Biochimica et Biophysica Acta 1832 (2013) 2173–2179

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

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

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ARTICLE INFO

Article history:
Received 18 April 2013
Received in revised form 7 August 2013
Accepted 8 August 2013
Available online 15 August 2013

Keywords:
Na,K-ATPase
ATP1A2
Familial hemiplegic migraine
FHM2
α2 isoform
Domain interaction

ABSTRACT

Familial hemiplegic migraine (FHM) is a monogenic variant of migraine with aura. One of the three known causative genes, ATP1A2, which encodes the α2 isoform of Na,K-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,K-ATPase α2 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 subunit. Both mutants likely hamper the interaction between these subunits and thereby decrease enzyme activity. Mutants E174K, R548C and R548H reduce the Na^+ and increase the K^+ 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^+ and Na^+ affinities, as observed with these mutants. Functional consequences were not observed for ATP1A2 mutations found in two sporadic hemiplegic migraine cases (Y9N and R879Q) and in migraine without aura (R51H and C702Y).

1. Introduction

Migraine is a disabling common brain disorder typically characterized by attacks of severe headache and associated with autonomic and neurological symptoms [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 [2]. Familial hemiplegic migraine (FHM) is a rare monogenic variant of migraine with aura with some degree of hemiparesis during the aura [1]. Of the three known FHM genes, two encode α1 subunits of voltage-gated $Ca_V2.1$ calcium channels (*CACNA1A*; FHM1) or voltage-gated $Na_V1.1$ sodium channels (*SCN1A*; FHM3) [3–5]. ATP1A2 (FHM2) encodes the catalytic α2 subunit of the sodium potassium pump [6]. This pump transfers Na^+ out and K^+ 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 α2 is located in glial cells where it generates the Na^+ gradient that is essential for the re-uptake of the excitatory transmitter glutamate. Moreover, it also removes excess K^+ from the intracellular space [7].

Since the discovery of the first FHM2 mutation in 2003 [6], over 50 ATP1A2 mutations have been published, but only a minority have been functionally studied in detail [7]. 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 [7]. For most of the mutations, however, functional evidence supporting that these mutations are causal is lacking. Here we set out to investigate 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 ATP1A2 mutations and sporadic and familial hemiplegic migraine, but do not provide robust functional evidence for a relationship with other types of migraine.

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http://dx.doi.org/10.1016/j.bbadis.2013.08.003

2. Materials and methods

2.1. The Gateway system

The wild type and mutants of Na,K-ATPase α2 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,K-ATPase β1-subunit in the pFastBac Dual vector (Life Technologies, Breda, The Netherlands) after the p10 promotor 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, *Dpn*I 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.

2.2. Generation of recombinant viruses

The pFastBac Dual 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 [8]. 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.

2.3. Preparation of Sf9 membranes

Sf9 cells were grown at 27 °C in 175 cm^2 monolayers and later in 500-mL shaking flasks cultures. For production of Na,K-ATPase, $1.5.10^6$ 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 (BioWhittaker, Walkersville, MD) as described before [9]. After 3 days, the Sf9 cells were harvested by centrifugation at 2000 ×*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 60 W (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.

2.4. 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 [10]. For immunoblotting, the separated proteins were transferred to Immobilon polyvinylidenefluoride membranes (Millipore Corporation, Bedford, MA). The α-subunit of the Na, K-ATPase was detected with the polyclonal antibody C356-M09 [10].

2.5. Na,K-ATPase assay

The ouabain sensitive ATPase activity was determined using a radiochemical method [11]. For this purpose, 0.6–5 μg of Sf9 membranes were added to 100 μL of medium, which contained 10–200 μM [γ-^{32}P]-ATP (specific activity 20–100 mCi·$mmol^{-1}$), 1.2 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM EDTA, 0.1 mM ouabain, 1 mM Tris–N_3, 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 clear supernatant, containing the liberated inorganic phosphate ($^{32}P_i$), 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.

2.6. Ouabain binding capacity

Ouabain binding was determined as described before [12]. Sf9 membranes (100 μg) were incubated at 21 °C in 20 mM histidine (pH 7.0), and 5.0 mM $MgCl_2$ and 5.0 mM P_i in a volume of 50 μL. 10 μL of [^{3}H]-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.

