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Mutations in the Na^+/K^+-ATPase $\alpha 3$ Gene *ATP1A3* Are Associated with Rapid-Onset Dystonia Parkinsonism

Report

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Summary

Rapid-onset dystonia-parkinsonism (RDP, *DYT12*) is a distinctive autosomal-dominant movement disorder with variable expressivity and reduced penetrance characterized by abrupt onset of dystonia, usually accompanied by signs of parkinsonism. The sudden onset of symptoms over hours to a few weeks, often associated with physical or emotional stress, suggests a trigger initiating a nervous system insult resulting in permanent neurologic disability. We report the finding of six missense mutations in the gene for the Na^+/K^+-ATPase $\alpha 3$ subunit (*ATP1A3*) in seven unrelated families with RDP. Functional studies and structural analysis of the protein suggest that these mutations impair enzyme activity or stability. This finding implicates the Na^+/K^+ pump, a crucial protein responsible for the electrochemical gradient across the cell membrane, in dystonia and parkinsonism.

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Introduction

Rapid-onset dystonia-parkinsonism (RDP, *DYT12*, OMIM 128235) is an autosomal-dominant disease characterized by the sudden onset of dystonia and parkinsonism (Dobyns et al., 1993; Brashear et al., 1997; Pittock et al., 2000; Linazasoro et al., 2002). Symptoms, which evolve over hours to days, usually start in late adolescence or early adulthood, although onset as early as 4 and as late as 58 years has been reported (Dobyns et al., 1993; Brashear et al., 1997; Pittock et al., 2000; Linazasoro et al., 2002). Most affected individuals have limb and cranial dystonia with dysarthria and dysphagia accompanied by bradykinesia, slow gait, and postural instability (Dobyns et al., 1993; Brashear et al., 1997; Pittock et al., 2000; Linazasoro et al., 2002). Seizures, paroxysmal dystonia and psychiatric symptoms have been observed in a few patients (Dobyns et al., 1993; Brashear et al., 1997; Pittock et al., 2000). Sudden onset or worsening of symptoms occur after stresses such as fever, exposure to heat, prolonged exercise, childbirth, or emotional stress. Most patients remain stable or demonstrate slight improvement years after the abrupt onset of symptoms. Some patients have reduced levels of homovanillic acid in the cerebral spinal fluid yet little or no response to L-dopa. PET studies reveal no loss of the dopamine transporter (Brashear et al., 1998, 1999), and pathological exam of a single affected brain was unremarkable (Pittock et al., 2000). The disease was linked to an 8 cM region of 19q13 in three families (Kramer et al., 1999; Pittock et al., 2000) and was recently refined to a 5.9 cM minimal interval (Kamm et al., 2004).

There are at least fifteen different loci that cause inherited dystonias (de Carvalho Aguiar and Ozelius, 2002; Grimes et al., 2002). To date, five dystonia genes have been identified (de Carvalho Aguiar and Ozelius, 2002; Nolte et al., 2003); however, none of these genes involve ion pumps.

The Na^+/K^+-ATPases (sodium pumps) belong to the P-type ATPase group. Pumps in this group catalyze active transport of cations across cell membranes and maintain ionic gradients through hydrolysis of ATP. The α subunit is the catalytic subunit, and three isoforms ($\alpha 1$, 2, and 3) are expressed in the nervous system (McGrail et al., 1991). Mutations in sodium pumps are known to cause neuronal dysfunction and neurodegeneration in *Drosophila* (Palladino et al., 2003). Recently, missense mutations in the $\alpha 2$ isoform of the sodium pump (*ATP1A2*)

Table 1. Clinical Characteristics and Mutations in RDP Families

Family	Mutation[a]	Codon Change	Age of Onset in Probands in Years (Range in Family)	Onset Period in Proband (Range in Family)	Number Affected in Family	RDP Severity Scale Range in Family[b]	Reference
1	T821C	I274T	37	14 days	1	3	
2	G829A	E277K	20	1 day	1	4	
3	C1838T	T613M	17	2 days	1	2	1
4	C1838T	T613M	17 (16–28)	Several hours (mins to 30 days)	4	3	2
5	T2273G	I758S	14 (14–45)	30 days (1 hr to 30 days)	12	2–4	3
6	T2338C	F780L	35 (16–35)	30 days (30–90 days)	2	3–4	
7	G2401T	D801Y	23 (12–23)	2–3 days	4	2–4	4

References: 1, Linazasoro et al., 2002; 2, Zaremba et al., 2004; 3, Dobyns et al., 1993; 4, Brashear et al., 1997.

