Inhibition of Phosphorylation of Na\(^+\),K\(^+\)-ATPase by Mutations Causing Familial Hemiplegic Migraine*\(^5\)

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**Background:** Familial hemiplegic migraine type II (FHM2) is caused by mutations in the Na\(^+\),K\(^+\)-ATPase \(\alpha\)-isoform.

**Results:** Several FHM2 mutations inhibit phosphorylation or dephosphorylation.

**Conclusion:** These mutations cause FHM2 by local and long range effects on the catalytic site and not by reducing the affinity for external K\(^+\).

**Significance:** Insights into the pathophysiological mechanism of FHM2 and the molecular mechanism of the Na\(^+\),K\(^+\)-ATPase have been obtained.

The neurological disorder familial hemiplegic migraine type II (FHM2) is caused by mutations in the \(\alpha\)-isoform of the Na\(^+\),K\(^+\)-ATPase. We have studied the partial reaction steps of the Na\(^+\),K\(^+\)-pump cycle in nine FHM2 mutants retaining overall activity at a level still compatible with cell growth. Although it is believed that the pathophysiology of FHM2 results from reduced extracellular K\(^+\) clearance and/or changes in Na\(^+\),K\(^+\)-dependent transport processes in neuroglia, a reduced affinity for K\(^+\) or Na\(^+\) is not a general finding with the FHM2 mutants. Six of the FHM2 mutations markedly affect the maximal rate of phosphorylation from ATP leading to inhibition by intracellular K\(^+\), thereby likely compromising pump function under physiological conditions. In mutants R593W, V628M, and M731T, the defective phosphorylation is caused by local perturbations within the Rossmann fold, possibly interfering with the bending of the \(\beta\)-domain during phosphoryl transfer. In mutants V138A, T345A, and R834Q, long range effects reaching from as far away as the M2 transmembrane helix perturb the function of the catalytic site. Mutant E700K exhibits a reduced rate of \(E_P\) dephosphorylation without effect on phosphorylation from ATP. An extremely reduced vanadate affinity of this mutant indicates that the slow dephosphorylation reflects a destabilization of the phosphorylation transition state. This seems to be caused by insertion of the lysine between two other positively charged residues of the Rossmann fold. In mutants R202Q and T263M, effects on the A-domain structure are responsible for a reduced rate of the \(E_P\) to \(E_P^\text{2P}\) transition.

Hemiplegic migraine is a severe subtype of migraine with aura associated with transient motor weakness and sensatory as well as speech difficulties. It is autosomally dominantly inherited (familial hemiplegic migraine). Besides mutations in neuronal voltage-gated Ca\(^{2+}\) and Na\(^+\) channels (FHM1 and FHM3), familial hemiplegic migraine has been associated with mutations in the \(\alpha2\)-isoform of the Na\(^+\),K\(^+\)-ATPase (FHM2) (1). The Na\(^+\) and K\(^+\) gradients created across the cell membrane by the Na\(^+\),K\(^+\)-ATPase are of vital importance for cellular function and activities, including generation of action potentials and secondary active transport of ions, nutrients, and neurotransmitters. In the mammalian brain, three different isoforms of the catalytic \(\alpha\)-subunit are expressed, \(\alpha1\), \(\alpha2\), and \(\alpha3\). The \(\alpha2\)-isoform is mainly distributed in glial cells, whereas the \(\alpha3\)-isoform is found in neurons and is absent in glial cells (2). So far, we know of more than 50 Na\(^+\),K\(^+\)-ATPase \(\alpha2\) mutations associated with hemiplegic migraine. It has been suggested that the pathophysiology results from an impaired clearance of extracellular K\(^+\), producing a wide cortical depolarization (3, 4). Moreover, the gradients of both Na\(^+\) and K\(^+\) are important for the reuptake of the excitatory transmitter glutamate from the synaptic cleft via the glutamate transporter, and impaired Na\(^+\) transport would also lead to a rise of intracellular Ca\(^{2+}\) via the Na\(^+\)/Ca\(^{2+}\) exchanger with secondary effects on Ca\(^{2+}\) signaling (1, 5, 6). Cell survival studies of transfected cells, in which the wild type has been inhibited by ouabain, have demonstrated the inability of certain FHM2 mutants to sustain cell growth in accordance with the theory of haploinsufficiency, i.e. only the wild type allele encodes a functional enzyme (1, 7). Other FHM2 mutants have nevertheless been found to retain transport function (4, 8–11), and more functional mutants exist, as indicated by the present results. A crucial question is therefore whether and how their function deviates from that of the wild type. One study has reported a 2-fold reduced apparent K\(^+\) affinity for activation of the ATPase reaction of mutant T345A, which was suggested to give rise to a reduced rate of external K\(^+\) clearance in vivo (4).