2.7. Chemicals

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

2.8. 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. IC_{50} and $K_{0.5}$ 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 $Na^+{}_{0.5}$ and $K^+{}_{0.5}$ 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 (Vmax) is the activity in the presence of 50 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 0.1 mM ATP at pH 7.0.

2.9. 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_1Na^+ (2C9M), E_1Na^+-ATP (1T5S), E_1Na^+-P-ADP (1T5T), E_1Na^+P:ADP (3BA6), E_2P (3N23), E_2K^+-P ATP (3B9R), E_2K^+-Pi ATP (2ZXE), E_2K^+ 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 and selection of the best model. Minimization was done with the standard force field in YASARA [9].

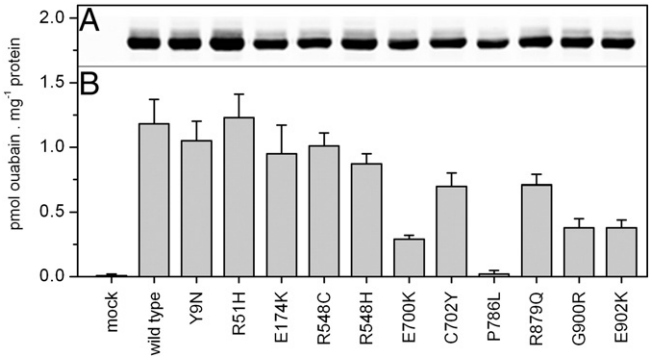

Fig. 1. Expression of wild type and mutant Na,K-ATPase. A: Western blot of the different Na,K-ATPase membrane preparations (10 μg) isolated from infected Sf9 cells. The presence of Na,K-ATPase α-subunit was detected with antibody C356-M09 [10]. B: Ouabain binding to wild type and mutant Na,K-ATPases. The assay was performed at 21 °C in 20 mM histidine buffer (pH 7.0), 5.0 mM $MgCl_2$, 5 mM P_i and 12 nM ^{3}H-ouabain. Average with SE for 3 different expression experiments.

3. Results

3.1. 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 subunit 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 [9,13,14]. The ouabain equilibrium saturation was analyzed and the affinity for ouabain was similar for all mutants (S1). However, the maximum binding (EOmax) 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.

3.2. Kinetic alterations

One of the most important properties of the 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 (S2). 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). In Fig. 2 the apparent affinity for Na^+ of the R548C mutant and wild type are shown.

In the presence of 100 μM ATP, 50 mM NaCl and varying concentrations of KCl, the activity of Na,K-ATPase was determined (S3). 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 and S4). We determined the maximal ATPase activity at 0.1 mM ATP, 50 mM NaCl and 5 mM KCl (S4, Table 1). Mutants Y9N and R51H had a similar Vmax as wild type enzyme. E174K, C702Y, and R879Q had a somewhat reduced Vmax (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 Vmax/EOmax is plotted in Fig. 3 and the calculated values are shown in Table 1. Most mutants had a similar turnover number as wild type enzyme. The

Table 1
Summary of the catalytic properties of wild type and mutant Na,K-ATPase from supplementary Figs. S1–S4. The values presented are the mean ± SE of 3–4 enzyme preparations. The values of the mutant enzyme preparations are compared to those of the wild type.

