the cell, in return transporting two K⁺ ions into the cell. This process requires the hydrolysis of ATP, to drive the transition into the high energy E₁P and E₂P states. Binding of DLCs, well-known inhibitors of Na+,K+-ATPase function, is only possible in the E₂P or E₂ state.

(B) Crystal structure of the Na+,K+-ATPase heteromer in the E₂ state (PDB: 3KDP), with the location of the six mutations studied here highlighted by spheres. Note how five mutations are located in or near transmembrane regions, whereas mutant D220N is located in the large cytosolic loop between transmembrane domains two and three. The six residues are conserved between the α1- and α3-isoforms, making the α1-isoform crystal structure suitable for representation. Blue is α-subunit, red is β-subunit, green is γ-subunit, and yellow is used to highlight the mutations studied here. Figure was created using YASARA software.
Phosphorylation was calculated by subtracting the amount of phosphate detected cold water the filters were added to 4 mL OptiFluor and analyzed by liquid scintillation collected by filtration through a 0.8 µm membrane filter. After repeated washing with ice-s at 0 °C. The reaction was stopped by adding 5% TCA and (phosphorylated) protein was detected using either the G36 (anti-α) or C385-M77 (anti-β) antibodies, respectively.20,21

To determine the ATPase activity in Na⁺ or K⁺ affinity studies, 20 µL of membranes was incubated in a final volume of 100 µL containing 50 mM Tris-Ac at pH 7.0, 0.1 mM EGTA, 1.25 mM MgCl₂, 1.0 mM Tris-N₃, 100 µM of radiolabeled ATP, and 50 mM NaCl or 50 mM KCl and increasing concentrations of either KCl or NaCl. After incubation of the samples for 30 min at 37 °C, the reaction was stopped by addition of 500 µL 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid and after 10 min at 0 °C the mixture was centrifuged for 30 s. Subsequently, 150 µL of the clear supernatant was mixed with 4 mL OptiFluor before analysis using a liquid scintillation counter to determine the amount of inorganic phosphate (Pi) formed, a measure for ATPase activity.22 The Na⁺,K⁺-ATPase specific activity was determined by subtracting the amount of Pi formed in the presence of 1.0 mM ouabain. Maximum ATPase values were determined by measuring the activity in the presence of 50 mM NaCl, 5.0 mM KCl, 1.25 mM MgCl₂ and 0.1 mM ATP at pH 7.0.

Ouabain binding experiments
Ouabain binding was determined by incubation of approximately 150-200 µg of membranes in the presence of 20 mM histidine, 5.0 mM MgCl₂, 5.0 mM H₃PO₄ at pH 7.0, (varying concentrations of KCl) and 25 nM of radiolabeled ouabain (Perkin-Elmer, Waltham, MA, USA) in a final volume of 60 µL at room temperature for 2 h. After incubation for 15 min on ice, the amount of bound ouabain was determined by washing the samples through a 0.8 µm ME27 filter using H₂O, retaining the enzyme-bound ouabain. The radioactivity retained on the filters was determined in a liquid scintillation counter after addition of 4 mL OptiFluor (Canberra Packard, Tilburg, the Netherlands).23

Data analysis
All data were analyzed using Graphpad Prism 5.02. Statistical analysis (ANOVA, with Dunnet's post test comparing to mock values, n ≥ 3 for all experiments and α=0.05) was performed to test for differences in maximum ouabain binding, apparent ouabain affinity, apparent Na⁺ and K⁺ affinities, maximum phosphorylation levels and maximum ATPase activity levels compared to mock. The Na⁺₀.₅ and K⁺₀.₅ values were calculated via the Hill equation using the averaged data and SEM values. The Hill coefficient obtained for wild type Na⁺,K⁺-ATPase was used also for calculation of the half-maximum Na⁺ and K⁺ concentrations of mutants.

Analysis of expression
Six single mutations associated with AHC (S137Y, D220N, I274N, D801N, E815K, and G947R, see Fig. 1B) were introduced in the α3-subunit of Na⁺,K⁺-ATPase. Wild type and mutant Na⁺,K⁺-ATPase were expressed in Sf9 insect cells using the baculovirus expression system. The membrane fractions of these Sf9 cells were isolated and used for biochemical characterization. Similar expression levels for wild type and mutant enzymes were detected by Western blot analysis using antibodies against the α- and β-subunits (Fig. 2).

Mutant D220N is catalytically active
The Na⁺,K⁺-ATPase activity of both wild type and mutants was determined in the presence of 100 µM ATP, 5.0 mM KCl and 50 mM NaCl. Five mutants did not show ATPase activity
Different from mock (Fig. 3). Significantly higher ATPase activities were observed for wild type enzyme and mutant D220N (p < 0.05). The activities of wild type enzyme (1.2 ± 0.1 µmol P, mg⁻¹ h⁻¹) and mutant D220N (0.98 ± 0.05 µmol P, mg⁻¹ h⁻¹) were not significantly different. The absence of ATPase activity for the other mutants indicated that these mutations result in a catalytically inactive enzyme.

Figure 2 Expression levels of mutants.

Western blot of Na⁺,K⁺-ATPase α- and β-subunits for six different Sf9 membrane preparations. Membranes (20 µg) were run on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The presence of α- and β-subunits was detected using antibodies G36 and C385-M77, respectively.

Figure 3 ATPase activity of mutant and wild type Na⁺,K⁺-ATPase.

Enzymes were incubated at 37 °C in the presence of 1.3 mM MgCl₂, 0.1 mM EGTA, 2.0 mM Tris-N₃, 50 mM Tris-acetic acid (pH 7.0), 100 µM radiolabeled ATP, 5.0 mM KCl and 50 mM NaCl for 30 min. Na⁺,K⁺-ATPase activity was corrected for background by subtracting mock values. Values are presented as mean ± SEM with N ≥ 3. * p < 0.05 versus mock, ANOVA with Dunnett’s post test.

Figure 4 Effect of Na⁺, K⁺ and ATP on the ATPase activity of wild type and mutant Na⁺,K⁺-ATPase.

Enzymes were incubated at 37 °C in the presence of 1.3 mM MgCl₂, 0.1 mM EGTA, 2.0 mM Tris-N₃, 50 mM Tris-acetic acid (pH 7.0), 50 mM or varying concentrations of NaCl (A), 50 mM or varying concentrations of KCl (B), and 100 µM or varying concentrations of ATP (C) for 30 min. Values are presented as mock corrected mean ± SEM with N ≥ 3.
Na⁺, K⁺, and ATP affinities for mutant D220N and wild type enzyme are similar

Next, we determined the effect of Na⁺, K⁺ and ATP on the ATPase activity. Since mutant D220N was the only protein that showed ATPase activity, these experiments were performed only for this mutant. The Na⁺ affinity was determined by measuring ATPase activity at increasing concentrations NaCl in the presence of 0.1 mM ATP and 5.0 mM KCl. ATPase activity increased with increasing Na⁺ concentration (Fig. 4A), and no significant differences in apparent Na⁺ affinity were observed between wild type and the mutant D220N (K₅₀ of 11 ± 1 mM and 12 ± 1 mM, respectively).

The K⁺ affinity was determined by measuring ATPase activity at increasing concentrations KCl in the presence of 0.1 mM ATP and 50 mM NaCl. As shown in figure 4B, there were no significant differences in apparent K⁺ affinity between wild type (K₅₀ = 0.62 ± 0.1 mM) and mutant D220N (K₅₀ = 0.56 ± 0.1 mM).

The ATP affinity was determined by measuring ATPase activity at increasing ATP concentrations in the presence of 50 mM NaCl and 5.0 mM KCl. Figure 4C shows no differences in apparent ATP affinity (K₅₀ of 55 ± 19 µM (wild type) and 86 ± 27 µM (D220N)).

Phosphorylation capacity

One of the features of the Na⁺,K⁺-ATPase reaction cycle is phosphorylation of the protein, forming an acid-stable intermediate. Both Na⁺ and ATP are necessary for the conformational change of the protein from the E₂ state to the phosphorylated E₂P state. In the absence of K⁺ and high levels of Na⁺, the enzyme will accumulate in this conformation (Fig. 1A). The phosphorylation capacity of all mutants was determined by measuring the amount of phosphoenzyme (EP) in the presence of 100 mM NaCl and 0.1 µM ATP, background-corrected for the amount of EP present when 10 mM KCl was added (allowing dephosphorylation to take place as well). Phosphorylation of mutants S137Y, I274N, D801N, E815K, and G947R was absent (Fig. 5). Mutant D220N showed a phosphorylation level of 1.8 pmol EP mg⁻¹, similar to wild type enzyme (1.8 pmol EP mg⁻¹).

Mutants S137Y, D220N and D801N bind ouabain

The conformational change from E₂ to E₂P-ouabain via E₂P (backdoor phosphorylation) could be determined in the presence of 5 mM Pi and increasing concentrations of [³H]-ouabain. Ouabain binding was observed for wild type Na⁺,K⁺-ATPase and three mutants: S137Y, D220N, D801N (Fig. 6A). The other three mutants (I274N, E815K and G947R) showed no ouabain binding. Mutant D801N showed an increase in maximum ouabain binding compared to wild type enzyme (6.6 pmol mg⁻¹ versus 1.5 pmol mg⁻¹). Mutant S137Y showed a decreased EOmax (1.0 pmol mg⁻¹), whereas mutant D220N exhibited ouabain binding capacity equal to wild type enzyme (1.5 pmol mg⁻¹). Calculated ouabain affinities (Kd) of mutants and wild type enzyme were not significantly different.

K⁺/ouabain antagonism is absent in S137Y and D801N

As described above, three mutants (S137Y, D220N and D801N) were able to bind ouabain (Fig. 6A), whereas only one of them (D220N) showed ATPase activity (Fig. 3). In order to

Figure 5 ATP phosphorylation level of wild type and mutant Na⁺,K⁺-ATPase.

Membranes expressing Na⁺,K⁺-ATPase were pre-incubated with 50 mM Tris-acetic acid (pH 7.0) 100 mM NaCl and 1.25 mM MgCl₂, at 0°C for 30 min. Then, 0.1 µM radiolabeled ATP was added for 10 seconds before the reaction was stopped. Values are presented as mean ± SEM with N ≥3. *p < 0.05 versus mock, ANOVA with Dunnett’s post test.

Figure 6 Ouabain binding experiments.

(A) Ouabain-binding capacity of wild type and mutant Na⁺,K⁺-ATPase. Membranes were incubated at room temperature in the presence of 50 mM histidine pH 7.0, 10 mM MgCl₂, 5 mM H₃PO₄, 25 nM radiolabeled ouabain and different concentrations of non-radiolabeled ouabain. Values are presented as mean ± SEM with N ≥3. Three mutants (I274N, E815K and G947R) did not bind ouabain and are not shown. (B) The effect of K⁺ on ouabain binding. Membranes were incubated at room temperature in the presence of 50 mM histidine at pH 7.0, 10 mM MgCl₂, 5.0 mM H₃PO₄, 25 nM radiolabeled ouabain and increasing concentrations of KCl for 2 h. Values are presented as mean ± SEM with N ≥3, corrected for values obtained in the absence of KCl.
In silico analysis of mutants

In silico analysis of three crystal structures representing α1β1 Na,K-ATPase in various conformations (E1P-ADP, E2P-ouabain, and E2K⁺·Pi) resulted in identification of various H-bond interactions involving the residues corresponding to Ser137 and Glu815. In the E2P-ouabain crystal structure, Ser137 was predicted to form a hydrogen bond with Gln85, whereas Glu815 was involved in a network of H-bonds involving residues Asn761, Lys928, Asn937, and Asn941 in multiple crystal structures. Finally, Gly947 was observed to have limited space due to the close proximity of Val947 and Pro948 as observed for the E₂P-ouabain conformation.

Discussion

Alternating hemiplegia of childhood (AHC) is a rare, severe neurodevelopmental disorder caused by de novo mutations in the ATP1A3 gene, coding for the α3-isoform of Na⁺,K⁺-ATPase. In this study we examined the functional consequences of six single mutations in ATP1A3 associated with AHC. The amino acids affected in all six mutations are highly conserved in Na⁺,K⁺-ATPase, suggesting an important impact of the mutations on enzyme function. Here, we discuss the relationship between functional activities of the mutants and the AHC phenotype. A summary of the experimental findings obtained for the mutants and wild type Na⁺,K⁺-ATPase is shown in Table 1.

D220N possesses biochemical characteristics similar to wild type enzyme

In general, AHC mutations are located near or inside one of the transmembrane domains. One exception to this rule appears to be D220N, located in the cytosolic loop between transmembrane domains 2 and 3 (Fig. 1B). We could not identify any statistically significant differences between D220N and the wild type enzyme. Protein expression, ATPase activity, apparent Na⁺, K⁺, and ATP affinities, phosphorylation, ouabain binding, as well as the ouabain/ K⁺ antagonism were all similar to those of wild type enzyme. These results imply that this mutation is possibly not pathogenic, although we have not investigated protein localization. Further studies are required to explain the severe phenotype reported for this mutation.

### Table 1 Summary of catalytic properties of wild type and mutant Na⁺,K⁺-ATPase.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>ATPase activity</th>
<th>Ouabain binding studies</th>
<th>Phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺ K⁺</td>
<td>Na⁺ K⁺</td>
<td>ATPase activity</td>
</tr>
<tr>
<td>wild type</td>
<td>0.94 ± 0.11</td>
<td>11 ± 1</td>
<td>0.64 ± 0.01</td>
</tr>
<tr>
<td>S137Y</td>
<td>-0.12 ± 0.07</td>
<td>0.51 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>D220N</td>
<td>0.07 ± 0.05</td>
<td>-0.03 ± 0.06</td>
<td>0.03 ± 0.06</td>
</tr>
<tr>
<td>I274N</td>
<td>0.13 ± 0.06</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>D801N</td>
<td>-0.07 ± 0.02</td>
<td>-0.09 ± 0.06</td>
<td>-0.09 ± 0.06</td>
</tr>
<tr>
<td>E815K</td>
<td>-0.09 ± 0.06</td>
<td>-0.09 ± 0.06</td>
<td>-0.09 ± 0.06</td>
</tr>
<tr>
<td>G947R</td>
<td>-0.09 ± 0.06</td>
<td>-0.09 ± 0.06</td>
<td>-0.09 ± 0.06</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM of three enzyme preparations corrected for background values. * = p < 0.05 versus mock, - = not tested, >> = exceeding tested range.