To fully understand the functional implications of the FHM2 mutations and the structural basis, it is necessary to reveal the mutational effects on the individual partial reaction steps of the pump cycle and the interaction with Na\(^+\) and K\(^+\) and to try to relate the observed effects to known structural features. We present here the functional consequences of nine Na\(^+\),K\(^+\)-ATPase \(\alpha2\) mutations, including T345A, which all have been found in patients exhibiting the characteristic symptoms of hemiplegic migraine (12–17). The \(\alpha\)-subunit carrying the

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\(^{1}\) This article contains supplemental Table S1.

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**Na⁺,K⁺-ATPase Mutations Causing Familial Hemiplegic Migraine**

FIGURE 1. **Na⁺,K⁺-ATPase structure with indication of the FHM2 mutations studied.** The structure shown has Protein Data Bank code 2ZXE (E[K₆]) with bound MgF₄ as phosphate analog (19). Color codes for P, A, N, and M-domain are gray, green, yellow, and cyan, respectively. The bound K⁺ ions are shown as purple spheres and MgF₄ (MgF) in the catalytic site as one green sphere with four cyan spheres. The mutated residues (numbered according to the human α2-isof orm) are highlighted as sticks colored according to the elements (carbon, gray; oxygen, red; nitrogen, blue; sulfur, yellow).

EXPERIMENTAL PROCEDURES

FHM2 mutations were introduced into full-length cDNA encoding an ouabain-resistant version of the human α2-isof orm (4). Mutants and wild type were expressed in COS-1 cells under ouabain selection pressure (20). The plasma membrane fraction was isolated and made leaky to allow access of incubation media from both sides of the membrane, and Na⁺,K⁺-ATPase activity was studied at 37 °C by following the liberation of Pi (21, 22). Measurements of phosphorylation and dephosphorylation were performed using either a manual mixing technique at 0 °C or a QFM-5 quench-flow module (BioLogic Instruments, Claix, France), allowing transient kinetic studies at 25 °C (21, 22). To eliminate the contribution of the endogenous Na⁺,K⁺-ATPase, ouabain was included in the reaction media (21). Data processing was performed using SigmaPlot (SPSS, Inc.) for linear regression analysis (21), and the results are reported as average values ± S.E. (shown by error bars in figures when larger than the size of the symbols). The number of independent determinations is indicated by n in Tables 1 and 2. Structural figures were prepared using PyMOL.

RESULTS

**Overall Function**—The nine FHM2 mutations were introduced into the ouabain-resistant version of the human α2-isof orm, and the mutants were expressed in mammalian COS-1 cells and subjected to ouabain-selection pressure (20), taking advantage of the >100-fold difference between the ouabain affinities of the exogenous Na⁺,K⁺-ATPase and the endogenous COS cell enzyme. Like the wild type, all mutants were able
to confer ouabain resistance in growth medium containing 5 μM ouabain, indicating that the rate of transport of Na⁺ and K⁺ is sufficiently high to sustain cell growth. Determination of the ouabain dependence of Na⁺,K⁺-ATPase activity showed 2- to 9-fold reduction of the apparent affinity for ouabain for mutants V138A, T263M, T345A, R593W, V628M, M731T, and R834Q, wild-type-like apparent affinity for R202Q, and 2-fold increased apparent affinity for E700K (Table 1). Hence, all the functional studies described below were carried out in the presence of ouabain to selectively inhibit the endogenous Na⁺,K⁺-ATPase (Kₐ₉₅ = 1 μM).

The catalytic turnover rate determined in the presence of 130 mM Na⁺, 20 mM K⁺, and a saturating concentration of MgATP was found reduced in all mutants, most severely for R593W, V628M, M731T, and R834Q, displaying less than one-third the turnover rate of the wild type. T263M, T345A, E700K, and V138A displayed a turnover rate around 50%, whereas R202Q showed ~20% reduction (Table 1).

**K⁺ Interaction**—A critical issue is whether the affinity for external K⁺ is lowered, thereby resulting in reduced ability to clear K⁺ from the extracellular space. The interaction with K⁺ was investigated by determining the K⁺ dependence of ATPase activity (Fig. 2). In the wild type, K⁺ at a submillimolar concentration activates ATP hydrolysis by binding at extracellularly facing sites of the E₂P form, thereby stimulating dephosphorylation (cf. Scheme 1, Reaction 6). The majority of the mutants displayed a K₀,₅ value for K⁺ activation similar to or lower (i.e., corresponding to higher affinity) than that determined for the wild type, with E700K being the only exception, exhibiting a slight 1.6-fold increase in the K₀,₅ value for K⁺ activation relative to wild type (Fig. 2 and Table 1). K⁺ concentrations above 15 mM inhibited the wild type somewhat, which is explained by K⁺ binding in competition with Na⁺ at the cytoplasmically facing sites of the enzyme in E₁ conformation, leading to conversion of E₁ back to the K⁺-occluded E₂ state (cf. Scheme 1, Reaction 1) (23, 24).