	Max ouabain pmol/mg protein	Ouabain affinity nM	ATPase activity μmol/mg protein/h	Na^+ affinity mM	K^+ affinity mM	ATP affinity μM	Turnover (min^{-1})
wt	3.15 ± 0.40	18.6 ± 2.9	0.55 ± 0.10	7.1 ± 0.47	1.35 ± 0.15	25.0 ± 4.2	3032 ± 637
Y9N	2.67 ± 0.45	16.5 ± 2.0	0.50 ± 0.12	8.1 ± 0.97	1.54 ± 0.14	23.3 ± 6.1	3110 ± 362
R51H	3.58 ± 0.87	18.5 ± 3.4	0.65 ± 0.13	7.6 ± 0.73	1.33 ± 0.14	26.0 ± 2.3	3229 ± 652
E174K	2.66 ± 0.65	19.7 ± 4.5	0.27 ± 0.07	15.7 ± 2.38*	0.86 ± 0.18	29.8 ± 5.5	1755 ± 270
R548C	2.87 ± 0.41	21.6 ± 1.8	0.12 ± 0.04**	17.0 ± 2.22**	0.81 ± 0.13*	31.3 ± 6.9	713 ± 200*
R548H	2.46 ± 0.11	21.5 ± 1.9	0.17 ± 0.02*	13.8 ± 0.59***	0.45 ± 0.05**	23.0 ± 10.2	1160 ± 168*
E700K	0.75 ± 0.06***	19.4 ± 1.9	0.06 ± 0.01**	n.d.	n.d.	n.d.	1449 ± 318
C702Y	1.61 ± 0.25*	14.8 ± 1.4	0.26 ± 0.04*	6.2 ± 1.07	1.17 ± 0.15	36.9 ± 8.3	2827 ± 405
P786L	n.d.	n.d.	−0.01 ± 0.00**	n.d.	n.d.	n.d.	n.d.
R879Q	1.77 ± 0.26*	16.4 ± 2.0	0.35 ± 0.08	8.5 ± 0.76	1.43 ± 0.14	35.2 ± 5.9	3593 ± 948
G900R	0.75 ± 0.15**	11.8 ± 1.1	0.16 ± 0.04*	7.8 ± 2.18	1.26 ± 0.16	29.7 ± 5.7	3854 ± 875
E902K	0.76 ± 0.08**	12.6 ± 2.5	0.18 ± 0.04*	10.8 ± 1.58	1.23 ± 0.15	42.6 ± 4.6*	4873 ± 1657

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.005$.

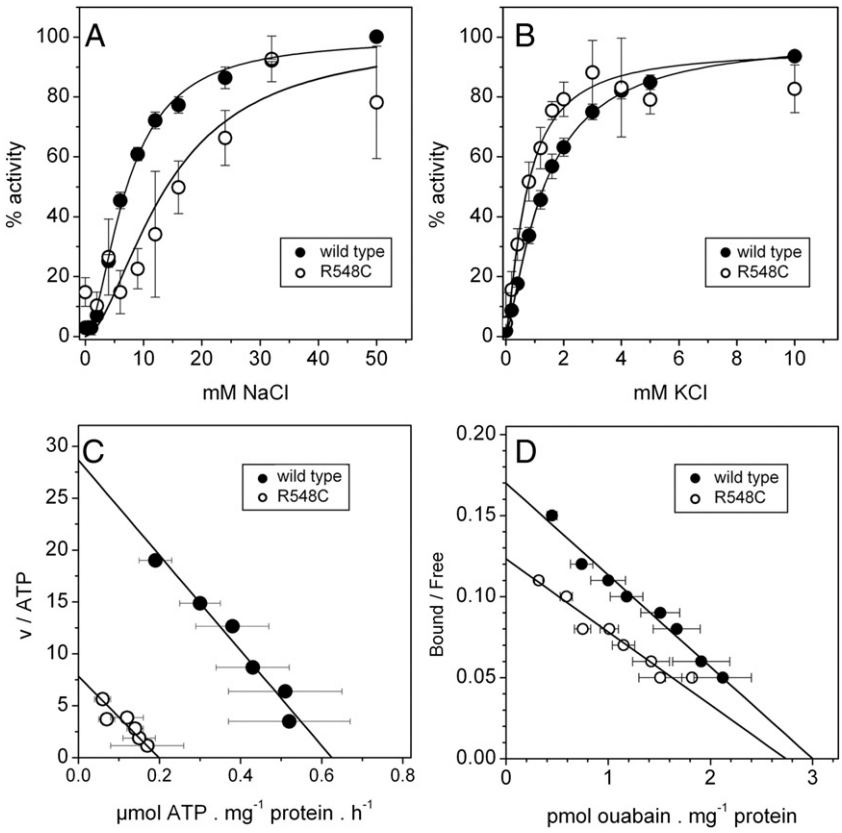

Fig. 2. The properties of the wild type Na,K-ATPase and the R548C mutant. Na^+ dependence (A), K^+ dependence (B), and ATP dependence (Scatchard plot) (C) are shown in the ATPase activity assay. In panel D the ouabain binding affinity and capacity are shown in a Scatchard plot. The assay conditions were described in the Materials and methods section.

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.

