Impaired plasma membrane targeting or protein stability by certain ATP1A2 mutations identified in sporadic or familial hemiplegic migraine

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Abstract: Mutations in three different genes have been implicated in familial hemiplegic migraine (FHM), two of them code for neuronal voltage-gated cation channels, CACNA1A and SCN1A, while the third encodes ATP1A2, the α2-isofrom of the Na+/K+-ATPase's catalytic subunit, thus classifying FHM as an ion channel/ion transporter disorder. The Na+/K+-ATPase maintains the physiological gradients for Na+ and K+ ions and is therefore critical for the activity of ion channels and transporters involved in neurotransmitter uptake or Ca2+ signaling. Diverse functional abnormalities have been identified for disease-linked ATP1A2 mutations, which reach far beyond simple loss-of-function. We have shown recently that ATP1A2 mutations frequently lead to changes in the enzyme's voltage-dependent properties, kinetics or apparent cation affinities. Here, we present functional data on a so far uncharacterized set of ATP1A2 mutations (G301R, R908Q and P979L) upon expression in Xenopus oocytes and HEK293FT cells, and provide evidence for a novel pathophysiological mechanism. Whereas the G301R mutant was inactive, no functional changes were observed for mutants R908Q and P979L in the oocyte expression system. However, the R908Q mutant was less effectively expressed in the plasma membrane of oocytes, making it the first missense mutation to result in defective plasma membrane targeting. Notably, the P979L mutant exhibited the same cellular expression profile as the wild-type protein, both in Xenopus oocytes and in transfected HEK293FT cells grown at 28°C, but much less P979L protein was found upon cell growth at 37°C, showing for the first time that temperature-sensitive effects on protein stability can underlie ATP1A2 loss-of-function.

Introduction

Familial hemiplegic migraine (FHM) is a rare and severe autosomal-dominant form of migraine with aura, which is characterized by some degree of reversible motor weakness during the aura phase. FHM type 2 (FHM2) is caused by mutations in ATP1A2, the gene coding for the human Na+/K+-ATPase α2-subunit.1 Mutations in two other genes have also been implicated in the disease, namely in CACNA1A (FHM1)2 and in SCN1A (FHM3),3 which code for neuronal voltage-gated Ca2+ or Na+ channels, respectively, suggesting that perturbations in neuronal excitability underlie the neurological phenotype. Besides the involvement in FHM, ATP1A2 mutations have been described in sporadic forms of hemiplegic migraine (SHM) and also in more general types of migraine with or without aura (MA/ MO) or even in basilar migraine (BM). Moreover, there is significant clinical overlap to other neuropathologies involving mental retardation, seizures or forms of epilepsy like benign familial infantile convulsions (BFIC) (refer to the paper by Morth and colleagues4 for a complete list). It is assumed in current disease models that most of the aura symptoms are directly linked to the phenomenon of cortical spreading...
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Results and Discussion

Upon expression of WT and mutant human Na+/K+-ATPase α3-subunits together with the β1-subunit in Xenopus oocytes, we measured stationary Na+/K+ pump currents and ouabain-sensitive uptake of Rb+. Figure 1A shows that the stationary pump currents of the P979L mutant were slightly smaller compared to WT, whereas a reduction by about 80% was found for the R908Q mutant, and the currents measured on oocytes expressing the G301R mutant were indistinguishable from baseline fluctuations. Rb+ uptake was nearly identical for ATP1A2 WT and P979L, and largely reduced for mutants R908Q and G301R. To explore more subtle functional consequences, we measured the voltage and [K+]ext dependence of K+-stimulated pump currents, and determined kinetics and voltage dependence of change movement from ouabain-sensitive transient currents mediated by Na+/K+-ATPase under extracellularly high [Na+] and K+-free conditions. Although stationary currents of the R908Q mutant were small, a sufficient number of cells could be analyzed for this construct. Compared to the WT properties determined previously (see Fig. 3A in Tavraz et al.10), the stationary I-V curves of the R908Q mutant (Fig. 1C) were very similar. The same was true for the P979L mutant (Fig. 1D), and also the voltage-dependent K0.5 values for [K+]ext dependence of pump currents were very similar to WT for R908Q (Fig. 1E) and P979L (Fig. 1F). Furthermore, the parameters V0.5 and z from fits of a Boltzmann function to the Q-V distributions of translocated charge and turnover numbers were not different from the WT values for mutants R908Q and P979L (Table 1). For the R908Q mutant, the reciprocal time constants from transient currents were also identical to WT (Fig. 1G), whereas slightly elevated rate constants were found for P979L (Fig. 1H). In summary, ion transport activity, as assessed by pump current and Rb+ uptake measurements, was almost completely lost for mutant G301R, largely reduced for R908Q (albeit without detectable functional changes on a molecular level), but unaltered for mutant P979L.