### TABLE 1
ATPase activity parameters

|                        | Ouabain-affinity* | Turnover ratea | Kₐ₉₅(K⁺)b | Kₐ₉₅(VO₄)³⁻b | Kₐ₉₅(ATP)b |
|------------------------|-------------------|----------------|-----------|---------------|------------|
| Wild type a2           | 297 ± 29          | 6843 ± 380     | 716 ± 19  | 27 ± 2        | 104 ± 6    |
| V138A                  | 693 ± 38          | 3768 ± 157     | 763 ± 18  | 290 ± 18      | 47 ± 2     |
| R202Q                  | 288 ± 40          | 5420 ± 277     | 748 ± 33  | 113 ± 6       | 67 ± 4     |
| T263M                  | 1323 ± 95         | 3233 ± 168     | 465 ± 19  | 73 ± 4        | 21 ± 1     |
| T345A                  | 657 ± 48          | 3419 ± 50      | 747 ± 20  | 283 ± 21      | 31 ± 2     |
| R593W                  | 2409 ± 518        | 1029 ± 56      | 263 ± 35  | >1000         | 19 ± 1     |
| V628M                  | 1282 ± 206        | 1885 ± 63      | 273 ± 15  | >1000         | 71 ± 6     |
| E700K                  | 146 ± 23          | 3669 ± 229     | 1145 ± 36 | >1000         | 28 ± 1     |
| M731T                  | 2708 ± 597        | 1238 ± 84      | 216 ± 9   | 778 ± 14      | 26 ± 1     |
| R834Q                  | 2134 ± 534        | 1236 ± 46      | 332 ± 21  | >1000         | 24 ± 2     |

*The rate of ATP hydrolysis was determined at 37 °C in the presence of 130 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM MgCl₂, 30 mM histidine buffer (pH 7.4), 1 mM EGTA, and various concentrations of ouabain. The data points were fitted using a function with the ouabain-inhibited enzyme represented by the sum of two hyperbolic components corresponding to the exogenous enzyme (affinity constant Kᵢ) and the endogenous COS cell enzyme (affinity constant Kₛ ≈ 1 μM), respectively (24): Vₕmax = 1 – aₒ[ouabain]/(Kₒ + [ouabain]) – aₛ[ouabain]/(Kₛ + [ouabain]). The values determined for Kₒ are indicated in the table.

The Na⁺,K⁺-ATPase activity was determined at 37 °C in the presence of 30 mM histidine buffer (pH 7.4), 130 mM NaCl, 3 mM ATP, 3 mM MgCl₂, 1 mM EGTA, ouabain to inhibit the endogenous enzyme, and 20 mM KCl. The catalytic turnover rate was calculated as the ratio between the Na⁺/H⁻-ATPase activity (maximum phosphorylation from [γ-³²P]ATP measured at 0 °C in the presence of 150 mM NaCl and oligomycin) (21, 22).

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FIGURE 2. K⁺ dependence of Na⁺,K⁺-ATPase activity. ATPase activity was measured at 37 °C in 40 mM NaCl, 3 mM ATP, 3 mM MgCl₂, 30 mM histidine (pH 7.4), 1 mM EGTA, 10 μM ouabain, and K⁺ concentrations as indicated. K₀,₅ values for K⁺ activation are listed in Table 1.
Because the variable inhibitory phase in the K\(^+\) dependence of ATPase activity inevitably will influence the apparent affinity for K\(^+\) activation corresponding to the rising phase, the activation by extracellular K\(^+\) was also examined more directly by determining the rate of E\(_1\)P dephosphorylation (Fig. 3 and Table 2). At a nonsaturating K\(^+\) concentration of 1 mM (Fig. 3, open symbols), T345A, R593W, V628M, M731T, and R834Q displayed a dephosphorylation rate constant almost identical to that of the wild type, indicating that these mutations did not weaken the interaction with extracellular K\(^+\). R202Q exhibited a higher rate constant of dephosphorylation than wild type. By contrast, the dephosphorylation rate constant was reduced for V138A and E700K relative to wild type (1.3- and 2.5-fold, respectively). To determine whether this was related to a reduction in affinity for external K\(^+\), similar experiments were performed at a saturating K\(^+\) concentration of 20 mM (Fig. 3, filled symbols). The calculated ratios between the dephosphorylation rate constants at 1 and 20 mM K\(^+\) are 0.29 \(\pm\) 0.02, 0.42 \(\pm\) 0.04, and 0.32 \(\pm\) 0.02 for V138A, E700K, and wild type, respectively. Hence, at 1 mM K\(^+\), the saturation of the external sites is similar to wild type for V138A and is actually higher for E700K, indicating that the reduced dephosphorylation rate is caused by a lower \(V_{\text{max}}\) of phosphoenzyme hydrolysis rather than a lower affinity for K\(^+\).