Catalytic activity

All six ATP1A3 mutants were expressed at protein levels comparable to that of wild type enzyme. However, no significant ATPase activity or phosphorylation level was detected for S137Y, I274N, D801N, E815K and G947R. Moreover, I274N, E815K, and G947R could not bind ouabain, which indicates that these mutations result in a severely affected protein with a complete loss of function.

Whereas almost all AHC-linked mutations are de novo, I274N was reported in a family with several members suffering from a form of alternating hemiplegia. Kirshenbaum et al. modeled the structure of Na⁺,K⁺-ATPase with I274N, which showed a loss of interaction between Glu776 with its interacting K⁺ atom and also a disturbance of the side-chain interaction between Thr272 and Ile274. These changes might seriously impact protein functionality, thereby explaining the lack of ATPase activity.

The absence of ATPase activity in mutant E815K was reported previously in a study using COS-7 cells. In three crystal structures, representing the E₁P-ADP, E₂P-ouabain and E₂K⁺·Pi conformations (4HQT, 4HYT, and 2XXE, respectively), we could identify an extensive interaction network between the side chains of Glu451 with Asn451, Lys451, Asp451, and Asn451, which is possibly affected by replacement of the negatively charged glutamic acid by a positively charged lysine.

Mutant G947R is located in transmembrane domain 9. Being located in an α-helix, a change from glycine to arginine might strongly disturb the helix structure. Based on a crystal structure representing the E₂P-ouabain conformation (4HYT), we found that the side chain of arginine at this position has limited space, as residues Val947 and Pro948 (in transmembrane domain 6) are located near Gly947. Also, Gly457 is located close to Glu457, important in regulating the C-terminal ion pathway. In addition, a recently published crystal structure of the Na⁺-bound state suggested that G947R affects the IIIα ion binding site of Na⁺,K⁺-ATPase, which is important for the release of Na⁺ ions following transition from the E₁P-ADP state to the E₂P state.
Ouabain and K⁺ binding

S137Y and D801N failed to show ATPase activity, whereas significant ouabain binding was present. We conclude that these two mutations result in correctly folded protein, capable of binding ouabain, yet with an impaired reaction cycle. It appeared that they can transit into the ouabain binding E₃P conformation, indicating that the conformation that is affected involves the Na⁺ binding E₁ state. The absence of phosphorylated protein also points in this direction, as ATP phosphorylation occurs via the E₁ state of the enzyme.

The ouabain binding capacity of these mutants allows us to evaluate the K⁺ binding capacity. It is known that ouabain and K⁺ act as antagonists, and a change in K⁺ affinity should therefore be reflected by a shift in the K⁺/ouabain competition curve. Although the amount of ouabain bound enzyme was decreased for wild type and D220N upon addition of K⁺, we could not observe this phenomenon for S137Y and D801N. This indicates that the latter mutations decrease the affinity for K⁺, which together with the absence of phosphorylation suggest that S137Y and D801N affect cation binding in general.

Whereas this study reports on the biochemical effects of the S137Y mutation, low ATPase activity for S137F was reported before in COS-7 cells. Serum is located within the second transmembrane domain where it forms an H-bond with Gln⁸⁵ as based on our in silico analysis of the recently published E₃P-ouabain structure. Gln⁸⁵ is located just before the characteristic αM₁ kink, important in the N-terminal ion pathway of the enzyme, through which the cations pass. Replacing the serine with a tyrosine might have a strong impact on the interaction between serine and glutamine, due to the bulky aromatic ring in the side chain of tyrosine.

Mutant D801N belongs to the most frequent mutations in AHC cases. This mutation has been reported to reduce ATPase activity in COS-7 cells, in accordance with our results. In addition, we have previously shown a lack of phosphorylation for the corresponding residue (Asp⁸⁰⁴) in rat α₁-isofrom Na⁺,K⁺-ATPase. Contrary to our results, Pedersen et al. described phosphorylation of mutant D804N after Na⁺ stimulation in yeast cells. Structural modeling of the D801N mutation predicted a direct effect on binding of potassium ions, reflecting the highly important role of Asp⁸⁰₁ in coordination of ion binding sites I and II in K⁺-occlusion and Na⁺-coordination, and also binding of a Mg²⁺ ion in site II in the high resolution ouabain bound structure. We speculate that replacing aspartic acid with asparagine at this position increases the preference of the enzyme for the E₃P-DLC conformation by decreasing the preference for the E₃P conformation. For the rat Na⁺,K⁺-ATPase α₁-isofrom it was already known that Asp⁸⁰₁ (corresponding to Asp⁸⁰₁ in human) plays a major role in the cation binding pocket.

Mechanism of disease

Unfortunately, the exact mechanism of disease for ATP1A3 mutations remains to be understood. It is known that the α3-subunit of Na⁺,K⁺-ATPase is found mainly in the central and peripheral nervous system, and therefore it is not surprising that these mutations have a neurological effect. The first study to look at the functional consequences of AHC at the cellular level was published recently, reporting a structural and functional granule defect in platelets and fibroblasts of AHC patients. Furthermore, increased cathepsin B-dependent apoptosis in fibroblasts was observed, linking lysosomal defects to decreased ATPase activity.

It is highly challenging to correlate changes in biochemical characteristics to the frequency and duration of attacks in patients suffering from AHC. However, due to the high frequency of D801N and E815K, responsible for most AHC cases, it was possible to show that E815K causes a more severe phenotype than D801N. Although both enzymes are catalytically inactive, we found that D801N is able to bind ouabain, whereas E815K is not. Possibly, the lack of ouabain binding might link E815K to its severe phenotype. Previous studies have shown that ouabain and other digitalis-like compounds are produced endogenously, both in adrenal cells as well as in the hypothalamus. The role of Na⁺,K⁺-ATPase as a signal transducer might provide an explanation for the less severe phenotype observed for D801N compared with E815K. A possible role of endogenous ouabain in AHC should be the subject of future studies.

Conclusion

Our study is the first to show in detail the biochemical properties of six AHC-causing mutations, and reveals that mutations in ATP1A3 affect the functionality of Na⁺,K⁺-ATPase, which might explain why they are associated with AHC. However, no differences in protein function were observed for mutant D220N compared to wild type Na⁺,K⁺-ATPase. Mutants I274N, E815K, and G947R did not result in functional protein as reflected by a complete loss of ouabain binding and ATPase activity, rendering them unsuitable for further studies. Mutants S137Y and D801N could bind ouabain, but showed no ATPase activity, no phosphorylation, and an impaired K⁺ binding. The presence of ouabain binding might provide an explanation for the less severe phenotype observed for D801N as compared to E815K.

Disclosure statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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References

Alternating hemiplegia of childhood mutations affect Na⁺,K⁺-ATPase differentially

Chapter 6

Chapter 7

\[ \text{Ile}^{679} \text{ and Pro}^{692} \text{ are responsible for the low activity associated with the human Na}^{+},\text{K}^{+}-\text{ATPase \( \alpha \)}{-}\text{isoform} \]

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Manuscript in preparation
Abstract

Na⁺,K⁺-ATPase plays an important role in the maintenance of membrane potential and ion-gradients across the cellular membrane. It serves this role at the expense of ATP consumption: for every molecule that is hydrolyzed, three sodium and two potassium cations are transported out of and into the cell, respectively. A functional enzyme consists of at least two subunits: a catalytic α-subunit and a β-subunit that is necessary for proper trafficking of the enzyme to the cellular membrane. In the human body, four isoforms of the Na⁺,K⁺-ATPase catalytic subunit exist, each with a specific tissue expression pattern. Previous unpublished studies by our lab have revealed that upon expression of all four α-isoforms in Spodoptera frugiperda (Sf9) cells, maximum ATPase activity was lowest for the α4-isoform, and highest for the α2-isoform. Interestingly, it has been suggested that the α4-isoform gene originated from the α2-isoform gene. Therefore, we aimed to elucidate the structural features determining the difference in maximum ATPase activity between the isoforms. By designing and producing various chimeras consisting of residues of both isoforms, we found that Ile⁶⁷⁹ and Pro⁶⁹² in the α4-isoform are responsible for the low activity, as compared to the corresponding Met⁶⁷⁰ and Thr⁶⁸³ in the α2-isoform. The two amino acids identified are located in the P-domain of Na⁺,K⁺-ATPase, which harbors a Rossmann-fold: a structural motif characteristic for nucleotide binding proteins. By using homology modeling, in silico analysis of the identified residues revealed that hydrogen-bonds predicted for the α2-isoform are not present in the α4-isoform. The physiological role of the α4-isoform probably has emerged from that of the α2-isoform, associated with a decreased need for enzyme activity.

Introduction

Na⁺,K⁺-ATPase plays an essential role in cellular life: ATP-mediated transport results in transfer of Na⁺ and K⁺ over the plasma membrane against steep concentration gradients. These gradients are the driving force for secondary active transporters and help maintain the membrane potential. Na⁺,K⁺-ATPase consists of at least two subunits: a large catalytic α-subunit and a β-subunit that together constitute a functional protein. The human genome encodes four α-isoforms, which are expressed tissue specifically. The α1-isoform is expressed in all human cell types, whereas the α2- and α3-isoforms are found in a limited number of tissues, including muscle cells, astrocytes, leukocytes and neurons. The α4-isoform is restricted to germ cells in the testis, playing an essential role in sperm motility and hyperactivation, hence, mice lacking this isoform show complete loss of fertility. In general, the similarity of all four α-isoforms of Na⁺,K⁺-ATPase is high. Interestingly, the α4-isoform shows the lowest degree of similarity (~78%) with the other three, which are even more closely related to each other (~87%). The lower similarity of the α4-isoform might reflect a different physiological role as opposed to the other isoforms. Analysis of evolutionary patterns of the various isoforms indicated that the α4-isoform possesses the highest similarity to the α2-isoform. Unpublished studies by our laboratory have shown that expression of all four human α-isoforms together with the β1-subunit in insect cells resulted in the highest ATPase activity for α2β1-isoform, whereas the α4β1-associated ATPase activity was almost negligible. The goal of this study was to understand how the difference in ATPase activity between these two isoforms emerged. Using baculovirus-mediated expression of a large number of chimeras and single mutants of the α2- and α4-isoforms in insect cells lacking endogenous Na⁺,K⁺-ATPase, we were able to identify two α4-specific amino acids that are responsible for the difference in ATPase activity between these two isoforms.

Methods

The Gateway system

The cDNA for the α2- and α4-isoforms was obtained by purchasing vectors (Thermo Scientific MHS1011-9199751 and MHS1010-98075446) containing the cDNA obtained from a human teratocarcinoma cell line or human testis (cDNA: NM_000702 and NM_14469). The desired chimeras were obtained by performing PCRs on human wild type α2- and α4-isoform encoding cDNA using either a 5’- or 3’-primer together with a chimera/mutation specific primer (Biolegio, Nijmegen, the Netherlands) annealing at the transition(s)/mutation site. Using Gateway cloning (Life Technologies, Breda, the Netherlands), the chimera/single mutant cDNA was then transferred to an entry vector containing a kanamycin resistance cassette using BP Clonase II enzyme according to the manufacturer’s instructions. After successful transformation of entry clones into Escherichia coli DH5α cells and overnight
Generation of recombinant baculoviruses

The expression clones generated using the Gateway system were transformed to competent DH10Bac Escherichia coli cells (Life Technologies, Breda, the Netherlands) harboring the baculovirus genome (bacmid) and a transposition helper plasmid, conferring resistance against kanamycin, gentamycin, and tetracycline. Upon transposition between the Tn7 sites present in both the bacmid and expression clone, recombinant bacmids were selected on LB-agar plates containing kanamycin, gentamycin, and tetracycline. Subsequently, resistance-medium (Sigma, Bornem, Belgium) at 27 °C in 175 cm² monolayers using Cellfectin reagent harvested and used directly to infect fresh Sf9 cells at a multiplicity of infection of 0.1. After (Life Technologies, Breda, the Netherlands). After successful transformation of expression clones into Escherichia coli DH5α cells and overnight selection on ampicillin selection plates, colonies were inoculated in LB medium containing ampicillin, grown overnight and subsequently isolated using the Genelute™ mini-prep isolation kit (Sigma-Aldrich). Throughout the cloning procedures, YFP was used as a negative control, resulting in an expression clone encoding YFPβ1.

Protein production using recombinant virus

Sf9 cells grown at 27 °C in 175 cm² monolayers and later in 500 mL shaking flasks in Xpress medium (Sigma, Bornem, Belgium) were infected at a density of 1.5 ·10⁶ cells mL⁻¹ in the presence of 1% (v/v) ethanol, as described before. After three days of infection, the cells were harvested by centrifugation at 2,000 × g. Subsequently, 150 µL of the clear supernatant was mixed with 4 mL OptiFluor trichloroacetic acid and after 10 minutes at 0 °C, the mixture was centrifuged for 30 s (10,000 × g). Subsequently, 150 µL of the clear supernatant was mixed with 4 mL OptiFluor before analysis using a liquid scintillation counter to determine the amount of formed P_i (inorganic phosphate), a measure of ATPase activity. Na⁺,K⁺-ATPase specific activity was determined by subtracting the amount of P_i formed in the presence of 1.0 mM ouabain. [γ-³²P]ATP (3000 Ci mmol⁻¹) was obtained from Perkin-Elmer (Waltham, MA, USA).

Western blotting

Approximately 10 µg of membranes was treated with SDS-PAGE solubilization buffer overnight at room temperature before loading on a 10% polyacrylamide gel, as described previously. After separation, the proteins were transferred overnight to a polyvinylidene difluoride (PVDF) membrane using dry blotting (iBlot, Life Technologies). Both subunits were detected using the polyclonal antibodies C356-M09 (α-subunit) and C385-M77 (β-subunit) antibodies.

