Kinetic Alterations due to a Missense Mutation in the Na,K-ATPase α2 Subunit Cause Familial Hemiplegic Migraine Type 2*

Received for publication, July 6, 2004, and in revised form, August 2, 2004
Published, JBC Papers in Press, August 11, 2004, DOI 10.1074/jbc.M407471200

Laura Segall‡, Rosemarie Scanzano⁶, Mari A. Kaunisto⁷, Maija Wessman§, Aarno Palotie¶, J. Jay Gargus§, and Rhoda Blostein+++ From the ①Departments of Biochemistry and Medicine, McGill University, Montreal, Quebec H3G 1A4, Canada, the ②Department of Physiology and Biophysics and Section of Human Genetics, Department of Pediatrics, University of California, Irvine, California 92697-4034, ③Biomedical Helsinki and the Department of Clinical Chemistry, University of Helsinki, Helsinki 00029 HUS, Finland, and ④The Finnish Genome Center, University of Helsinki, Helsinki 00085-7008, Finland, and the Departments of Pathology and Human Genetics, University of California, Los Angeles, California 90095-1361

A number of missense mutations in the ATP1A2 gene, which encodes the Na,K-ATPase α2 subunit, have been identified in familial hemiplegic migraine with aura. Loss of function and haploinsufficiency have been the suggested mechanisms in mutants for which functional analysis has been reported. This paper describes a kinetic analysis of mutant T345A, recently identified in a detailed genetic analysis of a large Finnish family (Kaunisto, M. A., Harno, H., Vannolkot, K. R., Gargus, J. J., Sun, G., Hamalainen, E., Liukkonen, E., Kallela, M., van den Maagdenberg, A. M., Frants, R. R., Farkkila, M., Palotie, A., and Wessman, M. (2004) Neurogenetics 5, 141–146). Introducing T345A into the conserved rat α2 enzyme does not alter cell growth or catalytic turnover but causes a substantial decrease in apparent K⁺ affinity (2-fold increase in Kᵢ(K⁺)). In view of the location of Thr-345 in the cytoplasmic stalk domain adjacent to transmembrane segment 4, the 2-fold increase in Kᵢ(K⁺) is probably due to T345A replacement altering K⁺ occlusion/deocclusion. Faster K⁺ deocclusion of the mutant via the E₃(K⁺) + ATP → E₃′ATP + K⁺ partial reaction is evidenced in (i) a marked increase (300%) in K⁺ stimulation of Na₀K₋ATPase at micromolar ATP, (ii) a 4-fold decrease in KᵢATP, and (iii) only a modest increase (∼3-fold) in Iₘ for vanadate, which was used as a probe of the steady state E₂/E₃ conformational equilibrium. We suggest that the decreased apparent K⁺ affinity is the basis for a reduced rate of extracellular K⁺ removal, which delays the recovery phase of nerve impulse transmission in the central nervous system and, thereby, the clinical picture of migraine with aura. This is the first demonstration of a mutation that leads to a disease associated with a kinetically altered but fully functional Na,K-ATPase, refining the molecular mechanism of pathogenesis in familial hemiplegic migraine.

Familial hemiplegic migraine (FHM) is a rare autosomal dominant form of migraine with aura. This disorder is usually associated with hemiparesis and can be accompanied with clinical features ranging from ataxia to epileptic seizures. This genetically heterogeneous disease has been traced to at least two loci, FHМ1 and FHМ2. FHМ1, which accounts for over 50% of all FHМ families, has been traced to chromosome 19p13 and associated with missense mutations in the CACNA1A gene encoding the α1 subunit of the voltage-dependent neuronal (P/Q type) calcium channel. A recent breakthrough in migraine genetics is the discovery of missense mutations in the ATP1A2 gene on chromosome 1q23 that encodes the α2 isoform of the Na,K-ATPase. This finding gives strong support to the notion that FHМ is caused by disruption of normal cation transport. (For a recent update on familial hemiplegic migraine, see Ref. 1.)