**Na\(^+\)-Interaction**—Phosphorylation from ATP is triggered when three Na\(^+\) ions have bound to intracellularly facing high affinity sites of the E\(_1\) form and become occluded in the E\(_1\)[Na\(_3\)] form (Scheme 1, Reaction 2) (25). To assess the Na\(^+\) affinity of the E\(_1\) form of the mutants, we studied the Na\(^+\) dependence of phosphorylation from ATP in the absence of K\(^+\) and presence of oligomycin to support occlusion (Fig. 4). The mutants displayed affinities similar to that obtained for the wild type; only R834Q exhibited a more than 2-fold reduced apparent affinity for Na\(^+\) relative to wild type.

**Rate of Phosphorylation**—Because oligomycin stabilizes the Na\(^+\)-occluded E\(_1\)[Na\(_3\)] form, thereby promoting phosphorylation (25, 26) and blocking the E\(_1\)P-E\(_1\)P transition (Scheme 1, Reaction 4), the presence of oligomycin enables the phosphoenzyme to build up a maximum level. Table 2 lists the level of phosphoenzyme accumulated at 150 mM NaCl in the absence of oligomycin relative to the maximum level obtained in the presence of oligomycin (\(EP/EP_{\text{oligo}}\)) for mutants V138A, T345A, R593W, V628M, M731T, and R834Q. Mutant R593W displayed a markedly reduced \(EP/EP_{\text{oligo}}\) ratio, and V138A and T345A showed a slight reduction relative to wild type, whereas T263M and E700K displayed higher \(EP/EP_{\text{oligo}}\) ratios than the wild type. Such effects can be due to changes in either the rate of phosphorylation or the rate of dephosphorylation. In the presence of oligomycin, the level of phosphoenzyme built up was sufficiently high to allow rapid kinetic studies of the rate of phosphorylation at a millisecond time scale at 25°C, and thus the time course of phosphorylation in the presence of 2 mM ATP was determined (Fig. 5). Mutants V138A, T345A, R593W, V628M, M731T, and R834Q all displayed a reduced rate constant relative to wild type, most pronounced for M731T, suggesting that either the phosphorylation reaction or the binding of ATP is affected in these mutants. To determine the maximal rate of phosphorylation and the apparent affinity of these mutants for ATP we performed the analysis at varying ATP concentrations. E700K was included, because the dephosphorylation studies (Fig. 3) indicated that the catalytic site might be defective in this mutant. In Fig. 6, the results are depicted as double-reciprocal plots of the initial phosphorylation rate per ATPase molecule as a function of the concentration of ATP. The linear dependence allows extraction of the maximal phosphorylation rate per ATPase molecule, \(V_{\text{max}}\), and the apparent affinity for ATP (the Michaelis constant \(k_m\)). M731T exhibited a most severe 6-fold reduction of \(V_{\text{max}}\) relative to wild type. Mutants V138A, T345A, R593W, V628M, and R834Q showed 2–3-fold reduction, whereas E700K was wild type-like (Table 2). None of the mutations increased the


**TABLE 2**

Phosphorylation parameters

| $K_{m}$ (Na$^{+}$) | $EP/EP_{0}$ | $k_{phos}$, 2 μM ATP | $V_{max}$, μM | $E_{P}$ | Rate of $E_{P}$ dephosphorylation$^{b}$ |
|-------------------|-------------|----------------------|---------------|--------|-------------------------------------|
| $\mu$M          | %           | $s^{-1}$             | $s^{-1}$      | $s^{-1}$ |
| Wild type α2     | 538 ± 15    | 76 ± 2               | 22 ± 1        | 83 ± 10 | 6.7 ± 0.8                          |
| V138A            | 807 ± 25    | 56 ± 3               | 12 ± 1        | 45 ± 8  | 5.1 ± 1.0                          |
| R202Q            | 541 ± 13    | 76 ± 3               | 17 ± 2        | ND     | ND                                  |
| T263M            | 633 ± 20    | 85 ± 5               | 23 ± 1        | ND     | ND                                  |
| T345A            | 814 ± 34    | 59 ± 3               | 13 ± 1        | 39 ± 2  | 4.5 ± 0.2                          |
| R593W            | 694 ± 23    | 16 ± 2               | 10 ± 1        | 28 ± 10 | 5.1 ± 2.0                          |
| V628M            | 890 ± 29    | 18 ± 2               | 12 ± 1        | 45 ± 11 | 5.9 ± 1.5                          |
| E700K            | 621 ± 19    | 85 ± 2               | 20 ± 1        | 69 ± 4  | 5.3 ± 0.3                          |
| M731T            | 218 ± 10    | 30 ± 1               | 5 ± 1         | 14 ± 3  | 3.0 ± 0.7                          |
| R834Q            | 2496 ± 89   | 21 ± 4               | 15 ± 1        | 35 ± 13 | 5.9 ± 2.3                          |

$^{a}$ Extracted from the data in Fig. 4.