[a] Nucleotide numbering is based on using the A in the start ATG as position 1. The reference cDNA sequence (NM_152296) has 116 bps of 5′ UTR before the start ATG.

[b] 0, unaffected; 1, limb dystonia only; 2, affected arm and bulbar, gait normal; 3, same as 2, with leg involvement but walking unassisted; 4, same as 2, walking with walker or in wheelchair.

were associated with familial hemiplegic migraine (FHM) type 2 (De Fusco et al., 2003; Vanmolkot et al., 2003). The absence of α2 resulted in akinesia and failed respiratory rhythm generation in newborns in a knockout mouse model (Moseley et al., 2003), and adult heterozygote mice displayed degeneration in the amygdala and enhanced anxiety behavior (Ikeda et al., 2003).

Here, we report mutations in the α3 subunit of the sodium pump gene (*ATP1A3*) in patients with RDP and assess the potential pathological role of these mutations through protein structural analysis, expression studies in cell culture, and subcellular localization experiments.

Results and Discussion

Several candidate genes in the minimal linked region were studied based on their putative functions: *DPF1*, *RYR1*, *GMFG*, *SUPT5H*, *PRX*, *NUMBL*, *RABAC1*, *ATP1A3*, and, as previously reported, *GRIK5* (Kamm et al., 2004) (human chr 19:43,394,196–47,261,797; July 2003 assembly, www.genome.ucsc.edu). We identified six different missense mutations in seven unrelated RDP families in the *ATP1A3* gene (Table 1). In the cases with family history (4 through 7; Table 1), the mutations segregated in all affected and obligate carrier members. We identified de novo mutations in two of the sporadic cases for which we had parental DNA samples (2 and 3; Table 1) and excluded nonpaternity using several polymorphic markers on different chromosomes. In addition, cases 3 (de novo) and 4 had the same mutation (T613M; C1838T), but haplotype analysis across the disease gene region confirmed that these were independent events. All six mutations are located in highly conserved regions of the protein (Figure 1) and were not identified in 500 northern European control chromosomes. The I758S mutation is located adjacent to a residue in the homologous region of *ATP1A2* mutated (L746P) in a FHM2 family (De Fusco et al., 2003) (Figure 1).

The Na^+/K^+-ATPase is closely related to the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA). SERCA1 (the skeletal muscle isoform) has been crystallized in two different conformations: E1 (Ca^{2+}) and E2 (thapsigargin) (Toyoshima et al., 2000; Toyoshima and Nomura, 2002). A structural model of α3 was generated based on its homology to SERCA1a (Sweadner and Donnet, 2001; Ogawa and Toyoshima, 2002) and on known structures for one domain of the α1 and α2 Na^+/K^+-ATPase (Hilge et al., 2003; Hakansson, 2003). SERCA and Na^+/K^+-ATPase are 30% identical and 65% similar, and 88% of Na^+/K^+-ATPase residues are scored as aligned with SERCA by gapped BLAST (Sweadner and Donnet, 2001). There is also considerable biochemical and mutational evidence that Na^+/K^+-ATPase adopts the same fold (Sweadner and Donnet, 2001; Ogawa and Toyoshima, 2002). Na^+/K^+-ATPase α1 and α3 are 90% identical and differ very little in these conserved regions. The disposition on the crystal structures of the SERCA1 residues corresponding to the mutations is thus highly predictive of their locations in the Na^+/K^+-ATPase. Figure 2 summarizes the locations of all six mutations in the Na^+/K^+-ATPase in the E1 conformation.