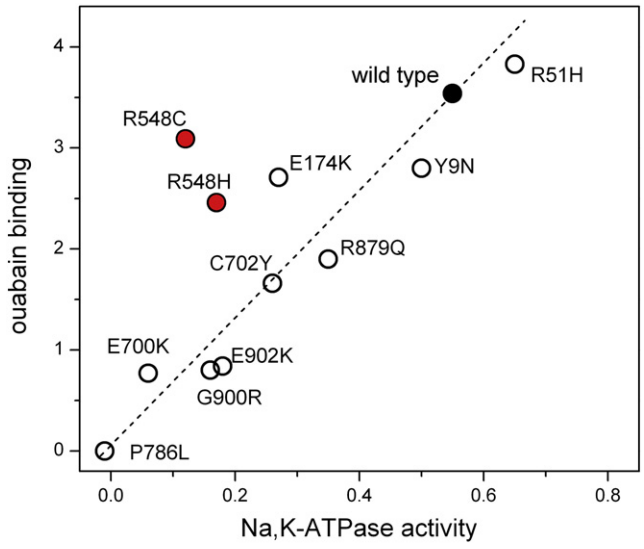

Fig. 3. Relationship between Na,K-ATPase activity and ouabain binding. The maximal ouabain binding (pmol per mg protein) and the Na,K-ATPase activities (µmol ATP hydrolyzed per mg protein per h) as determined from Supplementary Figs. 1 and 4 were plotted for each mutant and wild type enzyme. The dotted line indicates a catalytic turnover number that is equal to that of the wild type enzyme.

4. Discussion

Familial hemiplegic migraine type 2 (FHM2) is caused by mutations in *ATP1A2* that encodes the α2 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 α2 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 α2 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_1P conformation Glu^{174} is predicted to be in close proximity to Lys^{432} in the N-domain (Fig. 4). In this conformation a salt bridge between both residues might stabilize the E_1P 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 Glu^{221} that stabilizes the interaction between the A and N

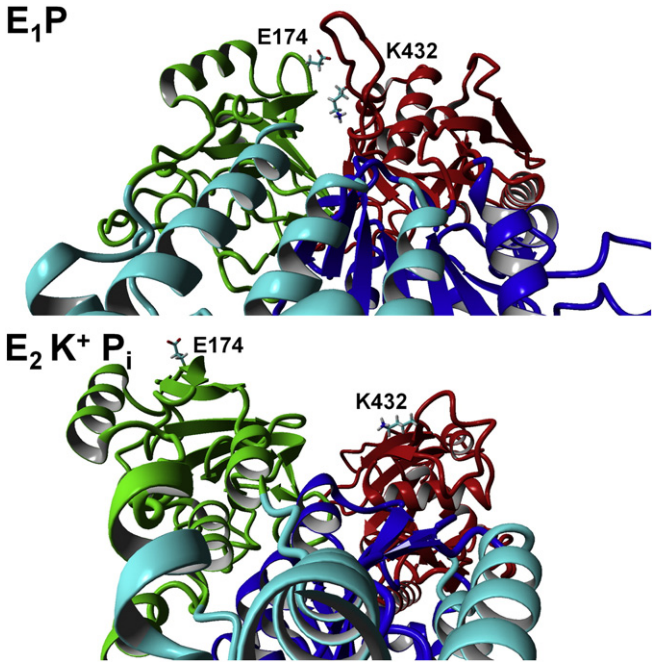

Fig. 4. Interaction between E174 and K432 visualized in Na,K-ATPase in the E_1P conformation (homology model based on 3BA6) and $E_2K{\cdot}Pi$ state (2ZXE). View is from outside to inside. Cyan is transmembrane domain; blue is phosphorylation domain, red is nucleotide-binding domain; green is actuator domain.

domains in the E_2P conformation (Fig. 5) [20]. 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 [21], whereas substitution with Lys was shown not to affect ATP binding, but reduced ATPase activity to 30% of the wild type enzyme [21]. Nucleotides might disrupt the Arg^{548}–Glu^{221} salt bridge, aiding dissociation of the A and N domains and accelerating K^+ release [20]. 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 for this mutation. 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 $E_2K{\cdot}Pi$ conformation (Fig. 6). Recently, it was shown that E700K exhibits a reduced rate of E_2P dephosphorylation and a reduced vanadate affinity [22]. These observations are in agreement with our observed reduced activity. Moreover, the disruption of the salt bridge might destabilize the E_2P conformation and thereby inhibit the E_1P to E_2P transition. In contrast to Schack et al. (2012), we did not observe an increased ouabain affinity.