$^{b}$ Ratio between phosphorylation levels without (EP) and with oligomycin (EP$_{0}$). Phosphorylation was carried out for 10 s at 0°C in 20 mM Tris (pH 7.5), 3 mM MgCl$_2$, 2 μM [γ-32P]ATP, 10 μM okazaki, 150 mM NaCl, and with 20 μg of oligomycin/ml.

$^{c}$ Extracted from the data in Figs. 5 and 6. The kinetic parameters were not determined (ND) for R202Q and T263M, because the phosphorylation rate at 2 μM ATP was close to wild type.

$^{d}$ Extracted from the data in Fig. 7.

$^{e}$ Extracted from the data in Fig. 3. For T263M, the $E_{P}$-P dephosphorylation rate constant was not determined (ND), because the high extent of accumulation of $E_{P}$, even at 25°C, precluded such a determination. The dephosphorylation rate at 20 mM K$^{+}$ was determined only when the dephosphorylation at 1 mM K$^{+}$ was slower than that of the wild type.

$K_{m}$ value. A significant reduction of $K_{m}$ is noted for M731T (Fig. 6 and Table 2), likely reflecting the marked reduction of $V_{max}$, which leads to increased accumulation of nonphosphorylated enzyme with ATP bound. According to the equation $K_{m} = (k_{-1} + k_{2})/k_{1}$, where $k_{1}$ and $k_{-1}$ are the respective rate constants for binding and dissociation of ATP, and $k_{2}$ equals $V_{max}$ (measured in units of rate per ATPase molecule), the $K_{m}$ value will decrease with $k_{2}$, unless $k_{-1}$ is much higher than the $k_{2}$ value, which is not the case here, because Na$^{+}$,K$^{+}$-ATPase binds the nucleotide rather tightly (27). In an analogous way, the accumulation of $E_{P}$ explains the increased apparent affinity for Na$^{+}$ in M731T (Table 2).

**$E_{P}$-$E_{P}$-P Distribution**—The phosphorylated form of the Na$^{+}$,K$^{+}$-ATPase exists in $E_{P}$ and $E_{P}$-P states that can be distinguished by their different sensitivities to K$^{+}$ and ADP (Scheme 1). To estimate the extent of each phosphoenzyme pool present at steady state, the dephosphorylation time course was examined following addition of ADP to the phosphoenzyme. A biexponential function could be fitted to the data, permitting extraction of a rapid and a slow decay component, reflecting the initial amounts of $E_{P}$ and $E_{P}$-P, respectively (Fig. 7). Table 2 lists the $E_{P}$ fraction. Notably, mutant T263M displayed a marked increase in the level of $E_{P}$-P (to 96% versus 44% for the wild type). The other mutants displayed a less pronounced shift of the $E_{P}$-$E_{P}$-P distribution toward the $E_{P}$ form or were wild type-like.

**Vanadate and ATP Affinities**—Vanadate is an inhibitor that acts as analog of the phosphoryl group in the transition state during ATP hydrolysis and binds exclusively to the enzyme in the $E_{2}$ conformation (28). The properties of the $E_{2}$ state were investigated by determining the apparent affinity for vanadate inhibition of ATP hydrolysis (Fig. 8). All the mutants exhibited a reduced apparent affinity for vanadate (Table 1). For R593W, V628M, E700K, M731T, and R834Q, the affinity was extremely low (≥30-fold reduced relative to wild type); in fact, no significant inhibition was seen for E700K within the vanadate concentration range examined (Fig. 8, right panel). A reduced sensitivity to vanadate inhibition can in principle arise from either a lowering of the intrinsic binding affinity of $E_{2}$ (possibly reflecting destabilization of the transition state of $E_{P}$-P hydrolysis) or from a shift of the $E_{1}$-$E_{2}$ distribution away from the vanadate binding $E_{2}$ form in favor of $E_{1}$. To examine the mutational effects on the $E_{1}$-$E_{2}$ distribution, the ATP dependence of ATPase activity was also studied (Fig. 9). Because ATP binds with higher affinity to $E_{1}$ than to $E_{2}$, an increase in the apparent affinity for ATP upon mutation reflects a shift of the $E_{1}$-$E_{2}$ distribution toward the $E_{1}$ form, whereas a decrease in the affinity for ATP reflects a shift toward the $E_{2}$ form. The results summarized in Table 1 show a significant increase of the apparent ATP affinity for all the mutants (2–5-fold), indicating a shift toward the $E_{1}$ form. However, for R593W, V628M, E700K, M731T, and R834Q, the effect on ATP affinity was much less spectacular than the reduction of vanadate affinity, indicating that the latter arises not only from a shift in $E_{1}$-$E_{2}$ distribution but also from a change in the intrinsic affinity of the $E_{2}$ form for vanadate.

