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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.

1. Introduction

Alternating Hemiplegia of Childhood (AHC) is a rare, severe neurodevelopmental disorder, reported for the first time in 1971 by Verret and Steele [1]. The incidence of AHC has been estimated at 1 in 1,000,000 births, with disease usually ensuing within the first six months [2]. AHC is characterized by episodes of hemiplegia on alternating sides of the body [3], 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 [4]. Patient studies have reported different provoking factors: water exposure, extreme temperatures, physical activity, bright light, and stress [5]. 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 Ca^{2+} influx inhibitor specific for vascular smooth muscle and neurons) has been reported to reduce symptoms [6,7]. However, the effectiveness and long-term effects of this treatment are unknown [5].

AHC was recently linked to de novo mutations in *ATP1A3*, the gene encoding the Na^+,K^+-ATPase α3 subunit [8,9]. 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 [10]. Previously, *ATP1A3* mutations were identified in patients suffering from Rapid-Onset Dystonia Parkinsonism (RDP) [11]. Until now, there has been only one mutation that is reported in AHC and RDP cases (D923N), indicating minor overlap between both diseases [12–14].

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 [15,16]. 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 [15]. During this catalytic cycle, Na^+,K^+-ATPase is present in the E_1 or the E_2 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 the phosphorylation of the protein in the E_1 state, which leads to a conformational change into

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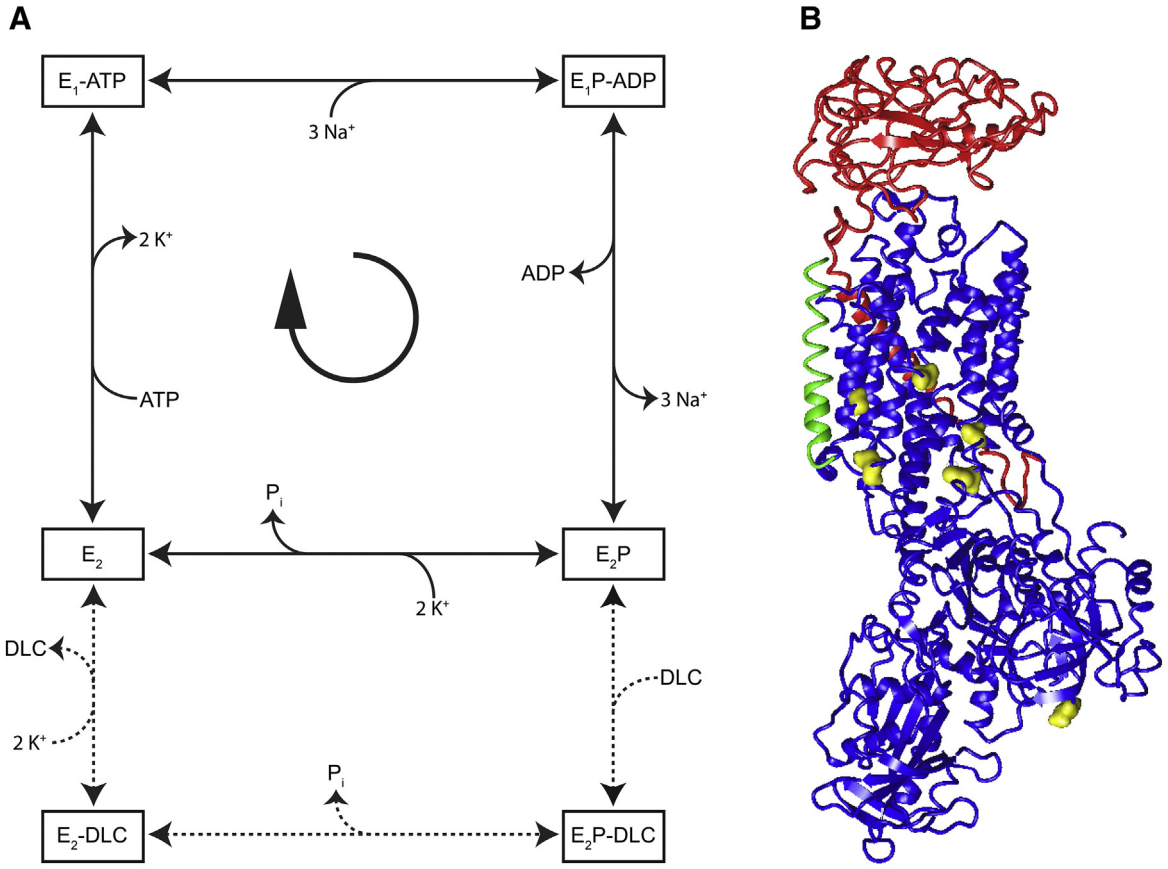

Fig. 1. (A) Albert-Post scheme of the reaction cycle of Na^+,K^+-ATPase. Transport of Na^+ and K^+ across the cell membrane is accomplished by a series of conformational changes of the Na^+,K^+-ATPase based on binding of either Na^+ or K^+, leading to phosphorylation or dephosphorylation, changing the 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 E_1P and E_2P states. Binding of DLCs, well-known inhibitors of Na^+,K^+-ATPase function, is only possible in the E_2P or E_2 state. (B) Crystal structure of the Na^+,K^+-ATPase heteromer in the E_2 state (PDB: 3KDP) [32], 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 alpha 1 and alpha 3 isoforms, making the alpha 1 crystal structure suitable for representation. Blue is alpha subunit, red is beta subunit, green is gamma subunit, and yellow is used to highlight the mutations studied here. Figure was created using YASARA software.

the Na^+-bound phosphorylated conformation (E_1P–ADP). During transition to the E_2P 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 E_2 state [17]. Binding of ATP drives the protein back into the E_1 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 determined the functional consequences of six de novo *ATP1A3* mutations (S137Y, D220N, I274N, D801N, E815K, and G947R, see Fig. 1B) in order to relate protein functionality to AHC phenotypes.

2. Materials and methods

2.1. 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 was cloned into an entry vector using BP Clonase II enzyme according to the manufacturer's instructions (Invitrogen). After a 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 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.

2.2. 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 the expression clone, recombinant bacmids were selected and isolated [18]. Subsequently, the obtained bacmids were transfected to Sf9 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 [19].