At this point, it was critical to determine protein expression levels and to monitor the relative amounts of ATP1A2 protein in the plasma membrane. Figure 2A shows that for the P979L mutant the amounts of α3-subunit protein in total membrane as well as plasma membrane fractions were similar to WT. However, protein abundance in the plasma membrane fraction was slightly smaller for the G301R mutant, and for R908Q severely reduced, although the amounts of α3-subunit protein in the total cellular membrane fractions were identical to WT (Fig. 2B).

Therefore, loss of activity for the G301R mutant can be attributed to a normally targeted but inactive enzyme, as found previously for mutations R764P and W887R.18 Gly301 resides in the central portion of transmembrane helix M3 (Fig. 3) where it closely approaches helix M4 around Leu322 and Ile325. Thus, mutation to a bulky arginine is likely to distort the local structure, and since the adjacent residue Glu332 is part of the cation binding pocket,15 this putative disruption may interfere with cation coordination or transport. For the R908Q mutation, deficient plasma membrane targeting can account for loss-of-function, although the mutant enzyme itself is fully functional. Reduced plasma depression (CSD), which—starting from a certain focus on the neocortex—is characterized by a spreading front of neuronal excitation (depolarization) that is followed by long lasting depression (hyperpolarization).3 Therefore, it is likely that FHM arises from transient neuronal hyperexcitability, very similar to epilepsy. The Na+/K+-ATPase is an electrogenic ion transporter, which utilizes ATP hydrolysis for uphill transport of 3 Na+ ions out of the cell and subsequent import of 2 K+ ions per reaction cycle. The enzyme consists of a large catalytic α-subunit and a smaller accessory β-subunit. Both subunit isoforms are expressed in a tissue- and developmental-specific manner.6 In the adult brain, the α3-subunit is mainly located in glial cells.7 The Na+/K+-ATPase maintains the physiological gradients for Na+ and K+ ions and is therefore crucial for proper activity of secondary active transporters, which utilize these gradients for transport of other solutes like e.g., Ca2+ ions or neurotransmitters, but also for ion channels, which are critical for action potential initiation or for setting the threshold of neuronal firing. Thus, neuronal hyperexcitability can be correlated to loss of Na+/K+-ATPase function in several ways: First, breakdown of the transmembrane K+ ion gradient would impair the repolarizing activity of voltage-gated K+ channels and therefore lower the threshold for action potential firing. Second, breakdown of the Na+ ion gradient would, on one hand, limit the activity of voltage-gated Na+ channels and therefore lower the threshold for action potential firing. Thus, neuronal hyperexcitability can be correlated to loss of Na+/K+-ATPase function in several ways: First, breakdown of the transmembrane K+ ion gradient would impair the repolarizing activity of voltage-gated K+ channels and therefore lower the threshold for action potential firing. Second, breakdown of the Na+ ion gradient would, on one hand, limit the activity of the neuronal Na+/Ca2+-exchanger to increase Ca2+ signaling, and on the other hand, impair the removal of glutamate from the synaptic cleft by the astrocytic Na+/glutamate symporter.