ATPase activity studies

Maximal ATPase activity was estimated from Na⁺-affinity studies by incubating 20 µL of membranes in a final volume of 100 µL containing 50 mM Tris-Ac pH 7.0, 0.1 mM EGTA, 1.2 mM MgCl₂, 1.0 mM Tris-N₃, 5.0 mM KCl, and 100 µM of radiolabeled ATP in the presence of increasing concentrations of NaCl. After incubation of the samples for 30 min at 37 °C, the reaction was stopped by addition of 500 µL 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid and after 10 minutes at 0 °C, the mixture was centrifuged for 30 s (10,000 × g). Subsequently, 150 µL of the clear supernatant was mixed with 4 mL OptiFluor before analysis using a liquid scintillation counter to determine the amount of formed Pi. Maximal ATPase activity was estimated from Na⁺-affinity studies by incubating 20 µL of membranes in a final volume of 100 µL containing 50 mM Tris-Ac pH 7.0, 0.1 mM EGTA, 1.2 mM MgCl₂, 1.0 mM Tris-N₃, 5.0 mM KCl, and 100 µM of radiolabeled ATP in the presence of increasing concentrations of NaCl. After incubation of the samples for 30 min at 37 °C, the reaction was stopped by addition of 500 µL 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid and after 10 minutes at 0 °C, the mixture was centrifuged for 30 s (10,000 × g). Subsequently, 150 µL of the clear supernatant was mixed with 4 mL OptiFluor before analysis using a liquid scintillation counter to determine the amount of formed P_i. Maximal ATPase activity was estimated from Na⁺-affinity studies by incubating 20 µL of membranes in a final volume of 100 µL containing 50 mM Tris-Ac pH 7.0, 0.1 mM EGTA, 1.2 mM MgCl₂, 1.0 mM Tris-N₃, 5.0 mM KCl, and 100 µM of radiolabeled ATP in the presence of increasing concentrations of NaCl. After incubation of the samples for 30 min at 37 °C, the reaction was stopped by addition of 500 µL 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid and after 10 minutes at 0 °C, the mixture was centrifuged for 30 s (10,000 × g). Subsequently, 150 µL of the clear supernatant was mixed with 4 mL OptiFluor before analysis using a liquid scintillation counter to determine the amount of formed P_i. Maximal ATPase activity was estimated from Na⁺-affinity studies by incubating 20 µL of membranes in a final volume of 100 µL containing 50 mM Tris-Ac pH 7.0, 0.1 mM EGTA, 1.2 mM MgCl₂, 1.0 mM Tris-N₃, 5.0 mM KCl, and 100 µM of radiolabeled ATP in the presence of increasing concentrations of NaCl. After incubation of the samples for 30 min at 37 °C, the reaction was stopped by addition of 500 µL 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid and after 10 minutes at 0 °C, the mixture was centrifuged for 30 s (10,000 × g). Subsequently, 150 µL of the clear supernatant was mixed with 4 mL OptiFluor before analysis using a liquid scintillation counter to determine the amount of formed P_i.

Data analysis

All data was analyzed using Graphpad Prism 5.02. Maximum ATPase activity was determined using non-linear curve fitting of log-transformed sodium concentrations. The ATPase activity of the α2-isoform was set at 100% and all other chimeras and single mutants were related to this value. Since all experiments were performed on two independent batches of protein, average ATPase activities are shown in the figures. Sequence alignments were performed using the web-based ClustalW2 program.

Generation of homology models

Crystal structures of the α1-isoform of Na⁺,K⁺-ATPase and sarcoendoplasmic reticulum Ca²⁺-ATPase (SERCA) were used as templates for four homology models representing the α4-isoform in the E1Na⁺ (2C9M13), E1P-ADP (3WGU14), E2P (4HYT15), and E2K⁺·Pi (2ZXE16) conformations. These homology models were built using YASARA software and the WHAT IF Twinset using default parameters. Minimization was performed using an automatic YASARA protocol and default settings.
Results

To identify the amino acids responsible for the observed difference in ATPase activities between the wild type α2- and α4-isofoms of Na⁺,K⁺-ATPase, we performed a series of chimera and single mutagenesis studies (Table 1). We were able to identify two residues in the P-domain of Na⁺,K⁺-ATPase that confer a tenfold increase in ATPase activity upon the α4-isofom following substitution by the corresponding α2-isofom residues.

The first series of experiments confirmed the difference in ATPase activity between both wild type proteins observed previously by our lab, with an eightfold difference in ATPase activity for the chimeras produced (Fig. 1). Replacement of the C-terminal part of the α2-isofom with the corresponding α4-isofom derived residues resulted in an increase in ATPase activity (chimera 4), whereas extension of the α4-isofom sequence towards αM4 decreased the activity dramatically (chimera 5). Introduction of the large cytosolic loop connecting αM4 and αM5 in the α4-isofom by the corresponding residues from the α2-isofom (chimera 10) increased ATPase activity to 1.6 µmol P i mg⁻¹ hour⁻¹ (171% of wild type α2), while protein expression was unchanged (Fig. 1). Hence, the amino acids responsible for the observed difference in ATPase activity between both isoforms are located in the loop connecting transmembrane domains αM4 and αM5.

Based on the high ATPase activity observed for chimera 10, five chimeras were designed (11-15) possessing αM4-αM5 loops that were partially substituted with α4-isofom residues (Fig. 2). Chimeras 12, 13, and 14 showed clearly increased (12 and 14) or mildly decreased ATPase activities, indicating that the amino acids replaced by α4-isofom residues are not responsible for the high activity associated with α2-isofom enzyme. Clearly, chimeras 11 and 15 possessed lower ATPase activities compared with wild-type α2-enzyme: 0.4 and 0.1 µmol P i mg⁻¹ hour⁻¹, respectively. Together, these results indicated that the amino acids responsible for the observed differences between both isoforms are located either in the N-terminal and/or the C-terminal segments of the αM4-αM5 loop.

In order to investigate the role of both the N-terminal and C-terminal segments of the αM4-αM5 loop, another series of chimeras was designed (Fig. 3). Since chimeras 16-19 showed high ATPase activity, the role of the N-terminal part was considered of minor importance compared to the C-terminal part. Chimeras 20 and 21 possessed increased ATPase activities, further pinpointing the high activity-associated amino acids to the very last segment of the αM4-αM5, confirmed by a marked decrease in ATPase activity observed upon

Table 1 Design of chimeras used in this study.

<table>
<thead>
<tr>
<th>Chimera</th>
<th>Composition on amino acid level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chim. 4</td>
<td>α2 1 – 750, α4 760 – 1029</td>
</tr>
<tr>
<td>Chim. 6</td>
<td>α2 1 – 348, α4 359 – 1029</td>
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<tr>
<td>Chim. 10</td>
<td>α4 1 – 358, α2 349 – 750, α4 760 – 1029</td>
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<tr>
<td>Chim. 11</td>
<td>α4 1 – 430, α2 421 – 750, α4 760 – 1029</td>
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<tr>
<td>Chim. 15</td>
<td>α4 1 – 358, α2 349 – 634, α4 644 – 1029</td>
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<tr>
<td>Chim. 17</td>
<td>α4 1 – 643, α2 635 – 750, α4 760 – 1029</td>
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<tr>
<td>Chim. 18</td>
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<tr>
<td>Chim. 22</td>
<td>α4 1 – 358, α2 349 – 420, α4 431 – 666, α2 676 – 1029</td>
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<td>Chim. 23</td>
<td>α4 with Ile679M, Q680T, K682E, Q686E, Q689K, P692T, L714Q, and V717I</td>
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</table>

Amino acid constitution of a selection of the chimeras designed and expressed for this study.
Introduction of α4-derived residues to this segment with chimera 22 (0.4 µmol Pi mg\(^{-1}\) hour\(^{-1}\)) possessed only eight amino acid substitutions compared with wild type α4-isoform, we concluded that one or more of these eight mutations present in the very last segment of the αM4-αM5 loop was responsible for the observed difference in activity.

Introduction of the α2-derived equivalents of all these eight amino acids into wild type α4-isoform increased maximal ATPase activity approximately tenfold, as shown in figure 4 (Chimera 23: 2.1 µmol Pi mg\(^{-1}\) hour\(^{-1}\) \textit{versus} α4 wild type: 0.2 µmol Pi mg\(^{-1}\) hour\(^{-1}\)). This clearly confirmed that the very last segment of the αM4-αM5 loop is involved in the difference in ATPase activity between both isoforms. To identify the role of each of these amino acids, single mutation studies were performed, which indicated that four amino acids were associated with the increased ATPase activity observed for chimera 22: α4-I679M (0.6 µmol Pi mg\(^{-1}\) hour\(^{-1}\)), α4-Q680T (0.2 µmol Pi mg\(^{-1}\) hour\(^{-1}\)), and α4-P692T (0.4 µmol Pi mg\(^{-1}\) hour\(^{-1}\)). Combination of the two mutations that conferred highest ATPase activity (I679M and P692T) resulted in an increase of activity to 112% of α2-isoform wild type enzyme. Maximum ATPase activity represents the average of two independently produced batches of protein.

Because chimera 22 possessed only eight amino acid substitutions compared with wild type α4-isoform, we concluded that one or more of these eight mutations present in the very last segment of the αM4-αM5 loop was responsible for the observed difference in activity.

A comparison of the eight amino acids present in chimera 23 across the human isoforms (Fig. 5) showed that several of the identified amino acids are exclusively present in the α4-isoform, including Gln\(^{686}\), Lys\(^{692}\), Pro\(^{692}\), Leu\(^{714}\), and Val\(^{717}\). The other three amino acids identified were not unique for the α4-isoform: Ile\(^{679}\) and Pro\(^{692}\) were found in at least two human isoforms. Next, a sequence alignment was performed to study the amino acid sequence of the α4-isoform across therian mammals, which are the only organisms possessing this isoform. As figure 6 shows, the two amino acids identified above were likely introduced into the α4-isoform upon the emergence of the \textit{Hominidae} family: man, chimpanzee, and orangutan share Ile\(^{679}\) and Pro\(^{692}\), whereas various other mammals (ranging from elephant, bat, mouse, guinea pig, green monkey, crab-eating macaque, and olive baboon) express different amino acids at these positions.

Finally, we constructed homology models representing the α2- and α4-isoforms in the four basic conformations (E\(_1\), E\(_{1}\)P, E\(_2\), and E\(_{2}\)) of Na\(^+,K^+\)-ATPase to identify possible intramolecular interactions, which might explain the effect observed upon replacement of Ile\(^{679}\) and Pro\(^{692}\) with methionine and threonine, respectively. Model calculations revealed for both isoforms...
that the identified residues are flanking an α-helix, which joins two β-sheets belonging to the Rossmann fold present in the P-domain. Interestingly, in the E_1 conformations of both isoforms the proximal β-sheet linked by the helix mentioned above is absent, whereas a hydrogen bond is predicted between the backbone of Pro692 and a loop that is organized into a β-sheet in the other three conformations for the α4-isoform, as shown in figure 7. For the α2-isoform, the same hydrogen bond was predicted for all conformations, except the E_1 conformation.

**Discussion**

In this study we investigated the difference in ATPase activity between the α2- and α4-isoforms of Na^+\text{-}K^+\text{-}ATPase. For this purpose, we designed and produced various chimeras and single mutants, which were tested in an ATPase activity assay. We identified residues Ile679 and Pro692 of the α4-isoform to be associated with the very low ATPase activity observed for this isoform. Proof for their involvement was obtained by the marked increase in ATPase activity observed upon replacement of these two residues with their α2-isoform derived counterparts.

The α4-isoform was discovered last of the four isoforms and was found to be expressed in testis tissue only.\footnote{Biochemical characterization of rat and human α4β1 and α4β3 (testis expresses both β-isoforms) enzymes expressed in insect cells showed that these two isoforms possess similar Na^+, K^+ affinities, whilst the β-subunit does not significantly affect the affinities for any of the parameters tested.\textsuperscript{8,9}}\textsuperscript{18,19} The latter study also showed that there are distinct differences between the α4- and α1-isoforms (testis expresses both β-isoforms); the α4-isoform possesses increased Na^+ affinities and decreased K^+ affinities, respectively, whereas the ouabain affinity was significantly increased compared with the α1-isoform.\textsuperscript{19} In fact, the latter is hypothesized to play a housekeeping role and the α4-isoform is specifically involved in sperm motility\textsuperscript{19}: α4-isoform knockout mice produce immotile spermatozoa incapable of (in vitro) motility.\textsuperscript{19}

**Figure 6** Sequence alignment of various mammalian α4-isoforms.

Grey-marked residues highlight the two identified amino acids across various mammalian species. HOMO: Homo sapiens (modern humans), PAN: Pan troglodytes (chimpanzee), PONGO: Pongo abelii (orangutan), MACACA: Macaca fascicularis (crab-eating macaque), PAPIO: Papio anubis (olive baboon), CHLOROCEBUS: Chlorocebus sabaeus (green monkey), CAVIA: Cavia porcellus (domestic guinea pig), MUS: Mus musculus (house mouse), MYOTIS: Myotis davidii (insectivorous bat), LOXODONTA: Loxodonta africana (African bush elephant).

**Figure 7** Homology models of the four enzymatic conformations of the α2- and α4-isoform of human Na^+\text{-}K^+\text{-}ATPase.