There are three distinct domains on the cytoplasmic face of the enzyme, and two of them undergo huge movements during catalysis, driving the rearrangement of the transmembrane spans. Directly above the membrane domain is the phosphorylation (or P) domain (yellow), which extends from transmembrane spans M4 and M5 and forms the core. The actuator (A) domain (red) extends from M1, M2, and M3 and undergoes large movements during the catalytic cycle, such that the three cytoplasmic domains are spread out in the E1 conformation and grouped compactly in E2. The nucleotide (N) domain (green) emerges from the P domain via a hinge and folds down over the P domain in the E2 conformation (Toyoshima et al., 2000; Toyoshima and Nomura, 2002). This is thought to position ATP bound to the N domain so that its terminal phosphate reaches the active site aspartate residue in the P domain. The terminal phosphate then is transferred covalently to the aspartate as the first step of ATP hydrolysis. One RDP mutation, T613M, lies at the edge of the P domain near the aspartate, and in the E2 conformation it forms a very close contact with G539 in the N domain. It seems likely that this bulky substitution would interfere with enzyme activity.

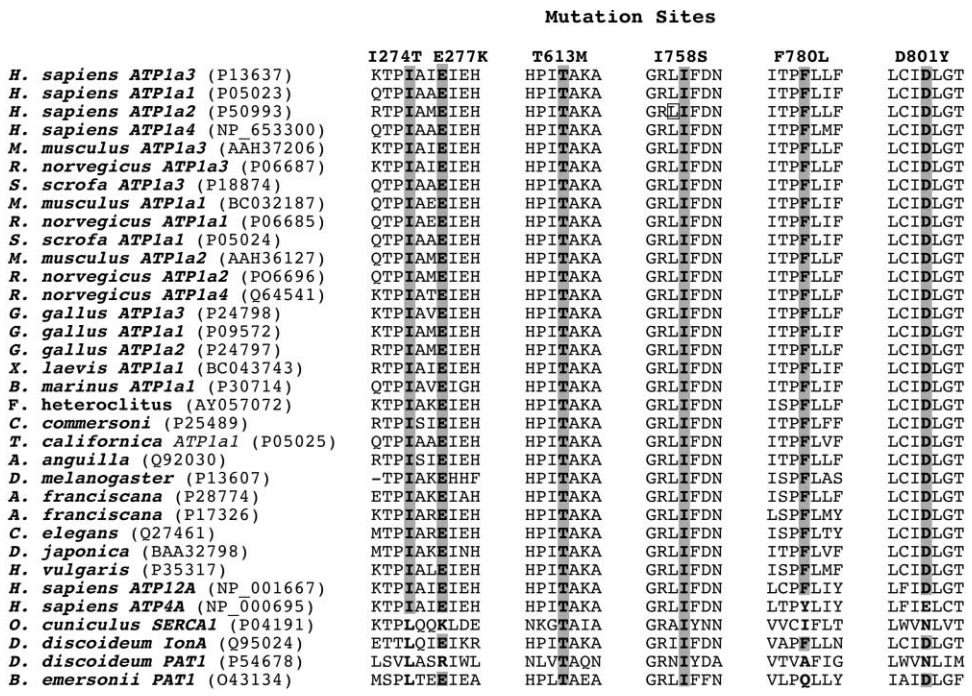

Figure 1. Protein Sequence Alignment of ATPases in the Regions Containing the RDP Mutations

Gray shading highlights the six mutated residues. The boxed amino acid in the human ATP1a2 sequence indicates the site of a mutation (L764P) associated with familial hemiplegic migraine type 2. All but the final six sequences are Na^+/K^+-ATPases. ATP12A is the closely-related nongastric X, K-ATPase; ATP4A is the gastric H^+, K^+-ATPase; SERCA1 is the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase with crystal structures. IonA and PAT1 are related ATPases of *Dictyostelium* (slime mold, protozoa); PAT1 of *Blastocladiella* (fungi) is an uncharacterized relative that shares 27% identity with PAT1 of *Dictyostelium*. SwissPro or GenBank accession numbers are indicated.