The Na,K-ATPase is an integral membrane protein complex that comprises a large catalytic α subunit of ∼110 kDa as well as a smaller, highly glycosylated β subunit that ensures the proper folding and mooring of α in the plasma membrane. This P-type ion pump catalyzes the ATP-driven exchange of intracellular Na⁺ for extracellular K⁺ ions across the plasma membrane of virtually all animal cells, with a stoichiometry of 3:2 for recent reviews, see Refs. 2 and 3). Thus, the sodium pump is essential to the maintenance of the electrochemical alkali cation gradients that are tapped by ion channels in the propagation of action potentials. During the course of its catalytic cycle, this P-type ion pump undergoes phosphorylation and dephosphorylation of a conserved aspartate residue in the active site of its catalytic subunit. This ion pump also undergoes conformational transitions of dephospho- and phospho-enzymes, commonly referred to as E₁ ↔ E₂ and E₁P ↔ E₂P transitions, respectively (see Scheme 1).

At present, four isoforms of α and three isoforms of β have been described. Both α and β are distributed in a tissue- and development-dependent manner. In adult mammals, the α2 isoform is located principally in skeletal muscle, and the brain (in particular glial cells), and to a lesser extent in the heart, adipocytes, and the eye (see Refs. 4–7). We have previously shown that the α2 enzyme differs from the ubiquitous α1 subunit, primarily in the steady state of the E₂/E₃ equilibrium. Thus, compared with α1, the E₂/E₃ poise of α2 is shifted toward E₃, and this shift is associated with an ∼2-fold increase in apparent affinity for ATP, a 50% decrease in

---

*This work was supported by Grant MT-3876 from the Canadian Institutes of Health Research (to R. B.) and NS37675-02 (to A. P.) from the National Institutes of Health and by The Research Funds of the Helsinki University Central Hospital, The Research Foundation of the University of Helsinki, The Juselius Foundation, the Academy of Finland, and a Young Scientist's Award (to A. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†‡§¶** To whom correspondence should be addressed: Montreal General Hospital Research Institute, 1650 Cedar Ave., Montreal, Quebec H3G 1A4, Canada. Tel.: 514-934-1934 (ext. 4501); Fax: 514-934-8332; E-mail: Rhoda.Blostein@mcgill.ca.

1 The abbreviations used are: FHM, familial hemiplegic migraine; WT, wild type; MES, 4-morpholineethanesulfonic acid.
catalytic turnover, a 3-fold increase in the $K^+$ deoclusion rate, and a 20-fold increase in the $I_{50}$ for vanadate inhibition of Na,K-ATPase (8–11).

Since the recent original observation that ATP1A2 alleles are associated with FHM2 (12), an increasing number of mutant alleles of the $\alpha_2$ subunit gene have been identified in families segregating FHM2. However, there have been extremely limited functional studies probing the mechanism by which the proposed pathogenic alleles act. These include L764P and W887R (12), M731T and R689Q (13), and most recently T345A (14), as well as several putative candidates presently under investigation (21). Thus far all alleles are missense, T345A (14), as well as several putative candidates presently under investigation (21). Thus far all alleles are missense, nearly all altering one amino acid in the large catalytic cytosolic loop between M4 and M5. Only the mutant W887R is found outside this domain in the extracellular loop between M7 and M8. No deletions, frameshifts, nonsense, or splice site alleles have been clearly identified. In the first two reported mutations, L764P and W887R, De Fusco et al. (12) implicated altered sodium pump activity and suggest that the disease is the result of haploinsufficiency because the mutant enzyme does not support the growth of cells in culture. Because the gene was not fully sequenced in this report, and because prior studies at 1q-linked FHM2 failed to reveal causal mutations in the gene (15), this functional attribute of the specific mutations was an essential link to ascribing pathogenicity. Although subsequent reports have included complete sequencing of at least all exons to ensure definition of a discrete mutation, none of the other reports of mutant alleles have included any functional analysis, leaving the link between the in vitro pump phenotype and pathogenicity insecure. In initial experiments, we tested the hypothesis that the in vitro growth phenotype would prove a robust means of discriminating the disease-associated alleles by introducing the five aforementioned disease-associated mutations (L764P, W887R, M731T, R689Q, and T345A) into the evolutionarily conserved (~95% identity) rat $\alpha_2$ enzyme and assessing their abilities to support cell growth. Cells bearing mutants L764P and W887R failed to grow, but the other three alleles supported growth, suggesting that either many alleles were missclassified or that the critical aspect of altered pump function remained to be identified.