Residues responsible for the observed ATPase activity differences highlighted in green. Under physiological conditions, Na^+\text{-}K^+\text{-}ATPase exerts its transport function by alternating between four different conformational states: E_i, E_iP, E_2P, and E_2. Panel A displays the localization of the two residues Met670 and Thr683 of the α2-isoform, with the formation of hydrogen bonds involving Thr683 in various conformations. Panel B highlights the localization of residues Ile679 and Pro692 of the α4-isoform, showing the formation of a hydrogen bond involving Pro692 in the E_1 conformation. Red represents the β-sheets that constitute the Rossmann-fold.
fertilization.2 The distinct biochemical characteristics of αβ1- and αβ3-isoforms have been linked to a highly specialized function proposed for the α4-isoform: co-localized with the Na’/H’ exchanger (NHE) its role supposedly lies in maintaining a Na’ gradient that is used for extrusion of protons, which are released from mitochondria in the sperm cell flagellum.20 In humans, an alternative regulator of intracellular pH in sperm is constituted by the proton-selective channel Hv1, which is activated by membrane depolarization as well, providing an alternative mechanism of action for α4-mediated intracellular pH maintenance.5

At the time of the discovery of the fourth isoform, its gene was shown to be physically linked to the α2-isoform encoding gene.5 Previous studies have highlighted the differences between the various isoforms of rat Na’,K’-ATPase: whereas the apparent Na’ and ATP affinities are similar for α2β1- and α4β1-isoforms, the apparent K’ affinity and ouabain affinity of the α4β1-isoform are decreased compared with the α2β1-isoform.18 Interestingly, we have observed before (unpublished studies) that these two isoforms exhibit different ATPase activities: the α2-isoform shows highest activity upon expression of all four isoforms in S9 insect cells, whereas the α4-isoform possesses lowest activity, approximately 0.1-0.2 µmol Pi mg⁻¹ hour⁻¹. Our goal was to identify the mutations that have conferred this low ATPase activity upon the α4-isoform in comparison with the closely related α2-isoform. The first series of chimeras resulted in identification of increased ATPase activity of an α4-based chimera where the αM4-αM5 loop sequence was replaced by the corresponding amino acids from the α2-isoform. This showed that the low ATPase activity observed for α4-isoform wild type enzyme is not due to differences in amino acid sequences in any of the ten transmembrane regions or extracellular domains of Na’,K’-ATPase, but rather is associated with differences in either the nucleotide binding (N-) or phosphorylation (P-) domains, which together constitute the large cytosolic loop connecting αM4 and αM5. Follow-up studies based on the design of chimera 10 revealed that the amino acids responsible for the observed ATPase activity differences were located at the beginning (N-terminal) and/or end (C-terminal) part of the αM4-αM5 loop, together constituting the P-domain. Upon replacement of the C-terminal residues of the αM4-αM5 loop with their corresponding α2-residues, the ATPase activity was increased dramatically compared with α4-isoform enzyme, highlighting the residues located within this region for further investigation. Eventually, we were able to narrow down the number of amino acids responsible for the observed effect to eight: the number of residues that are different between the α2- and α4-isoforms located within the 679-717 range of the α4-isoform sequence. Gain-of-function studies confirmed that indeed these amino acids were involved by showing that introduction of all eight α2-isoform derived amino acids into the α4-isoform resulted in a dramatic increase in ATPase activity compared with the α4-isoform wild type enzyme. Final experiments proved that the effect of introduction of these eight amino acids was mainly due to introduction of two mutations: I679M and P692T. Inclusion of two additional mutations (Q680T and K682E) resulted in only a minor increase in ATPase activity of α4 wild type enzyme, strengthening the hypothesis that I679M and P692T together are responsible for the observed differences in activity between the α2- and α4-isoforms.

As mentioned above, previous studies showed that the gene encoding the α4-isoform originates from the α2-encoding gene.5 Moreover, the former is hypothesized to be present only in therian mammals, based on analysis of evolutionary patterns of the various isoforms.7 Assuming that α2-isoform residues Met⁷⁰⁰ and Thr⁷⁴ⁱ were mutated into isoleucine and proline during evolution, we studied the α4-isoform evolutionary pathway for the two mutations identified in the present study. Investigation of the presence of these two amino acids across the various human isoforms showed that Ile⁶⁷⁹ and Pro⁶⁹² are both unique for the α4-isoform. The other three isoforms contain either a phenylalanine or methionine replacing the Ile⁶⁷⁹, while Pro⁶⁹² appears to have originated exclusively with the emergence of the α4-isoform. Comparison of α4-isoform sequences between species revealed that both Ile⁶⁷⁹ and Pro⁶⁹² are present in the sequence of all Hominidae family members including Pan troglodytes (chimpanzee), Gorilla gorilla (gorilla) and Homo sapiens (humans), whereas the Old World monkeys including Macaca fascicularis (crab-eating macaque), Papio Anubis (olive baboon), and Chlorocebus sabaeus (green monkey) differ in the amino acids located at these positions, suggesting introduction of these mutations upon the development of the great apes.

Both amino acids identified in the present study are located within the C-terminal part of the P-domain, the most conserved domain of Na’,K’-ATPase.20 The P-domain has a characteristic Rossmann-fold, a structural motif that is present in many nucleotide-binding proteins and in the case of Na’,K’-ATPase consisting of a central seven-stranded β-sheet flanked by various α-helices, including the cytoplasmic end of the fifth transmembrane domain (αM5).24 Within this cytoplasmic site, Ile⁶⁷⁹ and Pro⁶⁹² are flanking a small α-helix connecting the two Rossmann-fold β-sheets located at the cytoplasmic side of the protein in both the E₁ and E₂ conformations. Structural analysis did not result in identification of extensive hydrogen bond networks involving Pro⁶⁹² in the α4-isoform (except for a minor interaction in the E₂ conformation), excluding a direct role for these two residues in the conformational changes that take place during the Na’,K’-ATPase reaction cycle. Interestingly, the same hydrogen bond was predicted for all three conformations except the E₁ conformation of the α2-isoform. Moreover, no significant hydrogen bonds were predicted for Ile⁶⁷⁹ or its α2-equivalent in both isoforms, although this residue showed the largest increase in ATPase activity in the single mutant studies. Located closely to the phosphorylation site of Na’,K’-ATPase, one hypothesis concerning the observed effect on ATPase activity might be that replacement of the two residues resulted in an altered orientation of the Rossmann-fold, in particular involving an α-helix containing a conserved lysine residue (α4 Lys⁶⁸⁰) essential for the transition to the E₃ state by abstracting the negative charge of the Asp⁶⁷⁸ side chain and the γ-phosphate.25 In conclusion, we have shown that replacing two α4-isoform amino acids with their α2-isoform counterparts (I679M and P692T) resulted in a dramatic increase in ATPase activity. These two amino acids are located in a short α-helix connecting two β-sheets at the cytoplasmic side of the Rossmann-fold present in the Na’,K’-ATPase P-domain, and were not predicted to
form H-bond networks based on homology modeling. Both amino acid substitutions appear to have been introduced upon the emergence of the great apes, since they are not found in other mammalian species. Possibly, the α4-isoform has emerged from the α2-isoform, although with a decreased need for ATPase activity, which has given rise to the two mutations described here.

References

Chapter 8
General discussion
Introduction

Almost 60 years have passed since the discovery of Na⁺,K⁺-ATPase, and the scientific knowledge concerning this protein has been increasing steadily. Since 2009, when the project resulting in this thesis started, over 2500 articles have been published on the many functions of Na⁺,K⁺-ATPase. In these five years, the scientific community has taken a leap in understanding how this highly important protein is related to health and disease, through crystal structures of different conformations, newly discovered mutations in the isoforms, endogenous inhibitors, and an updated view on its role in signal transduction. The goal of the project was to investigate the various isoforms of Na⁺,K⁺-ATPase found in the human body. To this end a baculovirus expression system was used, taking advantage of the absence of endogenous Na⁺,K⁺-ATPase in insect cells used to produce different (mutant) isoforms. Not only was Na⁺,K⁺-ATPase itself studied, but also the effect of various inhibitors belonging to the ancient class of digitalis-like compounds (DLCs). Here, we discuss the results of the various studies published in this thesis in a broader context.

Na⁺,K⁺-ATPase and cellular signaling

Over the last ten years cellular signaling via Na⁺,K⁺-ATPase has received a lot of attention and is nowadays a firmly established concept. Various signaling pathways have been linked to Na⁺,K⁺-ATPase, including the EGFR-MAPK cascade, the PLC-γ pathway, and the PI 3K pathway. The MAPK pathway is perhaps most extensively studied: it is thought that upon stabilization of the E₂P-DLC conformation of Na⁺,K⁺-ATPase by binding of a DLC, the initiating event resulting in downstream MAPK signaling is alleviation of one of the Na⁺,K⁺-ATPase-Src direct interactions, releasing the Src kinase domain from the Na⁺,K⁺-ATPase N-domain, thereby inducing Src autophosphorylation. The phosphorylated Src transmits the signal to downstream signaling proteins, resulting in MAPK activation, subsequently leading to effects on various cellular functions. Na⁺,K⁺-ATPase involved in DLC-mediated signaling resides in caveolae, which are small invaginations of the plasma membrane. Reportedly, approximately 50% of total cellular Na⁺,K⁺-ATPase resides in caveolae, where the enzyme is dedicated to signaling and does not possess transport activity. However, over the last five years several studies have called into question the previously established view on the Na⁺,K⁺-ATPase-EGFR-MAPK cascade and the specific role of caveolar Na⁺,K⁺-ATPase as described above. At first, extensive comparative studies between caveolar and noncaveolar Na⁺,K⁺-ATPase pools reported no distinct differences between both pools: both contain active enzyme, and more importantly, both pools possess active Src that cannot be further activated by ouabain, casting doubts over the proposed direct interaction between Src and Na⁺,K⁺-ATPase. This result is in line with our unpublished studies in LLC-PK1 cells, where we were not able to induce Src phosphorylation upon addition of ouabain to the culture medium. In addition, the results described in Chapter 2 show that Src activation is not induced by DLC-mediated inhibition solely, since vanadate was capable of inducing Src phosphorylation as well in a test tube set-up.
using recombinant Src and purified Na⁺,K⁺-ATPase. This finding implies that Na⁺,K⁺-ATPase inhibition in general leads to local increases in ATP concentration, which subsequently result in Src autophosphorylation, a process that is inhibited when Na⁺,K⁺-ATPase is active (Fig. 1). This hypothesis is in sharp contrast with a study by Xie and coworkers, who reported that vanadate is not capable of inducing Src phosphorylation while using a similar experimental setup. This brings forward an important question: how can two similar assays result in such contradictory results? As mentioned above, the experimental setup used by our group was similar to the article by Xie and coworkers, and it is unlikely that small differences in experimental design led to opposite results. Our findings were recently confirmed by a study performed by another lab, again using a similar experimental setup, concluding as well that there is no evidence for a direct interaction between Src and Na⁺,K⁺-ATPase. Recently, the role of Src in cellular signaling was questioned with regard to the PI3K signaling pathway as well, stressing the need for further elucidation of the molecular mechanism of DLC-mediated signaling via Na⁺,K⁺-ATPase. Until then, the exact role of Src and the molecular mechanism involved in cellular signaling remains uncertain.

**Endogenous production of digitalis-like compounds**

Digitalis-like compounds are highly specific inhibitors of Na⁺,K⁺-ATPase, which are well-known in medical practice for their use in treatment of various heart conditions, including atrial fibrillation, atrial flutter and sometimes heart failure. Extracted from the leaves of *Digitalis purpurea* and isolated from the skin of DLC-resistant animals, these compounds have been used for both medicinal and hunting purposes. As mentioned in Chapter 1, various studies have pointed towards a role for endogenously produced DLCs in blood volume expansion. This has led to the identification of various endogenous DLCs, including ouabain, dihydro-ouabain-like factor, marinobufagenin, telocinobufagin, 19-norbufalin, and bufalin. It is now widely accepted that the human body produces small amounts of DLCs, resulting in circulating concentrations that are too low to exert Na⁺,K⁺-ATPase inhibition. Therefore, a role in cellular signaling as described above has been suggested for these compounds. However, a recent study using ultra-performance liquid chromatography tandem mass spectrometry method (UPLC-MS/MS), with a lower limit of detection of 1.7 pM designed for the detection of endogenous ouabain, could not detect the compound in plasma from patients with and without heart failure. The authors point out that identification of endogenously produced ouabain using immunoassay-based methods suffers from limited specificity due to cross-reactivity with structurally similar compounds, explaining how previous studies have reported concentrations in the high picomolar range. This calls for a careful approach regarding immunoassay-based identification of endogenously produced DLCs, for instance by combining multiple analytical assays as well as testing for the presence of DLCs by performing functional assays.

**DLC binding to Na⁺,K⁺-ATPase**

In recent years, the availability of crystal structures of enzymatic conformations has accelerated the pace of scientific developments concerning Na⁺,K⁺-ATPase. The first publication of a crystal structure of Na⁺,K⁺-ATPase with a DLC bound to its binding site described the low-affinity DLC bound state E₂-DLC obtained by ouabain soaking into previously formed E₂ state crystals in the presence of K⁺. More recently, a crystal structure representing Na⁺,K⁺-ATPase in the E₂P-DLC conformation obtained by co-crystallization of ouabain was published, representing the high-affinity ouabain bound state. This crystal structure revealed the presence of a magnesium ion in ion-binding site II and describes the possible involvement of this ion in long-range electrostatic interactions with the highly strained five-membered unsaturated lactone ring that is inserted deeply into the DLC binding site, 5 Å away from the ion-binding site. In addition, this study confirmed various previously published mutagenesis studies identifying key residues involved in binding of the apolar and polar sides of the steroid core of DLCs, strengthening the conclusions of these studies. Although the published crystal structures have provided valuable information on binding of ouabain, crystal structures of Na⁺,K⁺-ATPase binding other DLCs are not available yet. At the same time, the decline of digoxin and ouabain use in clinical practice, due to their low therapeutic index, has renewed the interest in binding affinities of other DLCs, especially with regard to the other isoforms of Na⁺,K⁺-ATPase. This idea is based on the presence of DLCs, for instance by combining multiple analytical assays as well as testing for the presence of DLCs by performing functional assays.