The other five mutations are found in the transmembrane domain (purple). Three of these (I274T, E277K, and I758S) are clustered in the stalk, a bundle of α helices that extends from the lipid bilayer to the P domain. All of these mutations are buried and close to a short α helix at the base of the P domain (dark blue) that forms the critical noncovalent contact between domains and all involve a change in polarity or charge. It is credible that these mutations would disrupt either activity or folding of the protein. The D801Y mutation of α3 is equivalent to N796 in SERCA1, which is one of the residues that ligates Ca^{2+} in the center of the membrane. A side chain oxygen atom of the asparagine forms part of the ion coordination site. The residue lies in M6, just one turn of the helix away from a kink that breaks M6 and forms a critical part of the ion binding pocket. Homology modeling of Na^+/K^+-ATPase predicts that the equivalent residue, an aspartate, contributes not only to Na^+ site II but to both sites for K^+ binding (Ogawa and Toyoshima, 2002). Mutations of this residue in SERCA1 are known to abolish activity (Ogawa and Toyoshima, 2002). The substitution with a bulky tyrosine may also disrupt folding. The last membrane domain mutation, F780L, lies closer to the extracellular surface. Although it is not as obvious why it should be damaging, mutations of the equivalent residue in *Xenopus* α1 (F790C) and of SERCA1a (I775A, I775S, but not I775V) are inhibitory (Chen et al., 1996; Rice and MacLennan, 1996; Guennoun and Horisberger, 2000). It is one turn of the helix closer to the extracellular surface than N773 and E776, which are predicted to coordinate ions; therefore, it may be on the pathway for ion movement through the protein.

Thus, all of the RDP mutations in α3 would be predicted to result in either loss of activity or loss of folding stability or both. This prediction was tested by expressing each mutant human α3 in human embryonic kidney (HEK) 293T cells. HEK293 has *ATP1A1* (α1) activity that can be inhibited by ouabain (Kockskamper et al., 1997). Therefore, to permit inhibition of the endogenous pump and analyze the viability of the mutant transfected α3 pump, we reduced the natural ouabain sensitivity of α3 by inserting two mutations known to confer ouabain resistance (Q108R and N119D) (Price et al., 1990). Many cell lines undergo rapid apoptosis when Na^+/K^+-ATPase is inhibited by ouabain, and this forms the basis for determining whether mutant and ouabain-resistant α3 Na^+/K^+-ATPases have enough expression and activity to functionally substitute for the endogenous, ouabain-inhibited enzyme. Cells transfected with ouabain-resistant α3 (α3-OR) were able to survive when cultured in medium containing 10 μM ouabain, and 48 hr after the ouabain challenge showed only 5.4% cell death compared to cells transfected with α3-OR in medium without ouabain (Figure 3). As expected, cells transfected with the wild-type ouabain-sensitive α3 (α3-WT) showed extensive cell death in ouabain-containing medium. Similar transfection experiments were performed using constructs containing each patient mutation in the α3-OR backbone. All presented significantly lower survival than α3-OR ($p < 0.001$), indicating that these mutations im-

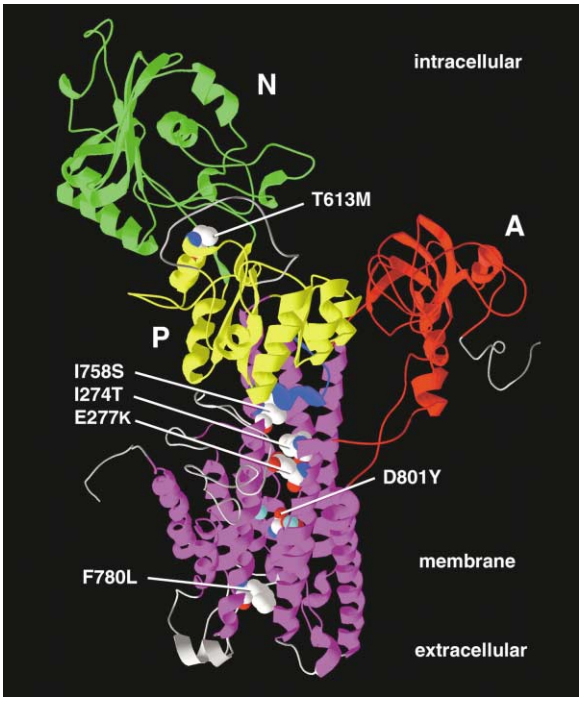

Figure 2. Location of RDP Mutations in Na^+/K^+-ATPase α3

A model of α3, shown in ribbon format, was computed based on homologous structures. The four domains of the protein (N, P, A, and membrane) are shown in the E1 (Na^+ binding) conformation. Two of the three predicted ion binding sites in the middle of the membrane domain are shown with Na^+ ions as cyan spheres. Loops that are colored gray have lower homology in SERCA1a, and thus their modeled structure is tentative. All mutated residues lie in highly conserved regions, however. The normal side chain of each disease residue is shown in spacefill format.

paired the function of the α3-ATPase. Greatest cell death was observed with the mutations I758S and D801Y and was similar to cells with ouabain-sensitive pump activity (α3-WT). Mutation I274T seemed to be the least severe, with the other three mutations displaying an intermediate level of cell death (Figure 3). It is unlikely that the mutations created in the constructs to confer ouabain resistance interact with the RPD mutations to reduce activity or enhance ouabain sensitivity, because equivalent mutations in α1, α2, and α3 have not been observed to impair function in other mutagenesis studies. Additionally, the ouabain binding mutations are at the extracellular aqueous surface, distant from all six buried RDP mutations.