In this paper we describe a detailed kinetic analysis of the mutant T345A because the previous study (14) identified all of the common polymorphisms in the exons and introns of the gene, showing that only the T345A mutation co-segregated with the disorder in a large family in which clinical data were available on 28 family members; the disorder was not present in 132 healthy Finnish control subjects.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Transfection, and Cell Culture**—The T345A, R689Q, M731T, L764P, and W887R mutations of the rat $\alpha_2$ cDNA were derived from the ouabain-insensitive rat $\alpha_2^*3$ cDNA developed by Jewell and Lingrel (16). Rat $\alpha_2^*$ cDNA was introduced into a modified pBlues vector, and mutations were introduced with the QuikChange mutagenesis kit (Stratagene). Clones containing the correct substitutions were identified by full-length sequencing of the mutated cDNA. The mutant cDNAs were then excised from the shuttle vector with HindIII and ligated into pcDNA3.1 (Invitrogen), and orientation was determined by restriction analysis.

HeLa cells were transfected with the pcDNA-$\alpha_2$ mutant constructs using the LipofectAMINE™ technique (Invitrogen), and cells expressing the relatively ouabain-resistant rat $\alpha_2$ enzymes were selected as described previously (16, 17). HeLa cells expressing the mutant $\alpha_2$ enzymes were amplified in Dulbecco’s modified Eagle’s medium plus 10% newborn calf serum, 100 units/mg penicillin G, 100 $\mu$g/ml streptomycin, and 1 $\mu$m ouabain as described previously (17).

**Growth Curves**—Cells were plated (5000 cells/well), grown in 24-well plates, trypsinized for 10 min at 37°C, and then counted using a hemocytometer.

**Membrane Preparation and Enzyme Assays**—NaCl-treated microsomal membranes were prepared from the mutant cells as described previously (16, 17). To determine maximal Na,K-ATPase activity (as in assays of catalytic turnover) and the effect of inorganic orthovanadate, assays were carried out with final concentrations of 1 mM ATP, 100 mM NaCl, 10 mM KCl, 3 mM MgSO$_4$, 20 mM histidine (pH 7.4), 5 mM EGTA (pH 7.4), and 5 $\mu$m ouabain (Sigma). Base-line hydrolysis was determined using 5 mM ouabain as described previously (17).

**Polyacrylamide Gel Electrophoresis and Western Blotting**—Unless otherwise indicated, transport assays were carried out as described by Munzer et al. (19). Kinetic constants were determined by fitting the data to a simple 1-site Michaelis-Menten model (K$_{ATP}^*$, or to a 2-site (K$_{ATP}^{2,5K}$) or 3-site (K$_{ATP}^{2,5K,5N}$) cooperative model, $v = V_{max}[cat] + K + [cat]^{-1}$) where $n = 2$ or 3 for the cation (cat), either K$^+$ or Na$^+$, respectively. Curve fitting was carried out using the Kaleidagraph computer program (Synergy). All measurements were carried out concurrently on mutant and control (wild-type) $\alpha_2^*$ enzymes, and each assay was performed in triplicate on at least two separate clones.

Unless otherwise indicated, transport assays were carried out as described by Munzer et al. (19). Kinetic constants were determined by fitting the data to a simple 1-site Michaelis-Menten model (K$_{ATP}^*$, or to a 2-site (K$_{ATP}^{2,5K}$) or 3-site (K$_{ATP}^{2,5K,5N}$) cooperative model, $v = V_{max}[cat] + K + [cat]^{-1}$) where $n = 2$ or 3 for the cation (cat), either K$^+$ or Na$^+$, respectively. Curve fitting was carried out using the Kaleidagraph computer program (Synergy). All measurements were carried out concurrently on mutant and control (wild-type) $\alpha_2^*$ enzymes, and each assay was performed in triplicate on at least two separate clones.
**Missense Na,K-ATPase α2 Mutant Causes Hemiplegic Migraine**

**RESULTS**

In initial experiments, we cloned the full-length cDNA of the rat α2 subunit expressing the T345A, R689Q, M731T, L764P, and W887R substitutions. To distinguish the activity of these exogenous pumps from endogenous pumps in the recipient cell line, mutations Q116R and N127D had been introduced in the growth of the cells in 1 mM ouabain, confirming the findings of De Fusco et al. (21) in their mutagenesis study of Na,K-ATPase.