**Figure 1** Schematic representation of the proposed mechanism of Src activation.

Under normal conditions (left side), Na⁺,K⁺-ATPase consumes ATP at a high rate, preventing Src phosphorylation. However, upon binding of a Na⁺,K⁺-ATPase inhibitor (1) as depicted on the right side, the rate of ATP consumption by Na⁺,K⁺-ATPase decreases (2), leading to a concomitant increase of local ATP concentration (3), which can then be used for phosphorylation of Src (pSrc), initiating a signaling cascade (4).
of multiple isoforms in heart muscle cells where the ubiquitous α1- and more restricted α2- and α3-isoforms are expressed. Since the inotropic effect of DLCs is attributed to a direct increase in cardiac contractility, identification of structural compound properties that confer a high selectivity to the α2- and α3-isoforms over the ubiquitously expressed α1-isoform would be of great interest for future drug development. This goal has been approached from the ligand point of view by testing a variety of DLCs with regard to their binding affinities, as well as from the receptor point of view by utilizing mutagenesis studies on Na’,K’-ATPase and mutants that confer increased or decreased affinities for specific DLCs. Based on an observation concerning the binding affinity of dihydro-ouabain for non-gastric H’,K’-ATPase, we set out to identify the interactions between hydrogenated forms of DLCs and amino acids present near the DLC-binding site as described in Chapter 3. Hydrogenated DLCs possess a fully saturated lactone ring, represented by absence of the double bond found in unsaturated lactone rings. Importantly, the unsaturated lactone ring was implicated in long-range electrostatic interactions with a magnesium ion in ion-binding site II as described above. We were able to obtain hydrogenated ouabain and digoxin by allowing ouabain and digoxin to react with H₂ gas for 16 hours, resulting in complete hydrogenation of these two compounds. Since these two amino acids play an important role in isoform-selectivity of these compounds. Since these two residues are located in the first extracellular loop linking transmembrane domains 1 and 2, their effect is most likely mediated by the initial binding and/or dissociation phase(s) of docking into the DLC binding pocket. We also observed another structural feature: the presence of a sugar moiety reduced the isoform selectivity. This is in contrast to a previous study, which found no such effect. 

**Na’,K’-ATPase and disease(s)**

As mentioned above, four isoforms of the α-subunit are encoded by the human genome, each having a characteristic expression pattern. In the last five years, many studies have been conducted on the link between Na’,K’-ATPase isoform mutations and various diseases, revealing associations between various disease phenotypes and the tissue-specific roles of different isoforms (Table 1). The α1-isoform has been linked only to aldosterone-producing adenomas by somatic mutations. The absence of familiarly associated disease for this ubiquitously expressed isoform most likely reflects the absolute importance of Na’,K’-ATPase function for cellular life. Also, there have been no reports of mutations in the α4-isoform leading to disease. A promising direction for future research would be to screen subfertile men for mutations in the α4-isoform of Na’,K’-ATPase, since this protein is associated specifically with sperm motility and therefore is an interesting candidate with respect to subfertility-related mutations.

In Chapters 4 and 5 we describe the biochemical characterization of various mutations of the α2-isoform leading to sporadic/familial hemiplegic migraine (SHM/FHM) type 2; a subtype of migraine characterized by neurological symptoms preceding the migraine attacks. In general, the proposed disease mechanism cause by these mutations is a loss of function of the α2-isoform that is expressed in astrocytes located at the synapses in the central nervous system. Astrocytes function as supporting cells, favoring the re-uptake of the neurotransmitter glutamate that is released upon arrival of a nerve impulse at the synaptic cleft. By uptake of extracellular potassium and extrusion of sodium, Na’,K’-ATPase provides the driving force necessary for reuptake of free glutamate via the glutamate transporter. However, there are still many questions that need answering regarding the pathophysiology of SHM/FHM type 2, including investigation of the exact sequence of events leading to a migraine attack, but also how specific mutations exert their effect on Na’,K’-ATPase function. Using baculovirus expression in insect cells, we were able to describe the biochemical characteristics regarding various SHM/FHM type 2 associated mutations.

In Chapter 4 we describe the biochemical characteristics of nine mutations associated with SHM/FHM type 2. For six of the mutations we detected a significant loss of catalytic activity, providing a possible explanation for the phenotype of these mutations in line with the suggested role of the α2-isoform in astrocytes at the synaptic cleft: loss of Na’,K’-ATPase function results in loss of glutamate reuptake, leading to disturbed signal transduction over the synapses in the central nervous system. At the same time, a group of researchers from the university of Århus published an article revealing a third half-channel present in Na’,K’-ATPase, located in the C-terminal half of the enzyme. This half-channel transports protons to the third (IIIb) ion-binding site in the E₂ conformation when potassium ions are bound at ion-binding sites I and II. Previous research had revealed that ion-binding sites I and II, located between transmembrane helices 4, 5, and 6, are accessible from the two sides of the membrane via two half-channels: one connecting these sites to the cytosol and the other linking the extracellular space with the core of the transmembrane region. One of the characteristic features of PII- and PIIMA-type ATPases is the presence of an additional

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C-terminal segment harboring four transmembrane helices. The aforementioned research group concluded from electrophysiological studies of various SHM/FHM type 2-associated mutations that an ion-pathway located within this C-terminal segment was affected. Our study included several mutations located within the region of the C-terminal half-channel as well, and indeed we confirmed for most of these mutants a severe loss of ATPase activity, whereas one mutant (R834Q) did possess a decreased ATPase activity compared with wild type enzyme. The work presented here and the discoveries of the Århus group provide crucial converging evidence for the mechanisms underlying diseases associated with SHM/FHM type 2-associated mutations.

Chapter 5 describes how various SHM/FHM type 2 mutations likely affect domain interactions necessary for the conformational changes of the reaction cycle. However, for the other mutations that were studied we could not identify an effect on Na⁺,K⁺-ATPase functionality for any of the biochemical characteristics that we tested, suggesting either non-pathogenicity or an alternative mechanism of disease for these mutations. Whereas SHM/FHM type 2 are associated with mutations in the α2-isofrom, mutations in the α3-isofrom of Na⁺,K⁺-ATPase have been linked to other neurological diseases, including rapid-onset dystonia parkinsonism (RDP) and alternating hemiplegia of childhood (AHC). RDP, which is characterized by an abrupt onset of symptoms including dystonia and parkinsonism, usually presents itself in teens and young adults and has been linked to a variety of α3-associated mutations. It was reported for the first time in the 1990s, and at least 10 mutations in the gene encoding the third isoform of Na⁺,K⁺-ATPase have been reported until today. More recently, mutations in the same isoform were linked to another severe neurological disease: AHC, which is characterized by episodes of (alternating) hemiplegia starting within the first year of life and associated with considerable co-morbidity. In contrast to RDP, AHC is characterized by seizures: symptoms usually disappear upon falling asleep, although attacks may last for several days. Patients may experience a high frequency of seizures, accounting for the devastating effects of this disease. On the other hand, the clinical severity of AHC cases has been reported to vary significantly. Since both RDP and AHC are now known to be caused by mutations in the α3-isofrom of Na⁺,K⁺-ATPase and symptoms may overlap between both diseases, it has been suggested that both diseases represent different manifestations of one underlying disorder. In this hypothesis, AHC represents the more severe phenotype, whereas RDP is associated with less severe manifestations. One of the more frequently observed AHC-associated mutations (D801N) affects an amino acid (Asp⁸⁰¹) that is associated with an RDP-causing mutation (D801Y) as well. Even more supportive of this hypothesis is mutation D923N, which has been detected in both RDP and mild AHC patients. In Chapter 6, we set out to investigate the biochemical characteristics of several frequently observed AHC-associated mutations because an extensive characterization was lacking and it became apparent that AHC showed a wide range of severity of disease. Indeed, using the baculovirus expression system to express six AHC-associated mutants (including D801N and E815K, responsible for ~60% of all cases), we were able to show differences in protein functionality, which improved our understanding of the relationship between disease severity and the various mutations. We showed that mutation E815K indeed resulted in a complete loss-of-function for the associated protein, whereas mutant D801N was able to bind ouabain and therefore might still function as a signaling protein, reflecting minor enzyme functionality for this mutation. These findings are in line with the less severe phenotype associated with the D801N mutation compared with the phenotype of E815K. This study is the first to describe the biochemical characteristics of AHC mutants in detail and provides important new information that might explain why the phenotypes of various mutations differ.

The fourth isoform

Whereas the expression profile of the first three α-isoforms was known already in the 80’s, it took until 1994 before it was clear that the fourth α-isoform encoding gene was expressed only in testis tissue. Until then, the role of the gene encoding this isoform (ATP1A2, later renamed to ATP1A4) was unknown, although a physical link with the α2-gene had been established before. Following extensive biochemical characterization of this isoform, further research highlighted expression mainly in germ cells of testes, with the highest amount of the α4-isoform present in spermatozoa, responsible for two thirds of the ATPase activity in gametes. This finding was confirmed in human tissue, where the amount of α4-isoform was shown to increase with sperm maturation, linking the enzyme to mature sperm physiology in humans. Moreover, the α4-isoform was shown to be localized within the sperm tail, predominantly in the mid-piece of the flagellum, and therefore was linked to sperm motility. The same study revealed that the human α4-isoform has a higher affinity for Na⁺ and a lower affinity for K⁺ and ouabain as compared to the α1-isoform also expressed in spermatozoa, suggesting a specialized role for the α4-isoform. More recently, sperm motility was shown to depend on the α4-isoform, since knock-out male mice were completely sterile and spermatozoa of these mice were unable to fertilize eggs in vitro. Together, these results point towards an important role for the human α4-isoform in male fertility. Unpublished studies by our lab had shown that ATPase activity observed for the α4β1-isoform expressed in insect cells was almost negligible, whereas the activity associated with the α2β1-isoform was the highest of all four isoforms. This led us to investigate how these two isoforms could differ so much in their ATPase activity. Via a series of mutagenesis studies, resulting in over 20 chimaeras and selected single mutations of both isoforms, we were able to identify two amino acids responsible for the low ATPase activity of the α4-isoform. Upon replacement of these residues with their α2-isoform counterparts (I679M and P692T), we could induce a tenfold increase in ATPase activity of the α4β1 enzyme. The role for these two amino acids remains to be established, but this finding is of importance for future studies on the effect of mutations in the α4-gene. These might be associated with subfertility, and the increase in α4-isoform ATPase activity upon replacement of the two amino acids mentioned above facilitates a large window of measurement for future loss-of-function mutations.
Conclusions and future directions
The work described in this thesis improves our understanding of Na⁺,K⁺-ATPase (patho) physiology. We have contributed to the scientific knowledge on cellular signaling via the α1-isoform, questioning the proposed role of the Src protein in DLC-induced cellular signaling. Also, we have established an important difference in hydrogenated-DLC affinities between the α1- and α2-isoform and have identified two amino acids located within the first extracellular loop responsible for this effect. In addition, we have studied mutant proteins associated with either SHM/FHM type 2 (α2-isoform) and AHC (α3-isoform), providing important information on biochemical characteristics associated with these mutations. Finally, studies into the low ATPase rate observed for the α4 wild type enzyme led to the discovery of two amino acids responsible for this low activity as compared to the α2-isoform.

For the future, several Na⁺,K⁺-ATPase related topics deserve further attention: (1) The important information on biochemical characteristics associated with these mutations associated with either SHM/FHM type 2 (α2-isoform) and AHC (α3-isoform), providing extracellular loop responsible for this effect. In addition, we have studied mutant proteins between the α1- and α2-isoform and have identified two amino acids located within the first signaling. Also, we have established an important difference in hydrogenated-DLC affinities α1-isoform, questioning the proposed role of the Src protein in DLC-induced cellular physiology. We have contributed to the scientific knowledge on cellular signaling via the Na⁺,K⁺-ATPase in the phenotypical characteristics of these diseases; (3) Finally, we hypothesize that it is worthwhile to screen subtelomere men for mutations in the gene encoding the α1-isoform. This isoform could be an interesting target for drug therapy since it plays a prominent role in sperm motility and since it is expressed exclusively in testis tissue.

All in all, this thesis contributes to the understanding of the firstly discovered active transport protein, although every new answer generates new questions. Or, to use the words of the illustrious discoverer of the subject of this thesis, Jens Christian Skou: "Looking for the answer, you hunt it, you catch it, you fool yourself; the answer, is always, a step ahead." Let this thesis be a small step along the way to answering all questions regarding the many functions of Na⁺,K⁺-ATPase isoforms in health and disease.