Expression of the protein was obtained in all of the mutants, but levels of expression were reduced and seemed to follow the same pattern observed in the cell viability assays (Figure 4). These observations are consistent with reduced stability and lower recovery of mutant forms. Consequently, both functional deficits and reduced level may contribute to the failure to replace α1 activity.

To assess whether the localization of mutant pumps was different from WT pumps, we conducted immunofluorescence studies using Xpress-tagged (Invitrogen) WT, WT-OR, and mutant OR α3 constructs transiently transfected into HEK293 cells and found no remarkable differences (data not shown). In accordance with other studies (Takeyasu et al., 1988) in which exogenous α subunit is expressed without additional exogenous β subunit, much of the nascent a3 appears in ER and Golgi, but some was observed at the plasma membrane for all mutant and WT constructs. Qualitatively, this indicates that none of the mutations disrupted the maturation of the Na^+/K^+-ATPase.

To date, there is no evidence of widespread neurodegeneration in patients with RDP, but further studies are necessary to assess this hypothesis. The clinical course of the majority of the patients, with acute onset of symptoms followed by stabilization, suggests an acute nonprogressive neuronal lesion. Some patients have paroxysmal dystonia and seizures; it is very likely that these mutations lead to abnormal electrical signaling in the brain. In *Drosophila*, mutations in the Na^+/K^+-ATPase α subunit were associated with neuronal hyperexcitability and seizure-like activity (Palladino et al., 2003), and mutations in *ATP1A2* have recently been associated with benign familial infantile convulsions (Vanmolkot et al., 2003).

There is considerable experimental evidence for oligomerization of the Na^+/K^+-ATPase, with the formation of either dimers or tetramers of α subunits, accompanied by β and γ subunits (Taniguchi et al., 2001; Ivanov et al., 2002; Arystarkhova et al., 2002). Such oligomerization would in principle make dominant-negative mutations possible. All of the RPD mutated residues, however, are predicted to be buried in one or both conformations, and nothing about them predicts a role in subunit interactions. The expression studies predict that, in RDP, α3 activity and expression will be reduced, with a result equivalent to haploinsufficiency. How this results in the

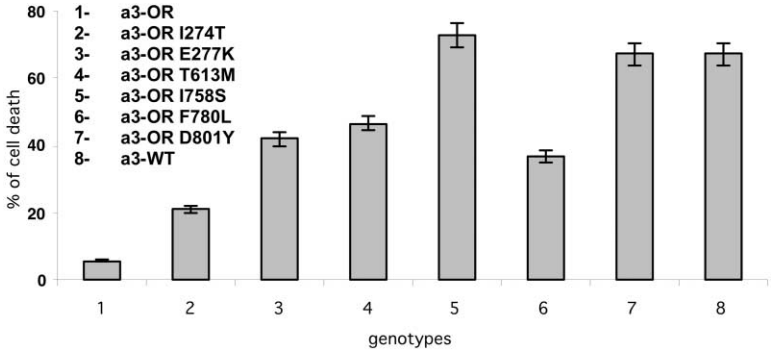

Figure 3. Ouabain Treatment of Transfected Cells

Histograms represent the percentage of cell death in HEK293T cells transfected with different *ATP1a3* constructs cultured in ouabain medium, based on the values of four experiments, with 5% error. Cells transfected with the RDP mutant constructs (lanes 2 through 7) show a higher percentage of cell death when compared to the control α3-OR construct (lane 1). Mutant constructs in lanes 5 and 7 show the highest percentage of cell death, similar to that of cells with inhibited ATPase activity (lane 8).