The P786L mutation is located within the transmembrane (TM) domain, more specifically TM5. P786L previously did not show survival of cells in our specialized assay [23]. Our present functional studies showed no ouabain binding or ATPase activity for this mutant. In the sarcoplasmic reticulum Ca^{2+}-ATPase substitution of the corresponding amino acid Cys^{774} with alanine caused inhibition of Ca^{2+} transport and ATPase activity [24]. This cysteine has been proposed to play a central role in proton transfer [25]. Homology modeling studies suggested that Pro^{786} in M5 might be necessary for the Na^+ ions to reach their binding site. This might be the reason why M5 possesses the sequence PEITP, similar

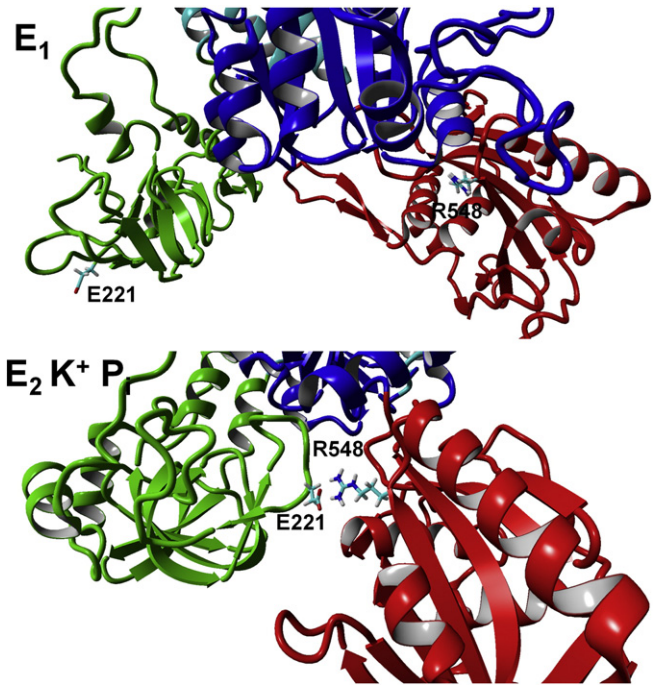

Fig. 5. Interaction between R548 and E221 visualized in the crystal structure of Na,K-ATPase in the E_1 conformation (homology model based on 2C9M) and $E_2K{\cdot}Pi$ state (2ZXE). Cyan is transmembrane domain; blue is phosphorylation domain, red is nucleotide-binding domain; green is actuator domain.

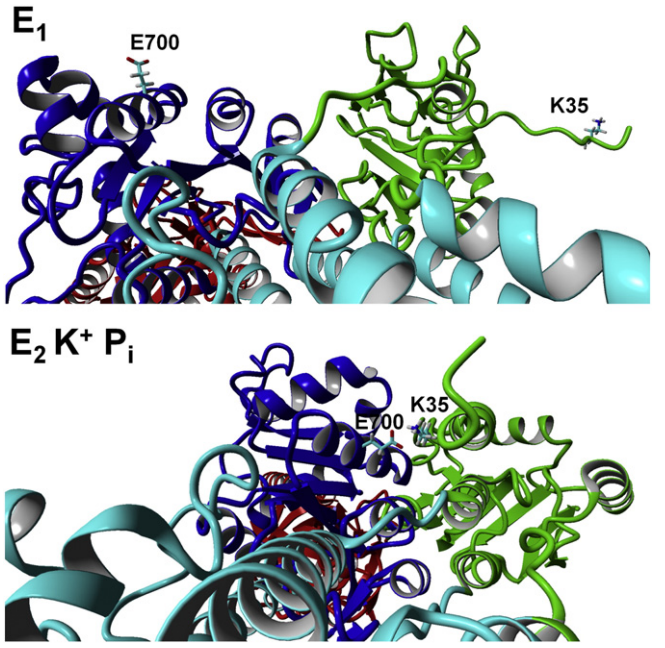

Fig. 6. Possible interaction between E700 and K35 visualized in the crystal structure of Na,K-ATPase in the E_1 conformation (homology model based on 2C9M) and $E_2K{\cdot}Pi$ state (2ZXE). K35 is located at the N-terminal region of Na,K-ATPase, due to which the modeling is rather hypothetical. View is from outside to inside. Cyan is transmembrane domain; blue is phosphorylation domain, red is nucleotide-binding domain; green is actuator domain.