Since 2003, about 50 ATP1A2 mutations have been described with clinical linkage to migraine, with FHM diagnosed in 36 cases.3 To understand the primary disease-causing mechanisms, it is mandatory to characterize the defects caused by ATP1A2 mutations on a molecular level. When novel ATP1A2 mutations are described in human genetic studies, ouabain survival assays are commonly carried out to probe for functional impairment.1 However, these experiments do not allow one to draw conclusions about the stage on which protein function is disrupted. The few studies that assayed the functional consequences of ATP1A2 mutations by electrophysiological8 or biochemical9 techniques have already hinted at a broad spectrum of molecular phenotypes. In a preceding publication10 we have investigated the effects of 11 FHM or SHM mutations, showing that, besides complete loss-of-function, subtle changes in the enzyme’s voltage-dependent properties or cation affinities are caused by the mutations. In addition, defects in protein expression or plasma membrane targeting were observed. Our previous study was focused on a certain subset of ATP1A2 mutations, which presumably cause disruptions of structurally critical H-bond patterns, and on carboxy-terminal mutations, including a stop codon mutation, which leads to an elongation of the carboxy-terminus by 28 amino acids. The Na+/K+-ATPase carboxy-terminus has been highlighted as a critical structural element for voltage-dependence and interaction with cations.10 In the present study, we analyze the molecular consequences of three additional ATP1A2 missense mutations, G301R11 (FHM), R908Q12 (SHM) and P979L13 (FHM), by measuring stationary pump currents and transient currents in Xenopus laevis oocytes, and monitoring protein expression in these and in mammalian HEK293FT cells at different temperatures.
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Membrane expression has previously been observed for the S966fs frame shift mutation and the deletion/insertion mutation del(K935-S940)ins(I),\textsuperscript{10} which represent severe disruptions of the primary structure and likely cause protein misfolding and degradation. Notably, R908Q is the first FHM2 missense mutation to result in defective plasma membrane targeting. Arg\textsuperscript{908} resides in the loop between transmembrane segments M8 and M9 and may be critical for structural integrity of the extracellular face of the \(Na^+\)/\(K^+\)-ATPase α-subunit, since the terminal amino group of this arginine is able to form an H-bond (~3.5 Å) to the backbone carbonyl oxygen of Leu\textsuperscript{797} in the M5-M6 loop (Fig. 3). Reduced plasma membrane trafficking can also explain the previously published results from ouabain survival assays, in which a severe impairment of cell viability was found for cells expressing the R908Q mutant, although the amount of total cellular \(\alpha\)-subunit protein was not changed compared to WT.\textsuperscript{12}

Since the P979L mutant did not show any functional alterations in our oocyte experiments at room temperature (20–22°C), we asked, whether a difference in the temperature dependence of the ion-pumping rate could lead to divergent activity at body temperature. This could happen either if the activation energy of the rate-limiting step for ion pumping by the P979L mutant protein would be different from that of the WT protein, or if at elevated temperature, another partial reaction of the catalytic cycle with a different temperature dependence would become rate limiting. To explore this possibility, we measured the temperature dependence of stationary pump currents between 18°C and 34°C on oocytes expressing ATP1A2 WT or the P979L mutant. Analysis of the resulting Arrhenius plots (data not shown) yielded very similar activation energies for WT and P979L. Two temperature regimes were generally observed with a transition temperature around 26°C (see Table 1). Such ‘breaks’ in Arrhenius plots have previously been observed for e.g., native \(Na^+\)/\(K^+\)-ATPase from rat cardiac myocytes\textsuperscript{16} and have tentatively been assigned to a phase transition within the lipidic cell membrane. However, also these measurements exclude the possibility that