References


 Chapter 8


Chapter 9
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Summary

The plasma membrane of animal cells consists of a lipid bilayer separating the intra- and extracellular environments, allowing for differences in the biochemical composition between both compartments. There are large differences across the membrane in the concentrations of ions, amino acids, proteins, and many other molecules. In order to fulfill its physiological role, each cell invests heavily in maintenance of its intracellular environment at the expense of cellular energy in the form of ATP. One of the prerequisites for cellular life is the presence of high intracellular K+ concentrations for neutralization of the negatively charged proteins present in the cell. The cell must employ a mechanism that ensures high intracellular K+ concentrations to allow proteins to function within their physiological range, while preventing swelling of the cell caused by the concomitant influx of water. Nature has solved this by elegantly balancing the influx of K+ by the efflux of another abundantly present cation: Na+. Cells go through great lengths in order to maintain an optimal intracellular environment, and the transport of Na+ and K+ across the cellular membrane is no exception. To maintain a high intracellular K+ and extracellular Na+ concentration the cell has to transport both cations in opposite direction across the membrane against their steep concentration gradients. This bidirectional transport is among the most energetically taxing processes that take place in the cell, accounting for approximately 30-40% of its ATP expenditure, or even higher percentages in certain tissues. In the 1950s, pioneering research by the Danish biochemist Jens Christian Skou led to the identification of an ATPase (ATP consuming enzyme) in the giant axons of squid tissue that was activated only in the simultaneous presence of Na⁺, K⁺, Mg²⁺, and ATP. At the time, the existence of active transport was still theoretical, and Skou's discovery of what is now known to be Na⁺,K⁺-ATPase proved for the first time that active transporter enzymes do exist. Later studies showed that Na⁺,K⁺-ATPase exists as a heterodimer, consisting of a catalytic α-subunit that together with a β-subunit (required for proper folding and insertion into the membrane) forms an enzyme that translocates Na⁺ and K⁺ across the cellular membrane in a fixed stoichiometry (3:2, respectively) at the expense of one molecule of ATP, and that this process can be inhibited by digitals-like compounds (DLCs). Nowadays, there is a wealth of information on the role of Na⁺,K⁺-ATPase within the human body, which is described in Chapter 1. The α-subunit is present in four different isoforms, and each isoform displays a tissue-specific expression pattern. This ranges from expression in all human cells as observed for the α1-isoform, to highly localized expression as observed for the α4-isoform, which is expressed solely in testis tissue. The other two α-isoforms display an intermediate expression pattern: whereas the α2-isoform is expressed by astrocytes located at the synaptic cleft (among other places), the α3-isoform is detected mainly in neurons of the central and peripheral nervous systems. All four α-isoforms have a physiological function that is reflected by their expression patterns. The α1-isoform, the most abundant of all four, is proposed to function as the housekeeping isoform. Present in every cell of the human body, it serves to maintain the essential ion gradients as discussed above. This isoform has
drawn renewed attention because of reports about the presence of pico- and nanomolar concentrations of various endogenously produced DLCs in blood plasma. Until then, DLCs were only known to exist in plants and certain animal species, mainly serving a protective role against predators. In fact, low concentrations of DLCs from plants have been used in medicine for the treatment of atrial fibrillation and heart failure: inhibition of Na⁺,K⁺-ATPase by DLCs results in inhibition of Na⁺ transport from the cell, eventually leading to increased contractility. With the discovery of endogenously produced DLCs in a concentration range that lies below the therapeutic range, it was hypothesized that these endogenous compounds are involved in cellular signaling events by inhibiting Na⁺,K⁺-ATPase only mildly. In Chapter 2, we investigated the role of Na⁺,K⁺-ATPase in one of the proposed DLC-induced signaling pathways where the enzyme is thought to be involved in a direct interaction with Src protein. In our experiments, we found no evidence for this proposed direct interaction. Using purified Na⁺,K⁺-ATPase and recombinant Src, we showed that phosphorylation of Src (suggested to be induced by a certain conformation of Na⁺,K⁺-ATPase) depended exclusively on the amount of ATP that was available in the test tube. We could induce Src phosphorylation not only by addition of digoxin, but also nonspecifically by depleting either Na⁺ or K⁺, or addition of another inhibitor, vanadate. These findings led us to conclude that there is not enough evidence for a mechanism of action including the proposed direct interaction between Na⁺,K⁺-ATPase and Src. We suggest that Src does indeed play a role in Na⁺,K⁺-ATPase-mediated signaling, but by means of a different mechanism, as shown by our findings. Recent publications from independent research groups have confirmed our findings regarding the role of Na⁺,K⁺-ATPase in Src-mediated signaling, which indicates that this pathway requires more investigation.

In Chapter 3 we also studied DLC binding to Na⁺,K⁺-ATPase, focusing on the binding affinities of (hydrogenated) ouabain and digoxin from both the ligand and the receptor perspective. To compare the various isoforms and their mutants, we made use of a baculovirus expression system, allowing us to obtain purified membrane fractions from insect cells that contained the desired (mutant) enzyme. We set out to investigate the binding affinities of various DLCs for the α1- and α2-isoforms. We observed that dihydro-ouabain showed a clear preference for the former, with an approximately tenfold difference in affinity between both isoforms. This was not seen for ouabain, although the structure of both DLCs only differs in a fully saturated (dihydro-ouabain) versus an unsaturated (ouabain) lactone ring moiety. Next, we investigated the specific amino acid residues responsible for the observed difference in affinity for dihydro-ouabain. We could identify two residues in the α2-isoform that upon conversion into their α1-isoform counterparts reduced the isoform selectivity that was present for the wild type enzymes: Met119 and Ser124 (α2-coding) reduced this difference significantly. The effect of hydrogenation of the lactone ring moiety was identical for digoxin and its hydrogenated form dihydro-digoxin: digoxin showed a preference for the α2-isoform, whereas dihydro-digoxin favored the α1-isoform, although the difference was less pronounced than observed with ouabain and dihydro-ouabain. Again, the same amino acids (Met119 and Ser124) appeared to play a role in the isoform selectivity. For digoxin, the preference for the α2-isoform could be reduced significantly upon replacement of Met119, whereas preference of dihydro-digoxin for the α1-isoform could be reversed by replacing Ser124. Together, these studies shed new light on the isoform selectivity for DLCs and their hydrogenated counterparts: isoform selectivity between the α1- and α2-isoforms is mediated via Met119 and Ser124.

Na⁺,K⁺-ATPase and disease

Over the last years, Na⁺,K⁺-ATPase has also received increasing attention for its involvement in development of disease. For instance, mutations in the α2-isoform have been linked to sporadic/familial hemiplegic migraine (SHM/FHM), rare subtypes of migraine with aura. Although the exact pathophysiological mechanism of these mutations remains to be determined, it is generally assumed that Na⁺,K⁺-ATPase expressed in astrocytes, located at the synaptic cleft as supporting cells, plays a role. Most likely, mutations in the α2-isoform hamper the catalytic functionality of the enzyme, thereby causing a decrease in the transmembrane gradients of both Na⁺ and K⁺. As a consequence, re-uptake of glutamate from the synaptic cleft is reduced, which contributes to the development of migraine attacks. Apparently, mutations associated with SHM/FHM affect the enzyme’s functionality. Therefore, we wanted to know what the exact biochemical effects of these mutations were. The results of our studies are described in Chapters 4 and 5.

Chapter 4 describes the role of nine SHM/FHM mutations in the development of disease. Based on assessment of one of the key parameters of Na⁺,K⁺-ATPase functionality, ATPase activity, we could identify six mutants with a complete loss of function: P796S, M829R, R834X, del 935-940 ins Ile, R937P, and D999H. All of these showed negligible amounts of ATPase activity. For mutant P796S, we speculated that introduction of a serine results in the formation of a hydrogen bond that might indirectly affect the association of the α- and β-subunits. Mutant M829R is suggested to have lost ATPase activity due to lack of important interactions stabilizing the interdomain region between the sixth and seventh transmembrane domains. As for the remaining four mutants showing complete loss of function, we hypothesize that these mutations all interfere with the integrity of a recently discovered half-channel, which is located within the C-terminal region of the enzyme and plays a very important role in the enzyme’s ion transport mechanism. Mutant T345A showed an insignificant increase in ATPase activity compared with α2 wild type enzyme, but this was paralleled by a minor increase in ouabain binding capacity. We could not provide an explanation for the role of this mutation in development of disease. The biochemical characteristics of mutant R879W also did not link to disease, and we could not identify any intramolecular interactions that might provide an alternative hypothesis. These two mutations could therefore constitute polymorphisms instead of disease causing mutations. Finally, we could identify a significantly decreased ATPase activity as well as maximum ouabain binding level for mutant R834Q. Although this mutation is also located within the C-terminal half-channel, apparently the resulting effect is not as detrimental as the other mutations within this region.
In Chapter 5 we characterized the biochemical effects of eleven additional SHM/FHM-linked mutations. Again, we used baculovirus expression to obtain membrane fractions expressing mutant enzyme. We found that the effects of these mutations varied considerably. For instance, we could not identify any differences in biochemical properties between wild type enzyme and mutants Y9N, R51H, C702Y, and R879Q, suggesting that they do not play a role in the development of SHM/FHM. On the other hand, mutant F786L showed a complete loss of function, and we could not determine any ATPase activity or ouabain binding, which are hallmarks of Na⁺,K⁺-ATPase activity. Two mutants, R548C and R548H, showed a decrease in turnover rate, indicating that these mutants function at a lower speed, resulting in less transport of Na⁺ and K⁺, which could therefore explain their link to development of SHM/FHM. Turnover rates of mutants E174K and E700K were also lower, although not statistically different. The remaining two mutants (G900R and E902K) did not reveal any biochemical explanation for their role in development of disease, but both affected residues are considered to play an important role in interactions between the α- and β-subunits, and might therefore result in decreased functionality of the Na⁺,K⁺-ATPase pool present at the astrocyte's plasma membrane.

Disease-causing mutations have also been linked to the α3-isoform. For instance, rapid-onset dystonia-parkinsonism (RDP), a severe neurological disorder, is associated with mutations in this isoform. More recently, it was shown that mutations located primarily in or near the transmembrane regions may lead to development of alternating hemiplegia of childhood (AHC), a disease characterized by episodes of hemi-paralysis that may occur already shortly after birth. Interestingly, it is hypothesized that these two different diseases (RDP and AHC) present two sides of a continuum of α3-isoform mutations. Chapter 6 provides an extensive biochemical characterization of six mutations found in AHC patients: S137Y, D220N, I274N, D801N, E815K, and G947R. Mutant D220N gave no changes in any of the tested parameters compared with the wild type enzyme, which suggests that this mutation may not be pathogenic, although we have not investigated protein localization. Three of the mutants (I274N, E815K, and G947R) lacked ATPase activity, ouabain binding, and phosphorylation, representative of a severe loss of function. Two mutants (S137Y and D801N) showed loss of ATPase activity and phosphorylation while both mutants were able to bind ouabain, suggesting that some protein functionality was still present. We hypothesize that the difference in protein functionality between mutants E815K and D801N (the two most frequently observed AHC-linked mutations) might reflect the difference in phenotype observed for these two mutations.

Chapter 7, the last experimental chapter of this thesis, describes the difference in ATPase activity between the α2- and α4-isoforms. We have found previously that membrane fractions expressing the α2-isoform possess high ATPase activity, whereas the α4-isoform displays only minor activity. Since both isoforms are hypothesized to be linked evolutionarily, we set out to identify the amino acids involved in the difference in ATPase activity. By designing and producing a series of chimeric proteins aimed at identifying the region of the proteins responsible for this difference, we were able to show that replacement of only two amino acids in the α4-isoform with their α2-isoform counterparts resulted in a dramatic increase in ATPase activity. Both amino acids are located in the P-domain of the large cytosolic loop connecting the fourth and fifth transmembrane domains. Here, Ile⁶⁷⁹ and Pro⁶⁹² (present in the α4-isoform) are located near the Rossmann-fold, which harbors the phosphorylation site of Na⁺,K⁺-ATPase, hypothetically affecting the ATPase activity through interference with the structural integrity of this region and thus leading to the significantly lower ATPase activity present for the α4-isoform.

Chapter 8 describes the broader context of the studies reported in this thesis, stressing the crucial role of Na⁺,K⁺-ATPase in health and disease. We argue that there will be an increase in the amount of mutations linked to Na⁺,K⁺-ATPase isoforms, which will require biochemical characterization to help understand the phenotype. In addition, it is desirable to focus on establishing the presence of endogenous DLCs using state-of-the-art techniques that have recently become available. Finally, it would be very interesting to screen subfertile men for mutations in the α4-isoform, since this isoform is expressed solely in testis and therefore provides an interesting target for therapy.

In summary, we have shown for the first time how AHC-linked mutations affect the protein's various functions, and have shed new light on the proposed role of Na⁺,K⁺-ATPase in its proposed role in cellular signaling. We have also investigated the ouabain binding pocket with regard to binding of hydrogenated DLCs and have increased the knowledge on the effects of various SHM2/FHM2-linked mutations. Finally, we have been able to increase the ATPase activity of the α4-isoform upon replacing only two residues. Together, the studies reported here add to our understanding of the role of Na⁺,K⁺-ATPase in health and disease, illustrating how this enzyme plays a crucial role in the human body.
Samenvatting

Het plasmamembraan van alle dierlijke cellen bestaat uit een dubbele lipidendraag die de intra- en extracellulaire milieus scheidt, waardoor verschillen in de biochemische samenstelling van beide compartimenten mogelijk zijn. Zo bestaan er grote verschillen in concentraties van bijvoorbeeld ionen, aminozuren, eiwitten en vele andere moleculen aan weerszijden van het membraan. Om zijn fysiologische rol te kunnen vervullen investeert elke cel grote hoeveelheden energie in de vorm van ATP in het handhaven van de intracellulaire samenstelling. Eén van de noodzakelijke voorwaarden voor het goed functioneren van de cel is de aanwezigheid van een hoge cytosolische concentratie van K⁺, waardoor de negatief geladen eiwitten worden geneutraliseerd. De cel heeft hiervoor een mechanisme nodig dat ervoor zorgt dat de intracellulaire concentratie van K⁺ hoog kan zijn, terwijl tegelijkertijd moet worden voorkomen dat het celvolume toeneemt door osmotische wateropname. De natuur heeft dit probleem opgelost door de cellulaire opname van K⁺ te compenseren met het uitpompen van Na⁺. Een belangrijk deel van de energie die de cel gebruikt voor het handhaven van het intracellulaire milieu, is nodig voor het transport van Na⁺ en K⁺ over het celmembraan. Doordat de extracellulaire Na⁺-concentratie en de intracellulaire K⁺-concentratie beide hoog zijn, moet de cel deze ionen in tegengestelde richting tegen hun concentratiegradiënt in transporteren. Dit is één van de meest energiekostende processen binnen de cel, dat naar schatting 30-40% van de totale ATP consumptie kost, terwijl dit percentage nog hoger ligt in bepaalde organen. In de jaren ’50 van de vorige eeuw leidde pionierswerk van de Deense biochemicus Jens Christian Skou tot de ontdekking van een ATPase (een ATP-verbruikend enzym) in axonen van de pijlinktvissen dat alleen geactiveerd kon worden wanneer zowel Na⁺, K⁺, Mg²⁺ en ATP aanwezig waren. Tot dat moment was het bestaan van actief transport vooral een theoretisch concept, maar Skou’s ontdekking van wat tegenwoordig Na⁺,K⁻-ATPase wordt genoemd bewees voor de eerste keer dat er inderdaad eiwitten bestaan die actief transport verzorgen. Latere studies hebben aangetoond dat Na⁺,K⁻-ATPase een heterodimer is: een eiwitcomplex dat bestaat uit een combinatie van een katalytische α-subeenheid en een β-subeenheid die betrokken is bij het vouwen, transporteren en invoegen van het enzym in het celmembraan. Na⁺ en K⁺ worden door het celmembraan heen getransporteerd in een vaste verhouding van 3:2 ten koste van één molecuul ATP, een proces dat geremd kan worden door de aanwezigheid van digitalis-achtige stoffen (DAS).