**Growth of the T345A mutant in ouabain** was shown previously by Jewell and Lingrel (16), wild-type (WT) α2*-transfected cells bearing these mutations can be selected, isolated, and grown in medium that contains ouabain added at low concentration (1 μM) to inhibit the endogenous recipient cell enzyme. Following transfection of T345A, R689Q, M731T, L764P, and W887R, neither L764P nor W887R supported the cell enzyme. Following transfection of T345A and the particularly clear identification of this mutation in a 2-fold increase in $K_{0.5}$ (Fig. 2B) and an ~4-fold decrease in $K_{ATP}$ (Fig. 2C). It is noteworthy that the decrease in apparent affinity for $K^+$ seen in unsided membrane preparations is also seen under more physiological conditions prevailing in intact cells. As shown in Fig. 3, the apparent affinity for extracellular $K^+$ is similarly decreased ~2-fold by the T345A mutation. Values of $K_{0.5}$ for α2* which are 0.63 and 1.13 mM for WT α2* and the T345A mutant, respectively (Fig. 3), were doubled when the flux experiments were carried out at 100 mM Na+ (experiment not shown).

**Sensitivity of Na-ATPase Activity**—Because $K^+$ binding/occlusion ($K_{ext} + E_dP → E_d(K + P)$) precedes $K^+$ deocclusion ($E_d(K + ATP) → ATP E_d + K_{cyst}$), a decrease in the latter reaction should be evidenced in an increase in $K^+$ stimulation of Na-ATPase at micromolar ATP concentration with an associated higher apparent affinity for ATP. This is indeed the case, as seen in Fig. 4; the associated decrease in $K_{ATP}$ was shown above (Fig. 2C). At 1 μM ATP, the maximal $K^+$ stimulation of Na-ATPase of T345A is near 300% compared with 120% for α2*. It is noteworthy that this ~2.5-fold increase in the percent stimulation is close to that estimated from a simple Michaelis-Menten model, where the ~4-fold decrease in $K_{ATP}$ of T345A compared with the WT α2* (Fig. 2C) should affect a similar increase in $E_d(K) → E_1$.

**Sensitivity to Vanadate**—To determine whether the relatively faster $E_d(K) → E_1$ sequence of T345A results in a shift in steady-state $E_d/E_{d0}$ poise toward $E_1$, we compared the vanadate sensitivities of the WT and T345A mutants. Inorganic orthovanadate is a transition-state analog of inorganic phosphate that binds to P-type ATPases in the $E_1$ conformation during steady state catalysis. Thus, Na,K-ATPase sensitivity to inhibition by vanadate is a measure of the proportion of enzyme in the $E_1$ state. In the representative experiment shown in Fig. 5, $I_{50}$ (μM) values were 6.40 ± 0.75 μM and 20.09 ± 0.40 μM for α2* and α2T345A, respectively. Because this shift is relatively small, it is not surprising that the T345A mutation did not significantly affect catalytic turnover measured as either the ratio of $V_{max}/E_{max}$ (not shown) or the ratio of immunoreactive α2 protein/unit of activity (Fig. 1).

**DISCUSSION**

The identification of ATP1A2 as a causative gene in FH2 is a major discovery in migraine genetics and has underscored the relevance of ion transport dysfunction to the pathophysiology of migraine. An increasing number of missense mutations in ATP1A2 that likely cause FH2 are
being identified, including L764P and W887R (12), R689Q and M731T (13), and most recently D718N, R763H, P979L (21), and T345A (14). Several others await validation (21).