Figure 1. Functional properties of ATP1A2 mutants. (A) Stationary currents at 10 mM \(K^+\) and -30 mV from oocytes expressing ATP1A2 WT and mutants. Data were obtained from the following number of cells (in brackets): WT (139), G301R (15), R908Q (31), P979L (17) from at least five oocyte batches. (B) Ouabain-sensitive \(Rb^+\) uptake calculated as the difference between the mean uptake measured in presence of 10 μM and 10 mM ouabain, respectively [N = 21–49 cells from four oocyte batches]. Data were normalized to the mean ouabain-sensitive \(Rb^+\) uptake of WT ATP1A2 for each experiment with the following normalization values (in pmole/oocyte/min): Exp. 1 (49), Exp. 2 (130), Exp. 3 (38), Exp. 4 (61). [C and D] \([K^+]_{\text{ext}}\) and voltage dependence of pump currents for ATP1A2 mutants R908Q (C), and P979L (D). Pump currents were measured in response to voltage steps upon addition of different \([K^+]_{\text{ext}}\) and amplitudes at \([K^+] = 10\) mM and 0 mV were used for normalization. Different \([K^+]\) are indicated by symbols. [E and F] Voltage-dependent \(K_{0.5}\) values for R908Q (E) and P979L (F) were determined from fits of a Hill function to the data in (C and D), the WT curve is superimposed as dotted line for comparison. [G and H] Voltage dependence of reciprocal time constants from transient currents measured under extracellularly high \([Na^+]\)/\(K^+\)-free conditions for mutants R908Q (E), and P979L (F). The dashed line indicates the course of the WT curve measured under identical conditions. Data were obtained from eight (R908Q) or 20 (P979L) oocytes from at least three frogs. All data are given as means ± S.E.
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Table 1  
Electrophysiological properties of ATP1A2 mutants

|       | $V_{0.5}$ (mV) | $z_q$   | Turnover (s$^{-1}$) | N  | $E_A$ (kJ/mol) 18°C–26°C | $E_A$ (kJ/mol) 26°C–34°C | N |
|-------|----------------|---------|---------------------|----|-------------------------|-------------------------|---|
| WT    | 4.3 ± 3.5      | 0.82 ± 0.03 | 12.8 ± 3.0          | 18 | 132 ± 14                | 87 ± 15                 | 5 |
| G301R | N/A            | N/A     | N/A                 | N/D| N/D                     | N/D                     | N/D|
| R908Q | 8.3 ± 3.5      | 0.82 ± 0.03 | 13.0 ± 4.7          | 8  | N/D                     | N/D                     | 5 |
| P979L | 3.8 ± 7.1      | 0.79 ± 0.04 | 18.6 ± 5.4          | 21 | 124 ± 6                 | 80 ± 2                  | 5 |

Parameters from fits of a Boltzmann function to the voltage-dependent distributions of charge from transient currents recorded in voltage jump experiments under extracellularly high [Na$^+$]/K$^+$-free conditions. $V_{0.5}$ values denote midpoint potentials, $z_q$ the slope factors (equivalent charges), and $E_A$ activation energies from Arrhenius plots derived from measurements of the temperature dependence of pump currents. N is the number of cells included in analysis. N/A, not applicable; N/D, not determined.

Figure 2. Expression of ATP1A2 constructs in Xenopus oocytes and HEK 293FT cells. (A and B) Representative western blots of samples from plasma membrane (PM) and total membrane (TM) fractions from oocytes expressing various ATP1A2 constructs. Samples equivalent to one oocyte were loaded per lane. Uninjected oocytes served as control. (C) Expression of ATP1A2 WT and mutants upon transient transfection in HEK 293FT cells and growth at permissive (28°C) and non-permissive (37°C) temperature. Expression of the Na$^+$/K$^+$-ATPase $\alpha_2$-subunit and $\beta$-actin (used as loading standard) was assessed by western blotting of whole cell lysate samples, each containing 15 μg of total protein. As an additional control for the consistency of the preparation, the non-disease-related polymorphism I883L$^{13}$ was also investigated using this assay. This mutant was expressed in a similar manner as the WT enzyme. Experiments were repeated three (A and B) or five (C) times, with identical results.