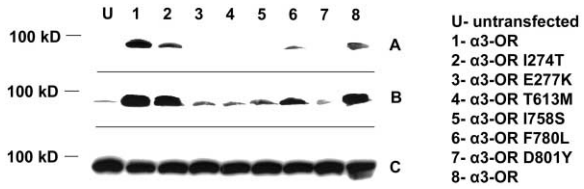

Figure 4. Western Blot of HEK293T Cells Showing Differences in Protein Expression of ATP1a3 between Transfected Constructs with Different Genotypes

(A) ATP1a3 with 20 s exposure; (B) ATP1a3 with 1 min exposure; (C) ATP1a1 used as a control for total amount of protein loaded in each lane, with 10 s exposure. Cells from lanes 2 through 8 were grown in ouabain-containing medium. Controls in lanes U and 1 were grown in regular medium. Cells transfected with the RDP mutant constructs (lanes 2 through 7) showed a lower expression of α3-ATPase when compared to the controls transfected with the α3-OR construct (lanes 1 and 8). Protein from E277K, T613M, I758S, and D801Y mutant constructs was only detectable at longer exposure time (B). Representative of four experiments.

acquisition of dystonic and/or parkinsonian symptoms after stressful events is likely to be related to an inability to keep up with a high demand for ion transport activity. Further, whether other mutations or polymorphisms in *ATP1A3* represent modifying factors for other forms of dystonia, parkinsonism, or epilepsy remains to be investigated.

Experimental Procedures

Patients

Seven probands clinically diagnosed with RDP according to the criteria defined by Brashear et al. (1998) and available family members were screened for mutations (Table 1). Families 3, 5, and 7 have been previously published (Linazasoro et al., 2002; Dobyns et al., 1993; Brashear et al., 1997). Clinical information on the probands and family members is summarized in Table 1. Informed consent was obtained, and the study was approved by the various institutional review boards.

Mutation Screening

DNA was extracted from white blood cells or buccal cells using standard methods. The 23 exons of the *ATP1A3* gene were amplified (primers and conditions in Supplemental Table S1 [http://www.neuron.org/cgi/content/full/43/2/169/DC1]) and the products sequenced by standard dideoxy nucleotide sequencing. For each identified mutation, we screened 500 control chromosomes by denaturing high performance liquid chromatography (DHPLC) using the WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE). Elution gradients and analysis temperatures for each exon were predicted based on the target sequence using the Transgenomic WAVEMaker software (Table S1). Mutation positive and negative controls were used in all runs.

Genotyping

For confirmation of potential de novo mutations, screening for nonpaternity was performed in the probands and their parents with the following polymorphic markers: *D19S223, D19S400, D19S 417, D19S 420, D19S 408, D17S785, D18S 976, GATA171C12, D4S2431, D4S3028, and D4S1607*. Primers and amplification conditions were as specified at www.gdb.org and http://www.chlc.org/. The same markers on chromosome 19 were used for haplotype analysis of patients who shared the same mutation. Products were analyzed on an ABI377 automated sequencer with Genescan and Genotyper 2.5 software (PerkinElmer, Wellesley, MA).

Modeling the Structure of the Na^+/K^+ Pump

A structure for the α3 isoform of the Na^+/K^+-ATPase was generated from the known structures of SERCA1a and the N domains of Na^+/K^+-ATPase α1 and α2 (Toyoshima et al., 2000; Hilge et al., 2003; Hakansson , 2003). The sequences of SERCA1a, α3, and structurally aligned portions of the α1 N domain were aligned, and the sequence alignment was further modified using the structural alignment reported in the crystallographic study of the N domain of α2 (Hakansson, 2003). We did not use coordinates from that study since they have not been made publicly available. Starting from the sequence alignment and from the published coordinates of SERCA1a (1EUL) and the NMR study of the N domain of α1 (1MO8), a structural model of Na^+/K^+-ATPase α3 was calculated using Modeller 6v2 (Marti-Renom et al., 2000). The figure was made using Deep View v3.7.