For at least two mutations (L764P and W887R), loss of function and haploinsufficiency are the suggested underlying defects based on a cell growth phenotype. However, because a myriad of critical physiological processes (from cell volume regulation to sodium gradient-dependent transport to the Nernst potentials for Na$^+$ and K$^+$ and their influence upon membrane potentials) all ultimately rely on normal pump function, the pathophysiological mechanism underlying FHM2 remains unresolved. In this study, we performed a detailed kinetic analysis of the T345A mutant pump cycle to reveal qualitative and quantitative changes associated with the disease allele, bringing focus to its reduced affinity for extracellular K$^+$. We also demonstrate that the growth phenotype may not be capable of discriminating many pathogenic alleles. It is clear that the growth rate of T345A-transfected HeLa cells is as high as WT control and that the T345A mutant enzyme is fully active, with no reduction in catalytic turnover ($V_{\text{max}}/E_{\text{Pmax}}$).

The glial cells that abundantly express the Na$^+$/K$^+$ isoform of the sodium pump have the key role in determining the resting extracellular K$^+$ concentration bathing neurons in the brain. This concentration directly alters $E_{\text{K}}$ and thereby the magnitude of neurons’ after-hyperpolarizations and rates of repolarization and pacing (22, 23). Accordingly, the lowered apparent affinity for extracellular K$^+$ affected by the T345A mutation should slow removal of K$^+$ from the extracellular space and thereby slow the recovery from neuronal excitation. It is noteworthy that the $K_{0.5\text{K}}^+$ values at a resting membrane potential of $-70 \text{ mV}$ may be even higher (~35%) (see Fig. 5B in Ref. 24.) than in the depolarized state (the conditions of the present study with porous membranes). The change in K$^+$ handling is thus a likely explanation for the altered cortical spreading depression in migraine associated with aura as hypothesized by Lauritzen (25). We suggest that the decreased removal of extracellular K$^+$ during the recovery phase of nerve impulse transmission is probably the critical manifestation of altered pump function that leads to disease. This may arise because of either a decrease in pump number (L764P and W887R) or a decrease in apparent affinity for extracellular K$^+$ (T345A).
an earlier analysis of the heterozygous α2 knock-out mouse, Moseley et al. (26) had similarly suggested that impaired clearance of extracellular K⁺ resulting from a loss of α2 function affects neuronal excitability in both neurons and glia. Under physiological conditions of high extracellular Na⁺ concentration, the apparent affinity of the T345A mutant for K⁺ is lowered sufficiently (see under “Results”). This delays the normal rate of recovery of extracellular K⁺ from values well above saturation to the resting level (3 mM) following action potential repolarization (27). It is less likely that the primary disease-causing effect of the T345A mutation results from altered subcellular localization or delivery to the plasma membrane rather than the kinetic effect on K⁺ affinity. Our experiments with intact cells indicate that the mutation neither decreases cell growth nor diminishes cell surface expression as evidenced in V_{max} of pump flux measured with intact cells (see legend to Fig. 3).

Decreased activity of α2 affected by either haploinsufficiency or reduced apparent K⁺ affinity may alter the Na⁺ gradient. As proposed by James et al. (28), if the α2 pump and Na⁺/Ca²⁺ exchanger are co-localized in microdomains of the same cell (astrocyte), a localized rise in cell Na⁺ (caused by the decreased K_{0.5K⁺}) would increase intracellular Ca²⁺ because of decreased Na⁺/Ca²⁺ exchange activity. Increased cell Ca²⁺ may, in turn, affect Ca²⁺-dependent processes in regions critical to the pathogenesis of migraine (cf. Ref. 1).

The T345A replacement likely decreases apparent K⁺ affinity directly affecting the cation binding pocket. Although this is a firm, physiological foundation for understanding how this mutation may lead to disease, the challenge for the future is to integrate the consequences of functional pump lesions with the consequences of the large number of FHIM mutations now recognized in the CACNA1A calcium channel gene, both of which produce a nearly identical phenotype in humans.

Acknowledgments—We thank Drs. E. Jewell and J. Lingrel for the α2 cDNA and Dr. Kathleen Sweadner for the gift of Mcb2 monovalent antibodies. We are grateful to Althina Zou for technical help and Dr. Pierre Drapeau for comments on the manuscript.

REFERENCES
1. Estevez, M., and Gardner, K. L. (2004) Hum. Genet. 114, 225–235
2. Kaplan, J. H. (2002) Annu. Rev. Biochem. 71, 513–535
3. Jorgensen, P. L., Kansson, K. O. H., and Karlish, S. J. D. (2003) Annu. Rev.