**DISCUSSION**

The nine FHM2 mutations studied here, which are found in various regions of the Na$^{+}$,K$^{+}$-ATPase α2-isoform, lead to a functionally altered but active enzyme capable of sustaining cell viability. Our results do not lend support to the hypothesis that...
a reduced affinity for external K\(^+\), causing a selective disturbance of K\(^+\) clearance, is an obligatory part of the mechanism underlying the disease (4). Neither can a defective Na\(^+\)/H\(^+\) interaction generally account for the pathophysiology of the disease, as only R834Q exhibited a significant reduction of the Na\(^+\)/H\(^+\) affinity. This is contrary to the neurological disorder rapid-onset dystonia parkinsonism, caused by mutation of the \(\alpha_3\)-isoform of Na\(^+\),K\(^+\)-ATPase, where all mutants characterized so far display markedly reduced Na\(^+\) affinity (21, 29, 30). At saturating Na\(^+\) and K\(^+\) concentrations, the FHM2 mutants studied here...
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A reduced turnover rate has previously been reported for mutants T263M, T345A, M731T, and R834Q. Our rapid kinetic studies of the phosphorylation from ATP identify a reduced \( V_{\text{max}} \) of phosphorylation as a major factor contributing to the reduction of the catalytic turnover rate of mutants V138A, T345A, R593W, V628M, M731T, and R834Q. These mutants displayed decreases in the \( V_{\text{max}} \) of phosphorylation of 2–6-fold (Fig. 6 and Table 2). To be able to carry out transient kinetic measurements, oligomycin was added to promote phosphorylation by stabilizing the Na⁺-occluded \( E_1[\text{Na}_3] \) form and blocking the \( E_i[\text{Na}_3] \rightarrow E_2P \) transition. In the absence of oligomycin, the relative level of phosphoenzyme at steady state was in fact very low for R593W, V628M, M731T, and R834Q (Table 2, “\( E_P/E_P_{\text{oligo}} \)”), suggesting that under physiological conditions without oligomycin the phosphorylation rate is even more dramatically affected than judged from the kinetic data obtained with oligomycin.

The low phosphorylation rate also accounts for the enhanced K⁺ inhibition of ATPase activity at high K⁺ concentrations seen particularly for R593W, V628M, M731T, and R834Q (Fig. 2). Hence, the low phosphorylation rate leads to increased availability of \( E_1 \) at steady state and consequently to enhanced K⁺ competition with Na⁺ at the intracellular \( E_i \) sites. In the living cell with cytoplasmic concentrations of ~150 mM K⁺ and only ~10 mM Na⁺, the enhanced K⁺ competition with Na⁺ may be highly relevant as a factor contributing to compromise pump function.

For R593W, V628M, E700K, M731T, and R834Q, the apparent affinity for vanadate was lowered to an extent that could not be accounted for by the observed shift of the conformational equilibrium toward \( E_1 \) (Fig. 8 and Table 1). Because vanadate binds at the phosphorylation site of the \( E_2 \) form as an analog of the phosphoryl group in the transition state between \( E_2 \) and \( E_2P \), the extraordinary low affinity for vanadate might be related to a perturbation of the phosphorylation site in \( E_2 \) and/or the transition state. For the mutants showing both a reduced \( V_{\text{max}} \) of phosphorylation from ATP in \( E_1 \) and an extraordinary low affinity for vanadate (R593W, V628M, M731T, and R834Q), the underlying structural changes in \( E_i \) and \( E_2 \) forms might be similar. By contrast E700K did not show a reduced phosphorylation rate with ATP, and for this mutant the phosphorylation site therefore seems not to be perturbed in \( E_1 \). Besides its extraordinary lack of sensitivity to vanadate, E700K was characterized by a relatively low maximal rate of dephosphorylation of \( E_2P \) (42 s⁻¹, versus 132 s⁻¹ for wild type), which probably causes the reduced catalytic turnover rate of E700K.

Both the disruption of vanadate binding and the reduced dephosphorylation rate can be explained by assuming that the transition state of \( E_2P \) is destabilized. It is furthermore of note that E700K exhibited an increased apparent affinity for ouabain, and even though E700K did not show reduced affinity for K⁺ activation of dephosphorylation (Fig. 3 and Table 2), a slight 1.6-fold reduction of apparent K⁺ affinity in activation of ATPase activity was noted (Fig. 2 and Table 1). Both of these effects might result from accumulation at steady state of the \( E_1[\text{Na}_3] \) phosphate analog (“\( E_P \)” cf. Refs 32 and 33). Thus, the evidence indicates that mutation E700K is most disturbing in the part of the pump cycle involving \( E_2P \) and \( E_2P \)-like states.