Figure 3. Location of mutated residues within the Na$^+$/K$^+$-ATPase structure. (A and B) Structural representation of the Na$^+$/K$^+$-ATPase according to protein data bank (PDB) structure entry 3B8E with amino acids numbered according to human ATP1A2. The numbering according to the rat Na$^+$/K$^+$-ATPase $\alpha_1$-subunit, which underlies the 3B8E structure, is given in brackets below. Residues mutated in this study are shown in ball and stick representation, two red spheres indicate Rb$^+$ ions within the cation-binding pocket as identified in the crystal structure. Structural elements outlined in color: Helix M3 (olive) contains Gly$^{301}$ (Gly$^{296}$), helix M8 (light orange) contains Arg$^{908}$ (Arg$^{904}$), the loop between helix M9 and M10 (magenta) contains Pro$^{979}$ (Pro$^{975}$). The central helix M5 is shown in green. It contains Leu$^{797}$ (Leu$^{793}$), whose backbone carboxyl group is the only candidate for the formation of an H-bond (red dashed line) to Arg$^{908}$ within a radius of 5 Å. (A) Side view of the transmembrane part of the Na$^+$/K$^+$-ATPase $\alpha$-subunit. (B) View from the extracellular side roughly along helix M5. In this panel, the $\beta$-subunit is also depicted (violet).
a change in the temperature dependence of the ion pumping rate underlies loss-of-function of the P979L mutant.

Since no changes in molecular function for the P979L mutant could be observed in oocytes we explored other expression systems to reveal abnormalities of the mutant protein. Preliminary experiments using HeLa cells to test whether the P979L mutant was able to confer cell survival in ouabain-containing media showed reduced cell viability compared to WT-transfected cells, yet this effect was subject to substantial batch variations (data not shown). However, we took this as first indication that expression in mammalian cells and/or cell growth at 37°C could aid to discriminate between ATP1A2 WT and the P979L mutant. Pro979 resides within the short extracellular M9-M10 loop, where it ensures a rather rigid local structure, which is likely to be relaxed upon substitution for a leucine. This could potentially lead to a less effective folding or even misfolding of the structurally important carboxy-terminal region. It has been shown that transmembrane helices M5-M10 are problematic for membrane integration of the Na+/K⁺-ATPase α-subunit upon biosynthesis at the ER. 17 These six helices probably have to be inserted into the membrane as a single block and may present several putative degradation signals when erroneously exposed to the cytoplasm. 18 However, we did not find any indication for misfolded P979L protein in Xenopus oocytes, but it is known that folding defects of mutant proteins can be revealed in a temperature-dependent fashion. The most thoroughly investigated example is probably the ΔF508 mutation in the CFTR chloride channel, the most frequent sequence variant in cystic fibrosis. Cellular quality control systems for correctly folded membrane proteins are stringently applied to nascent CFTR proteins. It has been shown that the ΔF508 mutant is efficiently and rapidly degraded before exiting the ER in mammalian cell cultures. 19 This effect was shown to be temperature-sensitive, 20 which easily explains why CFTR-ΔF508 could be functionally expressed in Xenopus oocytes. 21 We therefore investigated, whether temperature-dependent protein degradation could occur for the P979L mutant. For this purpose HEK293FT cells were transfected with cDNA of the WT or mutant Na+/K⁺-ATPase α-subunits plus β-1-subunits. Cell batches were split 24 hours after transfection and subsequently grown at 28°C and 37°C. Figure 2C shows a western blot of cell lysates from such cell cultures. These experiments consistently (N = 5) showed that the amount of P979L mutant α₂-subunit was identical to WT at 28°C, whereas a largely reduced protein abundance was found at 37°C. These results for the first time provide evidence, that a temperature-dependent effect on protein stability might underlie the loss of Na⁺/K⁺-ATPase function in FHM2 and expands the variety of abnormalities caused by mutations identified in this disease. It will be challenging to reveal the cellular mechanisms and pathways underlying the temperature-dependent degradation of the Na⁺/K⁺-ATPase α₂-subunit.