Physiol. 65, 817–849
4. Sweadner, K. J. (1998) Biochim. Biophys. Acta 988, 185–220
5. Moseley, A. E., and Kwan, P. S. (1994) Invest. Ophthalmol. Vis. Sci. 35, 3668–3689
6. Arguello, J. M., Whitis, J., and Lingrel, J. B. (1999) J. Biol. Chem. 274, 6341–6348
7. Lauritzen, M. (1994) J. Biol. Chem. 269, 19115–19120
8. Ransom, C. B., Ransom, B. R., and Sontheimer, H. (2000) J. Physiol. 524, 669–680
9. Sweadner, K. J. (1998) Biochim. Biophys. Acta 1391, 1–18
10. Sweadner, K. J. (2002) Science 297, 1161–1168
11. Segal, L. L., and Blostein, R. (1991) J. Biol. Chem. 266, 16925–16930
12. De Fusco, M., Marconi, R., Silvestri, L., Aterino, L., Rampoldi, L., Margante, L., Ballabio, A., Aragon, P., and Cattori, G. (2003) Nat. Genet. 33, 192–196
13. Ogawa, H., and Toyoshima, C. (2002) J. Biol. Chem. 277, 40915–40920
14. Kaunisto, M., Roks, E., Hottenga, J. J., Terwindt, G. M., Haan, J., Hoefnagels, W. A. B., and Sontheimer, H. (2000) J. Biol. Chem. 275, 36287–36292
15. Sweadner, K. J. (1998) Biochim. Biophys. Acta 1391, 1–18
16. Sweadner, K. J. (2002) Science 297, 1161–1168
17. Arguello, J. M., Whitis, J., and Lingrel, J. B. (1999) J. Biol. Chem. 274, 6341–6348
18. Ransom, C. B., Ransom, B. R., and Sontheimer, H. (2000) J. Physiol. 524, 669–680
19. Sweadner, K. J. (1998) Biochim. Biophys. Acta 1391, 1–18
20. Arguello, J. M., Whitis, J., and Lingrel, J. B. (1999) Arch. Biochem. Biophys. 367, 341–347
21. Ransom, C. B., Ransom, B. R., and Sontheimer, H. (2000) J. Physiol. (Lond.) 524, 427–442
22. Dambrosio, R., Gordon, S. D., and Winn, H. R. (2002) J. Neurophysiol. 88, 870–872
23. Horisberger, J. D., and Khodorov-Hess, S. (2002) J. Physiol. (Lond.) 539, 669–681
24. Lauritzen, M. (1994) J. Biol. Chem. 269, 19115–19120
25. Sweadner, K. J. (1998) Biochim. Biophys. Acta 1391, 1–18
26. Sweadner, K. J. (2002) Science 297, 1161–1168
27. Moseley, A. E., Lieske, S. P., Wetzel, R. K., James, P. F., He, S., Shelly, D. A., Paul, R. J., Hovin, G. P., Witte, D. P., Ramirez, J. M., Sweadner, K. J., and Lingrel, J. B. (2003) J. Biol. Chem. 278, 5317–5324
28. Xiong, Z. Q., and Stringer, J. L. (2000) Brain Res. 852, 113–117
29. James, P. J., Grupp, I. L., Grupp, G., Wun, A. L., Ashok, G. R., Creyle, M. L., Walsh, R. A., and Lingrel, J. B. (1999) Mol. Cell 3, 555–563
30. Blostein, R., Dunbar, L., Menke, M., Scanlan, R., Wilczynska, A., and Kaplan, J. H. (1999) J. Biol. Chem. 274, 18574–18581
31. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Nature 405, 647–655
32. Sweadner, K. J., and Donnet, C. (2001) Biochim. Biophys. Acta 1517, 685–704
33. Toyoshima, C., Nomura, H., and Sugita, Y. (2000) J. Biol. Chem. 275, 106–110
34. Ogawa, H., and Toyoshima, C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15977–15982
35. DeLean, A., Manson, P. J., and Rodbard, D. (1978) Am. J. Physiol. 235, E97–E102

Missense Na,K-ATPase α2 Mutant Causes Hemiplegic Migraine