Constructs and Site-Directed Mutagenesis

We obtained a full-length human *ATP1A3* cDNA clone in the expression vector pCMV6-XL5 (α3-WT) from OriGene (OriGene Technologies, Inc.) and used site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene) to derive various mutant constructs. To confer ouabain resistance (OR), we introduced two mutations, A323G (Q108R) and A355G (N119D), and obtained the α3-OR cDNA clone. Using α3-OR as template, we introduced one of the following patient mutations: T821C (α3-OR I274T), G829A (α3-OR E277K), C1838T (α3-OR T613M), T2273G (α3-OR I758S), T2338C (α3-OR F780L), or G2401T (α3-OR D801Y). To obtain tagged constructs, α3-WT, α3-OR, and all six α3-OR mutant constructs were amplified (Pfu Turbo Taq, Stratagene) with the following primers 5′-CACAGAATTCGGGGACAAGAAAGATG-3′ and 5′-CACACTCGAGT CAGTAGTAGGTTTCC-3′. The products were digested with EcoRI and XhoI (NEB) according to the manufacturer's instructions and subcloned into the pcDNA4/HisMaxC (Invitrogen) expression vector. All clones were sequenced in full length. Note: the reference cDNA sequence in the database (NM_152296) has 116 bps of 5′ UTR sequence before the start ATG. We numbered the mutations using the A in the start ATG as position 1.

Cell Culture and Transfections

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine in a 5% CO_2 environment at 37°C. Transient transfections were performed using PolyFect Transfection Reagent (QIAGEN) according to the manufacturer's instructions.

Ouabain Treatment

Cells were distributed in nine 100 mm plates and were transfected between 70%–80% confluence. One plate was transfected with the wild-type ouabain-sensitive (α3-WT) cDNA, two plates were transfected with cDNAs modified only to obtain ouabain resistance (α3-OR), and six plates were transfected with α3-OR cDNAs containing each one of the patient mutations described above. Transfection efficiency was assessed by cotransfection of pDsRed-Express-N1 Vector (Clontech) and estimated at 70%. To inhibit the endogenous sodium pump activity, transfected cells were fed medium containing 10 μM ouabain 36 hr after transfection. One plate transfected with α3-OR was fed medium without ouabain and was used as a control of the expected number of cells. Two days after the ouabain challenge, the surviving cells were resuspended and counted with a hemacytometer (Fisher Scientific, Co.), and an estimation of cell death in each group was obtained by comparing the number of surviving cells to those of the control group. The experiment was repeated four times. In order to verify that the differences in cell death numbers between the α3-OR cultured in ouabain and the other α3-OR mutants was significant, a χ^2 test was performed, and a p value ≤ 0.05 was considered significant.

Western Blot

Protein (50 μg) from transfected and untransfected cell lysates was loaded in 7.5% Tris-HCl polyacrylamide gels. SDS-PAGE was performed for 2 hr at 100 V. For *ATP1A3* detection, transblotted nitrocellulose membranes were incubated with the α3-specific XVIF9-G10 monoclonal antibody (Affinity Bioreagents, Golden, CO). Goat anti-mouse (GAM)-HRP conjugate (Bio-Rad) was used as a secondary

antibody. SuperSignal West Dura Extended Duration Substrate kit (Pierce) was used for chemiluminescent protein detection. Antibodies were then removed from the membranes with Restore Western Blot Stripping Buffer (Pierce), and membranes were further incubated with the α1-specific monoclonal antibody α6F (Developmental Studies Hybridoma Bank-Iowa) to detect the endogenous α1 protein.

Immunocytochemistry

Twenty-four hours after transfection with the various His tag constructs, cells were plated onto collagen-coated (Vitrogen) coverslips and incubated at 37°C for another 24 hr. Cells were washed in PBS for 5 min, fixed with 100% methanol for 5 min, rinsed 5 × 2 min in PBS, and then blocked with PBS in 10% FBS for 20 min at room temperature (RT). Cells were incubated with anti-Xpress monoclonal antibody (1:200, Invitrogen) and anti-CD40 polyclonal antibody (5 μg/ml, Stressgen) or BiP polyclonal antibody (1:200, Stressgen) for 1 hr at RT. After washing 2 × 5 min in PBS, the cells were incubated with secondary antibodies and Cy3 goat anti-rabbit IgG antibody (1:1200, Jackson Immunoresearch) and AlexaFluor 488-conjugated goat anti-mouse IgG (1:1000, Molecular Probes), for 1 hr at RT in the dark. Coverslips were rinsed 2 × 5 min in PBS, mounted onto slides using Gel/Mount aqueous mounting media with antifading reagent (Biomeda Corp), and visualized using a Ziess Axiomat microscope at 40× magnification with the appropriate filters.

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