The spectrum of molecular phenotypes of ATP1A2 mutations may still be incomplete. For instance, it has not been tested whether FHM2 mutations might disrupt interactions with e.g., adapter proteins. In this context, it is notable that the LQT4 subtype of the cardiac long QT syndrome is caused by mutations in ankyrin B, 22 which is required for recruitment of certain ion transporters (including the Na⁺/K⁺-ATPase α₂-subunit) to specific microdomains of cardiomyocyte membranes to coordinate spatio-temporal Ca²⁺ signalling. 23 Thus, it will be interesting to see whether, in addition to the already established complexity of molecular phenotypes, changes in subcellular localization can underlie ATP1A2 loss-of-function.

Concluding Remarks

Our results show that assays to investigate the consequences of FHM2 or SHM mutations in heterologous expression systems, which do not include determination of plasma membrane protein, are insufficient to characterize the origin of a possible loss-of-function at the level of the protein. To discriminate between molecular dysfunction and structure-folding-related phenomena, it would be helpful to combine ouabain survival assays, which are commonly utilized as a first test for functional defects in studies describing novel ATP1A2 mutations, with biotinylation studies that also quantify the amount of plasma membrane protein. The mutations R908Q and P979L are the first examples within the ATP1A2 protein that result in completely functional enzymes, which are most likely defective in targeting to the plasma membrane (R908Q) or subject to premature degradation at body temperature (P979L). These observations expand the spectrum of molecular phenotypes of ATP1A2 mutations in sporadic and familial hemiplegic migraine and call for the inclusion of cell biological assays to clarify the correlation between the cellular fate of the protein and primary disease-causing processes.

Materials and Methods

Xenopus oocytes were used for expression of the ouabain-insensitive (IC₅₀ in mM range achieved by mutations Q116R and N127(D)) ¹⁴ human Na⁺/K⁺-ATPase α₂-subunit (referred to as WT ATP1A2) and mutants thereof, together with human β₁-subunits. Measurements of stationary pump currents, pre-steady state currents, Rb⁺ uptake, and plasma membrane or total cellular membrane preparations were carried out as described. ¹⁰ Expression in HEK-293FT cells and temperature shift assays. Amino-terminally HA-tagged human Na⁺/K⁺-ATPase α₂-subunits were expressed together with human β₁-subunits in HEK-293FT cells upon transient transfection of 1:1 cDNA mixtures using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Cells were grown to about 70% confluence in 10 cm Petri dishes in DMEM/Glutamax medium + 10% fetal bovine serum and 10 μM ouabain. Water-transfected cells served as control. Twenty-four hours after transfection, the cell charges were split and incubated separately at 28°C or 37°C, respectively, for an additional 24 hours. Subsequently, cells were harvested and subjected to whole cell lysis. After determination of total protein concentration in these total lysates using a bicinconinic acid assay (Perbio, Bonn, Germany), samples containing 15 μg of total protein were separated on sodium-dodecylsulfate polyacrylamide gels by electrophoresis and subsequently transferred to nitrocellulose membranes. The HA-tagged α₂-subunits were detected with primary rat monoclonal anti-HA antibody 3F10 (Roche Molecular Biochemicals, Mannheim, Germany). β-actin was detected as a loading standard.
using mouse monoclonal anti-β-actin antibody AC-15 (Sigma, Deisenhofen, Germany). Secondary antibodies were horseradish peroxidase conjugates against rat or mouse (Promega, Mannheim, Germany), and the ECL chemiluminescence reagent (Pierce) was used for imaging on a Kodak Biomax film (Sigma, Deisenhofen, Germany).

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