From the crystal structure it is known how the residues studied here are positioned in the \( E_2[\text{K}_2] \) state with bound MgF₄⁻ as a phosphate analog (18, 19), and although crystal structures are static snapshots without the flexibility of the native enzyme, they are useful as a basis for analysis of the mechanisms under-
lying the observed mutational effects. Arg⁵⁹³, Val⁶²⁸, Glu⁷⁰⁰, and Met⁷³¹ are located in the Rossmann fold, which is a central feature of the P-domain, consisting of a seven-stranded parallel β-sheet associated with seven short α-helices (P1–P7) as indicated in Fig. 10. Asp⁷⁷⁴ (phosphorylation site) and residues involved in Mg²⁺ binding, Thr³⁷⁶, Asp⁷¹⁴, and Asp⁷¹⁸ are located centrally in the Rossmann fold (Fig. 10). The phosphorylation of Asp⁷⁷⁴ is triggered by an approach between the N-terminal end of the P3 helix in the Rossmann fold. It is part of a hydrophobic/van der Waals interaction network (Fig. 10, A and C), and the interaction with Pro⁶⁹⁴ and Val⁶⁹⁶ at the N-terminal end of the P2 helix may contribute to stabilize the positions of helices P2 and P3. The V628M mutation might lead to a shift of the position of Pro⁶⁹⁴, thereby disturbing the interactions of the adjacent Arg⁵⁹³ with Gly⁷³⁷ and Thr³⁷⁶ mentioned above.

The glutamate replaced in E700K is located in the P5 helix of the Rossmann fold. In the crystal structure of the Na⁺,K⁺-ATPase E[E₂K₂] state with bound MgF⁴⁻, Glu⁷⁰⁰ is positioned between two positively charged residues, Arg⁷⁰⁴ (at the end of the P5 helix) and Lys⁷²⁴ (P6 helix), with a distance of 4.6 Å to each (Fig. 10A). Moreover, Lys⁷²⁴ is within hydrogen bonding distance to Gln⁷⁰³ in the P5 helix (2.7 Å). It can be imagined that the extra positive charge introduced between Arg⁷⁰⁴ and Lys⁷²⁴ by E700K leads to a repulsion between the three positive charges disturbing the positioning of P5 and P6. Such a disturbance could lead to a shift of β5 and the loop between β5 and P6 containing Asp⁷¹⁴ and Asp⁷¹⁸ critical to Mg²⁺ binding (Fig. 10A). We wondered whether Glu⁷⁰⁰ and either Lys⁷²⁴ or Arg⁷⁰⁴ might actually be closer to each other in the vanadate-bound E₂ state than the 4.6 Å in the E[E₂K₂] crystal structure. The Na⁺,K⁺-ATPase residues Glu⁷⁰⁰, Lys⁷²⁴, and Gln⁷⁰³ are conserved as Glu⁷⁰⁰, Lys⁷²⁴, and Gln⁷⁰³ in the Ca²⁺-ATPase, which has been crystallized not only in the MgF⁴⁻-bound E₂ form...
similar to the Na\textsuperscript{+},K\textsuperscript{+}-ATPase crystal form but also in several other E\textsubscript{2} states, including the AlF\textsubscript{4}\textsuperscript{-}·bound form. The latter is considered the state closest to the E\textsubscript{2}P transition state with vanadate bound, as AlF\textsubscript{4}\textsuperscript{-} and vanadate are both believed to mimic the trigonal bipyramidal structure of the penta-coordinated phosphate in the transition state of E\textsubscript{2}P dephosphorylation (39, 40). Interestingly, among the AlF\textsubscript{4}\textsuperscript{-}-bound E\textsubscript{2} crystal structures of the Ca\textsuperscript{2+}-ATPase, 4 out of 7 have a distance between Glu689 and Lys713 of 4 Å, i.e. short enough to indicate the presence of a salt bridge between these residues in the E\textsubscript{2}P transition state. In contrast, in the MgF\textsubscript{4}\textsuperscript{2-}-bound Ca\textsuperscript{2+}-ATPase structures, the corresponding distance is 4–5 Å, consistent with the distance in the MgF\textsubscript{4}\textsuperscript{2-}-bound form of Na\textsuperscript{+},K\textsuperscript{+}-ATPase (cf. supplemental Table S1). If this scenario for the E\textsubscript{2}P transition state were extrapolated to the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, it would explain the very strong destabilization of the vanadate-bound state of E700K (Fig. 8). If in addition Glu700 and Lys724 were further apart in the E\textsubscript{1}[Na\textsubscript{3}] state, the lack of effect of the mutation on the phosphorylation from ATP would be understandable. The Ca\textsuperscript{2+}-ATPase E\textsubscript{1} structures do not provide a clear answer to this question, but it is noteworthy that 4 out of 8 E\textsubscript{1} structures show a distance larger than 4 Å (cf. supplemental Table S1).