Tegenwoordig is er veel bekend over de functies van Na⁺,K⁻-ATPase in het menselijk lichaam, zoals in Hoofdstuk 1 beschreven. De α-subeenheid is aanwezig in vier verschillende isovormen, waarbij elke isovorm een weefselspecifiek expressiepatroon heeft. Dit varieert van expressie in alle cellen voor de α₁-isovorm tot zeer gelokaliseerde expressie voor bijvoorbeeld de α₄-isovorm, welke alleen aanwezig is in testisweefsel. De andere twee isovormen worden tot expressie gebracht in meerdere, maar niet alle organen: waarbij de α₂-isovorm onder anderen voorkomt in astrocyten die gelokaliseerd zijn bij synapsovergangen, komt de α₃-isovorm vooral voor in neuronen, zowel van het centrale als het perifere zenuwstelsel. Het
is aannemelijk dat alle vier de isovormen een rol hebben die past bij hun expressiepatroon. Zo duidt de aanwezigheid van de α1-isovorm in alle humane celdtypen op een algemene, basale rol in de cellulaire fysiologie. Door de aanwezigheid van deze isovorm worden de essentiële ion-gradienten tussen de intra- en extracellulaire compartimenten gehandhaafd. In de afgelopen decennia heeft deze isovorm echter hernieuwde aandacht gekregen, vanwege studies die de aanwezigheid van pico- en nanomolaire concentraties van enkele endogene geproduceerde DAS aantoonden in het bloedplasma. Tot dan toe waren deze stoffen alleen aangetoond in planten en bepaalde diersoorten, die ze gebruiken als afweer tegen viraat en roofoorden. DAS verkeren uit planten werden al sinds lang tijd als geneesmiddel gebruikt ter behandeling van boezemfibrilleren en hartfalen: de remming van Na+,K+-ATPase minimaal te remmen. In Hoofdstuk 2 hebben we de rol van Na+,K+-ATPase in één van de mogelijke DAS-geïnduceerde signaltransductiecascade onderzocht, waarbij Na+,K+-ATPase verondersteld wordt een directe interactie aan te gaan met het Src-ewit. In onze experimenten vonden we echter geen bewijs voor een dergelijke interactie tussen beide eiwitten. Re combinant Src en gezuiverd Na+,K+-ATPase samen te voegen in een reageerbus ontdekten we dat Src-fosforylering (verondersteld te worden door recombinant Src en gezuiverd Na+,K+-ATPase) direct afhankelijk was van de hoeveelheid ATP die aanwezig was. Zo konden we Src-fosforylering niet alleen induceren door toevoeging van digoxine (een DAS), maar ook door het verminderen van de Na+ - of K+-concentraties of het toevoegen van vanadate, een andere (niet-DAS) remstof. Deze resultaten leidden ons ertoe te concluderen dat er geen bewijs is voor een signaltransductiereserveur mechanisme waarbij een directe interactie tussen Src en Na+,K+-ATPase betrokken is. Dit betekent niet dat Src geen enkele rol zou spelen in cellulaire signaltransductie via Na+,K+-ATPase. In de laatste jaren heeft Na+,K+-ATPase steeds meer aandacht gekregen met betrekking tot de rol van het enzym in verschillende ziekten. Zo is bijvoorbeeld bekend dat mutaties in de α2-isovorm kunnen leiden tot het ontstaan van sporadische/familiale hemiplegische migraine (SHM/FHM), zeldzame vormen van migraine met aura. Alhoewel het exacte moleculaire mechanisme van deze mutaties nog onbekend is, wordt over het algemeen aangenomen dat de aanwezigheid van α1+-ATPase in de astrocyten die aanwezig zijn als ondersteunende cellen bij de synapsen een belangrijke rol speelt. Waarschijnlijk leiden mutaties in α2+-ATPase tot een verminderde katalytische activiteit voor het handhaven van ion-gradienten van zowel Na+- als K+, beide belangrijk voor het heropnemen van glutamaat uit de synapsenplicht, waardoor migraine kan ontstaan. Daarom hebben we de effecten van verschillende mutaties op de biochemische eigenschappen van α1+-ATPase onderzocht. De resultaten van deze studies zijn beschreven in Hoofdstukken 4 en 5. In Hoofdstuk 4 wordt beschreven hoe negen verschillende SHM/FHM-mutaties mogelijk tot de ontwikkeling van deze ziekte kunnen bijdragen. Eén van de belangrijke parameters van Na+,K+-ATPase, de ATPase-activiteit, liet zien dat zes van deze mutaties een ernstig verlies van functionaliteit induceerden: P796S, M829R, R834X, del 935-940 ins Ile, R937P en D999H veroorzaakten vrijwel geen ATPase-activiteit. Voor de mutant P796S lijkt met de introductie van een constructe, deze stof vertoonde een tienvoudig verhoogde affiniteit ten opzichte van de α2-isovorm. Dit verschil in affiniteit was niet aanwezig voor ouabaine, terwijl beide stoffen slechts verschillen in de chemische structuur van de lactonring, namelijk de aan- (ouabaine) of afwezigheid (dihydro-ouabaine) van een dubbele binding.
M829R lijkt een verminderde ATPase-activiteit te veroorzaken door verlies van belangrijke interacties die de binding tussen het zesde en zevende transmembraandomein stabiliseren. De overige vier mutaties werden verondersteld de ATPase-activiteit te verminderen doordat ze de structurele integriteit van een recent ontdekt half-kanaal aantasten dat gelokaliseerd is in het C-terminale gedeelte van Na⁺,K⁺-ATPase en dat een belangrijke rol speelt in het ion-transport mechanisme. Mutant T345A liet een niet-significante toename zien in de ATPase activiteit, maar een evenredige toename van de ouabaïne bindingscapaciteit toonde aan dat er geen significante verstoring van de Na⁺,K⁺-ATPase functie is te verwachten door deze mutatie. Hierdoor kunnen we op grond van de gedane experimenten niet verklaren hoe deze mutatie dan wel tot ziekte kan leiden. Mutant R879W liet ook geen afwijkingen zien in de biochemische parameters zoals getest en ook kon geen verklaring worden gevonden in een mogelijk effect op intramoleculaire interacties. Het kan zijn dat het dan ook geen mutaties zijn die leiden tot ziekte, maar twee zeldzame polymorfismen. Voor mutant R834Q ontdekten we een significante verlaagde ATPase-activiteit en een verlaagde maximale ouabaïne bindingscapaciteit. Deze mutatie is tevens gelokaliseerd in het C-terminale half-kanaal zoals hierboven reeds beschreven, maar mogelijk is het effect van deze mutatie minder sterk dan de overige mutaties die dit half-kanaal aantasten.

In Hoofdstuk 5 bestudeerden we de biochemische effecten van elf additionele SHM/FHM-geassocieerde mutaties. Opnieuw werd gebruik gemaakt van dezelfde baculovirusgemediaande expressie zodat membraanfracties konden worden verkregen met daarin de te bestuderen mutaties. Biochemische karakterisering van deze mutanten liet grote verschillen in de effecten zien. Zo konden er geen effecten worden gezien voor de mutanten Y9N, R51H, C702Y, R879Q, waaruit geconcludeerd kan worden dat deze mutatnten mogelijk geen causale rol spelen in de ontwikkeling van SHM/FHM. Mutant P786L liet een verlies van functie zien door zowel een afwezig ATPase-activiteit alsook een minimale ouabaïne binding, verlies van twee zeer kenmerkende eigenschappen van Na⁺,K⁺-ATPase. Twee mutanten, R548C en R548H, vertoonden een lagere transportsnelheid, leidend tot minder transport van zowel Na⁺ als K⁺ en daardoor mogelijk een oorzaak van het ontwikkelen van migraine. Lagere transportsnelheden werden ook gezien bij mutanten E174K en E700K, maar deze afnames waren niet statistisch significant. De laatste twee mutanten (G900R en E902K) lieten weliswaar geen effect zien op de geteste biochemische eigenschappen, maar mogelijk spelen beide residuen een rol in de interactie tussen de α- en β-subeenheden, waardoor er minder functioneel eiwit aanwezig is in de celmembraan.

Ook mutaties in de α2-isoform zijn geassocieerd met het ontstaan van ziekte. Een voorbeeld hiervan is een speciale vorm van de ziekte van Parkinson. Van meer recente aard is de ontdekking dat een andere neurologische ziekte ook veroorzaakt wordt door mutaties in deze isovorm. Deze ziekte, pediatrische alternerende hemiplegie (PAH), wordt veroorzaakt door mutaties gelokaliseerd in of vlakbij de transmembraandomeinen en wordt gekarakteriseerd door aanvallen met halfzijdige verlamming, optredend vanaf de eerste levensjaar. Een interessante hypothese is het idee dat beide ziektes in elkaars verlengde liggen.

In Hoofdstuk 6 beschrijven we uitgebreid de biochemische eigenschappen van zes frequente mutaties die gevonden zijn in patiënten met PAH: S137Y, D220N, I274N, D801N, E815K en G947R. Mutant D220N liet geen enkele afwijking zien in vergelijking met het normale Na⁺,K⁺-ATPase, waardoor een causale rol voor deze mutatie vooralsnog onwaarschijnlijk is, alhoewel wij niet de lokalisatie van deze mutant hebben onderzocht. Drie mutatanten (I274N, E815K en G947R) vertoonden geen ATPase-activiteit, konden geen ouabaïne binden en konden niet worden gefosforyleerd, hetgeen aanmoedigt dat deze mutaties tot een ernstig functieverlies van Na⁺,K⁺-ATPase leiden. Twee mutatanten (D801N en G947R) lieten geen ATPase-activiteit en fosforylering zien, terwijl de ouabaïne bindingscapaciteit niet verloren was gegaan, wat impliceert dat deze mutaties tot een minder ernstig verlies van functionaliteit leiden dan de andere drie mutaties. Mogelijk verklaart het verschil in biochemische eigenschappen tussen de mutanten D801N en E815K (de meest voorkomende mutaties) het in de literatuur gepubliceerde verschil met betrekking tot het fenotype.

Hoofdstuk 7, het laatste experimentele hoofdstuk van dit proefschrift, beschrijft de resultaten van een serie experimenten die erop gericht was om de verschillen in ATPase-activiteit tussen de α2- en α4-isoformen beter te begrijpen. Eerdere studies in ons laboratorium hadden reeds aangetoond dat waar de α2-isoform de hoogste ATPase-activiteit vertoonde, de α4-isoform slechts in geringe mate actief was. Om de resultaten in de literatuur beide isoformen een voorkeur aan te kunnen geven, bestudeerden we welke aminozuren verantwoordelijk zijn voor het gevonden verschil in ATPase-activiteit. Door middel van de productie van een serie chimeren gericht op het ontdekken van de verantwoordelijke aminozuren, konden we aantonen dat de vervanging van twee aminozuren in de α4-isoform door de corresponderende α2-isoform residuen de ATPase-activiteit van de α4-isoform zeer sterk verhoogt. Beide residuen zijn gelokaliseerd in het P-domein dat zich bevindt in de grote cytosolische ruimte tussen het vierde en vijfde transmembraandomein. De α4-isoformresiduen Ile²⁷⁹ en Pro³⁰² bevinden zich hier vlakbij een zogenaamde Rossmann-structuur die ook het fosforylering residue bevatt. Mogelijk dat de aanwezigheid van de twee residuen de Rossmann-structuur aantast, waardoor de lagere ATPase-activiteit van de α4-isoform verklaart.

In Hoofdstuk 8 worden de resultaten van het onderzoek dat beschreven is in dit proefschrift beschouwd in een bredere context, waarmee duidelijk wordt dat Na⁺,K⁺-ATPase een belangrijke rol speelt in ziekte en gezondheid. Verder valt te verwachten dat in de komende jaren meer mutaties zullen worden ontdekt in Na⁺,K⁺-ATPase isoformen, waarvan biochemische karakterisering bij zal dragen aan het begrijpen van het bijbehorende fenotype. Daarnaast is het wenselijk om met de nieuwste analysetechnieken de aanwezigheid van endogene DAS verder te onderzoeken. Een andere interessante vraag betreft de aanwezigheid van mutaties in de α4-isoform bij subfertiele mannen, aangezien het expressiepatroon van deze isoform een interessant therapeutisch aanknopingspunt biedt.