to M4 (PEGLP) [26]. The prolines and polar amino acids in M5 are also responsible for the very inefficient signal anchor sequence [27]. Its correct membrane insertion is probably mediated by posttranslational hairpin formation with M6, which is favored by a proline pair in the connecting loop. The proline mutant had a decreased apparent Na^+ and K^+ affinities 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 *X. laevis* oocytes at 18 °C [27], 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 [17].

Several studied ATP1A2 mutations are located in the extracellular loop between transmembrane segment 7 and 8: R879Q [28], R879W [28], W887R [6], G900R [29], E902K [30], and R908Q [23]. This part of the Na,K-ATPase α-subunit has been implicated in binding of the Na, K-ATPase β-subunit [31,32]. We did not observe any effect on ligand affinity for the G900R and E902K mutants, although we observed a drastic reduction in the amount of functional ATPase molecules for these mutants. The overall effect for the patient will likely be that the amount of functional Na,K-ATPase molecules is reduced, which may hamper the restoring of ions and might enhance cortical spreading depression. The affected glycine 900 is conserved and part of the amino acid sequence SYGQ, which is directly involved in interactions between the α- and β-subunits of the Na, K-ATPase [32]. 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 [20,33]. For instance, Tyr^{899} binds to Lys^{250} and Gln^{901} binds to the backbone atoms of Ile^{185} (Fig. 7) [20,33]. Glutamic acid 902 is also well conserved (Asn or Glu) and forms a hydrogen bond with Arg^{183} of the β-subunit. Replacement of Gly^{900} or Glu^{902} 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 7–8 loop, that most likely is not involved in direct binding of the beta 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.

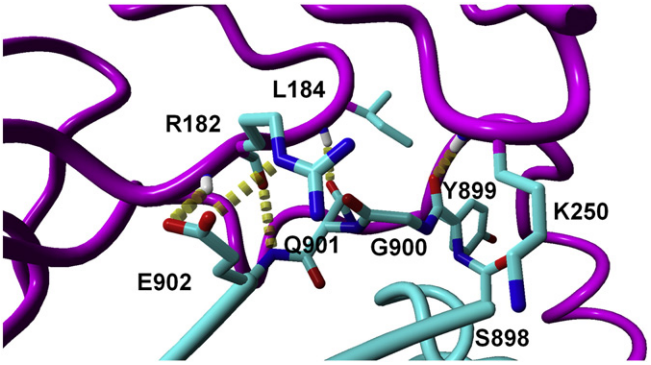

Fig. 7. Interaction between Na,K-ATPase α and β subunits visualized in the crystal structure of Na,K-ATPase in the $E_2K{\cdot}Pi$ state (2ZXE). Cyan is transmembrane domain of the α subunit; magenta is β subunit.

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 chromosomes [30,35,36], we could clearly show catalytic dysfunction of the Na,K-ATPase enzyme. The same is true for mutation G900R, that was observed in a family with patients having FHM, epileptic seizures or both [29], 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 Arg^{548} (R548H) was found in an Italian family that was reported suffering from basilar migraine and absent from a large group of controls [37]. Both Arg^{548} mutations resulted in similar functional effects and are likely disease-causing. The difference between FHM and basilar 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 basilar 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 [28]. It was not detected in the 92 controls and was found in several unaffected family members [28]. 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 [29]. Y9N was identified in two SHM patients and the healthy mother of one of the probands [28,38]. Moreover, Y9N has been observed in the unaffected mother of a carrier with the R583Q mutation [28]. Finally, a Portuguese three-generation family with migraine without aura harbored the R51H variant [39]. 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 assays [39] 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 as 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 as 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.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.08.003.

Disclosure statement

The authors declare that there are no conflicts of interest.

Acknowledgements

Financial support for this study was provided by a personal VIDI-grant from the Netherlands Organization for Scientific Research to J.B.K. There was no involvement of the funding source with any part of the research performed or decision to submit this article.

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