T345A in the cytoplasmic extension of M4 and R834Q in the L6–7 loop are both located at the boundary between the P-domain and the transmembrane region, and the reason that these mutations also reduce the phosphorylation rate significantly may be the participation of Thr345 and Arg834 in interaction networks involving helices connected with the Rossmann fold (Fig. 11). Thr345 might participate in van der Waals interactions with Val362 at the N-terminal end of the P-domain helix P1, from which the central β-strand of the Rossmann fold (β-strand “1” in Fig. 11) leads to the phosphorylation site with Asp374. Interestingly, mutation V362E has also been found in patients with FHM2 (41). Depending on the actual rotational state of the Thr345 side chain, the hydroxyl group might form a hydrogen bond with Glu761 of M5. Mutation R834Q seems to disrupt bonds from P1-helix residues Glu363 and Ser367 (C-terminal end of the P1-helix) to the L6–7 loop (19). R834Q furthermore disrupts the bond between Arg364 and Glu285 of M3 (Fig. 11), which may explain the reduction of Na\textsuperscript{+} affinity caused by R834Q, since mutation of Glu285 has been shown to reduce Na\textsuperscript{+} affinity, possibly a consequence of the involvement of Glu285 in control of the cytoplasmic entrance pathway for Na\textsuperscript{+} (22).
Surprisingly, the mutation V138A also had significant impact on the phosphorylation rate, even though Val138 is positioned in the transmembrane segment M2 far from the phosphorylation site, thus indicating a long range effect, which might be exerted through interference with the hydrophobic/van der Waals interactions between M1 and M2 (cf. Fig. 12A). These interactions seem to allow M1 to close the Na\(^+\) binding pocket through contact with the ion binding Glu132 of M4 (42), a conformational change believed to be propagated to the phosphorylation site in a yet undefined way and result in phosphoryl transfer (35). Indeed, a reduced rate of phosphorylation was previously observed following replacement of M1 residues Leu\(^{94}\) and Gly\(^{97}\) (42, 43).

For R202Q and T263M, the phosphorylation rate was wild type-like, and the reason for the reduced catalytic turnover rate seems to be a slow conversion of \(E_1P\) to \(E_2P\), in particular T263M showed a conspicuous accumulation of \(E_2P\). Furthermore, both R202Q and T263M appeared to shift the \(E_1\)-\(E_2\) distribution of the dephosphoenzyme in favor of \(E_1\). These mutations are both associated with the A-domain, which undergoes drastic structural rearrangements during the \(E_1\)-\(E_2\) and \(E_1P\)-\(E_2P\) transitions (35, 44). Arg\(^{202}\) is positioned in a \(\beta\)-strand of the A-domain, where it appears to form a hydrogen bond with the backbone carbonyl oxygen of Pro\(^{227}\) (2.7 Å distance, Fig. 12B). This bond could be important for stabilization of the loop containing the conserved TGES motif, and hence for interaction of TGES with the P-domain in \(E_2\) and \(E_2P\). Thr\(^{263}\) is located in the AM3 linker segment connecting the A-domain to M3 and appears to be involved in stabilization of the kinked \(\alpha\)-helix present in the AM3 linker in \(E_2\), but not in \(E_1\) (see Fig. 12, C and D). Hence, in \(E_2\) the side chain hydroxyl group of the threonine seems to form a hydrogen bond with the backbone amide nitrogen of Gly\(^{266}\) (2.8 Å distance). This structural arrangement is likely destabilized by the T263M substitution due to the bulkiness of the methionine side chain. The ability of the \(E_1\) conformation to better accommodate the methionine, due to a looser structure compared with the helical arrangement in \(E_2\) (Fig. 12D), explains that the conformational equilibrium is shifted toward \(E_1\) in the mutant. In line with this interpretation, mutation of Gly\(^{266}\) to alanine, disturbing the kinked helix arrangement, was previously shown to cause accumulation of \(E_1\) (45).

To summarize, if disturbance of K\(^+\) clearance by glial cells is the reason for development of FHM2, this disturbance must be attributed to a low maximum turnover rate of the Na\(^+\),K\(^+\)-ATPase and not to a reduced affinity for external K\(^+\). In several of the FHM2 mutants studied here, the function of the catalytic sites is impaired in a yet undefined way.
site in phosphorylation (V138A, T345A, R593W, V628M, M731T, and R834Q) or dephosphorylation (E700K) is affected, involving local effects on the catalytic assembly as well as helices connected with the Rossmann fold, and long range effects transmitted from as far away as the membrane domain. The last two mutations (R202Q and T263M) affect the maximum turnover rate by destabilizing the A-domain in the $E_2/E_2^P$ conformations.

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