Samenvattend hebben we als eerste laten zien hoe PAH-geassocieerde mutaties de verschillende eiwitfuncties beïnvloeden en hebben we tevens laten zien dat de veronderstelde rol van Na⁺,K⁺-ATPase in signaaltransductie verder onderzoek behoeft. Ook hebben we de
ouabainbindingsplaats onderzocht met betrekking tot de binding van gehydrogeneerde DAS en hebben we SHM/FHM geassocieerde mutaties bestudeerd. Daarbij hebben we de ATPase-activiteit van de α4-isovorm kunnen verhogen door vervanging van slechts twee aminozuren. Gezamenlijk dragen de resultaten van dit proefschrift bij aan het vergroten van de wetenschappelijke kennis omtrent de rol van Na⁺,K⁺-ATPase in ziekte en gezondheid en ze onderstrepen daarmee de belangrijke rol van dit enzym binnen het menselijk lichaam.
**List of abbreviations and glossary**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>A</td>
<td>alanine, nonpolar amino acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate, formed during ATP hydrolysis</td>
</tr>
<tr>
<td>AHC</td>
<td>alternating hemiplegia of childhood, disease resulting from mutations in ATP1A3</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine, nonpolar amino acid</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase, signaling protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance, statistical tool to measure differences between groups</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine, basic amino acid</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine, polar uncharged amino acid</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid, acidic amino acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate, cellular fuel; hydrolyzed to obtain energy</td>
</tr>
<tr>
<td>ATP1A1</td>
<td>gene encoding the α1-subunit</td>
</tr>
<tr>
<td>ATP1A2</td>
<td>gene encoding the α2-subunit</td>
</tr>
<tr>
<td>ATP1A3</td>
<td>gene encoding the α3-subunit</td>
</tr>
<tr>
<td>ATP1A4</td>
<td>gene encoding the α4-subunit</td>
</tr>
<tr>
<td>ATP1B1</td>
<td>gene encoding the β1-subunit</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cysteine, polar uncharged amino acid</td>
</tr>
<tr>
<td>CD</td>
<td>cytosolic domain</td>
</tr>
<tr>
<td>CG(s)</td>
<td>cardiac glycosides, alternative name for DLC(s)</td>
</tr>
<tr>
<td>Chimera</td>
<td>protein consisting of parts of multiple proteins</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>Conformation</td>
<td>structural arrangement</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine, polar uncharged amino acid</td>
</tr>
<tr>
<td>D</td>
<td>aspartic acid, acidic amino acid</td>
</tr>
<tr>
<td>de novo</td>
<td>new/unprecedented</td>
</tr>
<tr>
<td>DLC(s)</td>
<td>digitalis-like compound(s), inhibitor(s) of Na⁺,K⁺-ATPase activity</td>
</tr>
<tr>
<td>E</td>
<td>glutamic acid, acidic amino acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid, chelating molecule</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor, signaling protein</td>
</tr>
<tr>
<td>EOₘₐₓ</td>
<td>maximum amount of ouabain binding</td>
</tr>
<tr>
<td>EP</td>
<td>amount of phosphorylated intermediate</td>
</tr>
<tr>
<td>ERBB2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase, signaling protein</td>
</tr>
<tr>
<td>F</td>
<td>phenylalanine, nonpolar amino acid</td>
</tr>
<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FHM</td>
<td>familial hemiplegic migraine, disease resulting from mutations in ATP1A2</td>
</tr>
<tr>
<td>G</td>
<td>glycine, polar uncharged amino acid</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid, acidic amino acid</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine, polar uncharged amino acid</td>
</tr>
<tr>
<td>H</td>
<td>histidine, basic amino acid</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>H-bond</td>
<td>hydrogen-bond</td>
</tr>
<tr>
<td>His</td>
<td>histidine, basic amino acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase, used to detect presence of antibody</td>
</tr>
<tr>
<td>I</td>
<td>isoleucine, nonpolar amino acid</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>concentration at which 50% inhibition takes place</td>
</tr>
<tr>
<td>IFD</td>
<td>initial-fit docking</td>
</tr>
<tr>
<td>Ile</td>
<td>isoleucine, nonpolar amino acid</td>
</tr>
<tr>
<td>in vitro</td>
<td>in a test tube</td>
</tr>
<tr>
<td>in vivo</td>
<td>in a living organism</td>
</tr>
<tr>
<td>IP3R</td>
<td>inositol trisphosphate receptor, involved in Ca²⁺ release</td>
</tr>
<tr>
<td>K</td>
<td>lysine, basic amino acid</td>
</tr>
<tr>
<td>Kᵢₕ₀</td>
<td>concentration at which half-maximal Kᵢₕ₀ mediated ATPase activity is observed</td>
</tr>
<tr>
<td>Kᵢₖ</td>
<td>dissociation constant, measure for affinity of compounds</td>
</tr>
<tr>
<td>L</td>
<td>leucine, nonpolar amino acid</td>
</tr>
<tr>
<td>Leu</td>
<td>leucine, nonpolar amino acid</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>pig kidney cell line</td>
</tr>
<tr>
<td>Lys</td>
<td>lysine, basic amino acid</td>
</tr>
<tr>
<td>M</td>
<td>methionine, nonpolar amino acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase, signaling protein</td>
</tr>
<tr>
<td>Met</td>
<td>methionine, nonpolar amino acid</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>N</td>
<td>asparagine, polar uncharged amino acid</td>
</tr>
<tr>
<td>n.d.</td>
<td>not detectable</td>
</tr>
<tr>
<td>Na$_{0.5}$</td>
<td>concentration at which half-maximal Na$^+$ mediated ATPase activity is observed</td>
</tr>
<tr>
<td>nH</td>
<td>Hill-coefficient, quantifies cooperative binding</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-Methyl-D-Glucamine, used to compensate for loss of ionic strength</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>P</td>
<td>proline, nonpolar amino acid</td>
</tr>
<tr>
<td>PBS(-T)</td>
<td>phosphate buffered saline (-tween),</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data bank, crystal structure repository</td>
</tr>
<tr>
<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylalanine, nonpolar amino acid</td>
</tr>
<tr>
<td>P$_i$</td>
<td>phosphate, formed during ATP hydrolysis</td>
</tr>
<tr>
<td>P$_{i,K}$</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase, signaling protein</td>
</tr>
<tr>
<td>PLC-$\gamma$</td>
<td>phosphoinositide phospholipase C-gamma, signaling protein</td>
</tr>
<tr>
<td>Pro</td>
<td>proline, nonpolar amino acid</td>
</tr>
<tr>
<td>Q</td>
<td>glutamine, polar uncharged amino acid</td>
</tr>
<tr>
<td>R</td>
<td>arginine, basic amino acid</td>
</tr>
<tr>
<td>RAS</td>
<td>rat sarcoma protein, signaling protein</td>
</tr>
<tr>
<td>RDP</td>
<td>rapid-onset dystonia parkinsonism, disease resulting from mutations in ATP1A3</td>
</tr>
<tr>
<td>s</td>
<td>second(s)</td>
</tr>
<tr>
<td>S</td>
<td>serine, polar uncharged amino acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean, measure of variation within a group</td>
</tr>
<tr>
<td>Ser</td>
<td>serine, polar uncharged amino acid</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca$^{2+}$-ATPase</td>
</tr>
<tr>
<td>Si9</td>
<td>Spodoptera frugiperda 9, insect cell line</td>
</tr>
<tr>
<td>SH</td>
<td>SRC homology</td>
</tr>
<tr>
<td>SHM</td>
<td>sporadic hemiplegic migraine, disease resulting from mutations in ATP1A2</td>
</tr>
<tr>
<td>(p)Src</td>
<td>(phosphorylated) tyrosine-protein kinase Src, signaling protein</td>
</tr>
<tr>
<td>State</td>
<td>structural arrangement, similar to conformation</td>
</tr>
<tr>
<td>T</td>
<td>threonine, polar uncharged amino acid</td>
</tr>
<tr>
<td>TBS(-T)</td>
<td>tris buffered saline (-tween)</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine, polar uncharged amino acid</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan, nonpolar amino acid</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine, polar uncharged amino acid</td>
</tr>
<tr>
<td>V</td>
<td>valine, nonpolar amino acid</td>
</tr>
<tr>
<td>V$_{max}$</td>
<td>maximum velocity of an enzymatic reaction</td>
</tr>
<tr>
<td>Val</td>
<td>valine, nonpolar amino acid</td>
</tr>
<tr>
<td>Vanadate</td>
<td>phosphate analog, inhibits Na$^+$,K$^+$-ATPase nonspecifically</td>
</tr>
<tr>
<td>W</td>
<td>tryptophan, nonpolar amino acid</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine, polar uncharged amino acid</td>
</tr>
<tr>
<td>YASARA</td>
<td>yet another scientific artificial reality application, freeware to view crystal structures</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow-fluorescent protein, used as a negative control</td>
</tr>
</tbody>
</table>
Curriculum vitae


List of publications


Dankwoord

Ineens doemt uit de verte de finishstreep van mijn promotieonderzoek op. Ook al wist ik dat het eraan zat te komen, het voelt toch enigszins onwennig om te beseffen dat het nog maar zo'n klein stukje is. Heb ik de afgelopen jaren dan als een zware tocht ervaren? Gelukkig niet, integendeel zelfs: het waren jaren waarin ik niet alleen onderzoek heb mogen doen naar een zeer interessant onderwerp, maar bovendien het geluk heb gehad daarbij omringd te zijn door een aangenaam reisgenootschap.

Eén van mijn eerste herinneringen aan de start van het project is het gesprek dat gevoerd werd op de kamer van Frans, mijn promotor. Beste Frans, het is me altijd bijgebleven hoe jij me wist te overtuigen van mijn slagskansen op dit project. Dit bleek achteraf tekenend te zijn voor jouw stijl van leidinggeven: je integere houding, onuitputtelijke kennis wat betreft de farmacologie, gekoppeld aan het altijd beschikbaar zijn voor vragen (zelfs na middernacht) en de rust die je uitstraalt maken je tot een kundig afdelingshoofd en promotor.

Bij datzelfde gesprek was ook Jan aanwezig, de co-promotor van dit project. Jan, jouw nuchtere houding in de wetenschap en het leven daarbuiten maakte dat ik makkelijk bij je binnen liep, en waar nodig simpelweg tijd in jouw agenda reserveerde. Volgens mij begrijpen we elkaar goed, waardoor niet alles uitgesproken hoeft te worden, dat is wel zo efficiënt. Alles bij elkaar is het een prachtig project geworden, met als kers op de taart het door jou georganiseerde ATPase congres in Nederland, afgelopen zomer. Leuk dat we bij de verdediging een paar diehard ATPase onderzoekers konden uitnodigen. Deze geven zo'n verdediging enig "cachet", zoals ons voormalig afdelingshoofd Paul Smits zou zeggen.

Also, I would like to express my gratitude to our collaborators in Århus: Natasha Fedosova, Mette Laursen, and Poul Nissen. Thanks for all the discussions, and the purified protein, it was essential for chapter 2, whereas Mette's contribution to chapter 3 was just as significant!

Het was in dezelfde kamer dat ik Herman ontmoette, die als analist op hetzelfde project was aangesteld, al was het officieel maar voor de helft van de tijd. Herman, het was van het begin af aan duidelijk dat jij de ware vakman binnen het ATPase onderzoek bent, met een oneindige hoeveelheid ervaring. Dank voor alles (en dat is heel wat!) wat je aan het project hebt bijgedragen en alle gesprekken over het doen van onderzoek, maar ook over de geschiedenis van het biochemisch onderzoek in Nijmegen. Misschien dat we de koelkast op de derde verdieping maar tot monument moeten laten verklaren? Ik denk met plezier terug aan onze gesprekken aan het strand van Californië en ons bezoek aan San Francisco, waar we ons rot zochten naar een terras.
Julien (mon petit, Assange), qu’est-ce que je dois dire a vous? Dans les dernieres années tu es devenu plus qu’un collègue. La seule chose qui manque est ton néerlandais (bien que vous ayez passé le test avec le veló sous la pluie), mais mon Francais sonne comme quelqu’un un peut fort brouillé aussi. It was always nice to practice my French with you, and it’s a pity that our working spaces are now farther apart. Still, we meet regularly during “meatings” and discuss about the future. I still think we need to work on our plans for a certain TV-show with monsieur Schirris!

Op het lab mocht ik mijn eerste jaar plaatsnemen aan de bench bij Rachel (Rage), al werd er al snel met tape een duidelijke scheiding gemaakt tussen onze werkplekken. Elkaars tolerantiegrens passeerden we allebei graag en daardoor zorgden we samen toch voor het nodige leven in de brouwerij. Daarbij was het fijn dat er iemand was om slechte filmquotes mee te delen. Gelukkig was het niet alleen maar onzin, we hebben ook serieuze gesprekken gehad, toch?

In hetzelfde U-tje zat ook Hanneke (Mevrouw Wittgen), die als een soort van oudere zus fungeerde. Zo begon haar maandagochtend met het bijpraten met het hele lab, zodat ze weer op de hoogte was van het wel en wee van iedereen. Hanneke, onze gesprekken aan het einde van de werkdag heb ik altijd zeer gewaardeerd, en moesten nog wel eens worden afgebroken omdat we nu echt naar huis moesten, een goed teken lijkt mij.

De laatste U-genoot van het illustere U-tje 4 was Rick. Rick, met terugwerkende kracht mijn excuses voor alle onzin die je hebt moeten aanzien van mijzelf en Rachel, hopelijk heb je er niet teveel onder geleden. Onze taalliefde uitte zich in het adopteren van een beleggen woord, en je farmacokinetische formules op de achterkant van een bierviltje zal ik ook niet snel meer vergeten! Leuk dat we onze samenwerking nog even voortzetten, en jammer dat onze tocht naar Groningen met de mighty Mic niet doorging, die houd je tegoed.

Het is fijn om mensen om je heen te hebben die misschien wel niet de finesses van jouw onderzoek begrijpen, maar die je des te meer steunen op andere manieren. Bijvoorbeeld door het proofreaden van de inleiding, discussie en samenvatting (waarvoor dank, Mark!), maar ook door de vele goede gesprekken waarbij de lastiger onderwerpen niet geschuwd worden. Dank aan iedereen die geïnteresseerd bleef als je de vraag hoe het mij ging een antwoord kreeg over de stand van zaken rondom dit proefschrift! Ik houd me voor dat ik nu meer tijd zal hebben voor contacten met nog meer mensen, hopelijk maak ik dat waar.

Mijn opa leerde mij dat om goed te begrijpen waarom mensen bepaalde keuzes maken je iets zult moeten weten over hun geschiedenis. Mijn begint in een gezin met twee broers. Roeland en Victor (en Tirza!), niet alleen ben ik trots op wat jullie kunnen en doen, meer nog waardeer ik hoe we ons de laatste jaren hebben ontwikkeld tot volwassenen, met ongeveer dezelfde waarden, maar nog belangrijker: met oog voor de beweegredenen van de ander, en berusting in het feit dat we met hetzelfde sop overgaten zijn.

Mijn ouders wil ik bedanken voor alles wat jullie ons hebben gegeven (en nog steeds doen), en de bescheiden verwachtingen voor wat betreft onze studieresultaten: als je maar je best doet. Het is fantastisch om zoveel mogelijkheden te krijgen en daarbij je eigen keuzes te mogen maken. Zie hier het resultaat van één zo’n keuze, al zijn er (gelukkig) nog belangrijker keuzes in het leven.

Lieve Laura, wat kan ik nog zeggen dat jij niet allang weet? Het ware thuis is daar waar je niets hoeft te zeggen. Dank je voor het zijn van zo’n thuis. Je bent mijn lief! In Dei Nomine Feliciter.