Functional consequences of the CAPOS mutation E818K of Na\textsuperscript{+},K\textsuperscript{+}-ATPase

The cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss (CAPOS) syndrome is caused by the single mutation E818K of the α3-isofrom of Na\textsuperscript{+},K\textsuperscript{+}-ATPase. Here, using biochemical and electrophysiological approaches, we examined the functional characteristics of E818K, as well as of E818Q and E818A mutants. We found that these amino acid substitutions reduce the apparent Na\textsuperscript{+} affinity at the cytoplasmic-facing sites of the pump protein and that this effect is more pronounced for the lysine and glutamine substitutions (3–4-fold) than for the alanine substitution. The electrophysiological measurements indicated a more conspicuous, ~30-fold reduction of apparent Na\textsuperscript{+} affinity for the extracellular-facing sites in the CAPOS mutant, which was related to an accelerated transition between the phosphoenzyme intermediates E\textsubscript{1}P and E\textsubscript{2}P. The apparent affinity for K\textsuperscript{+} activation of the ATPase activity was unaffected by these substitutions, suggesting that primarily the Na\textsuperscript{+}-specific site III is affected. Furthermore, the apparent affinities for ATP and vanadate were WT-like in E818K, indicating a normal E\textsubscript{1}–E\textsubscript{2} equilibrium of the dephosphoenzyme. Proton-leak currents were not increased in E818K. However, the CAPOS mutation caused a weaker voltage dependence of the pumping rate and a stronger inhibition by cytoplasmic K\textsuperscript{+} than the WT enzyme, which together with the reduced Na\textsuperscript{+} affinity of the cytoplasmic-facing sites precluded proper pump activation under physiological conditions. The functional deficiencies could be traced to the participation of Glu-818 in an intricate hydrogen-bonding/salt-bridge network, connecting it to key residues involved in Na\textsuperscript{+} interaction at site III.

The Na\textsuperscript{+},K\textsuperscript{+}-ATPase (“Na\textsuperscript{+},K\textsuperscript{+}-pump”) forms and maintains essential gradients for Na\textsuperscript{+} and K\textsuperscript{+} across the plasma membranes of all animal cells (1, 2). It consists of α-, β-, and γ-subunits. Four human genes encode the four isoforms of the α-subunit (α1, α2, α3, and α4), which have distinct functional properties and tissue distribution (3, 4). In 2003 and 2004, it was discovered that the neurological disorders familial hemiplegic migraine and rapid-onset dystonia parkinsonism (RDP)\textsuperscript{2} are associated with mutations in the α2- and α3-isofoms of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, which are expressed in glial and neuronal cells of the brain, respectively (5, 6). Since then, mutations in the α3-isofom have been shown to cause a spectrum of related neurological disorders, including, in addition to RDP, also alternating hemiplegia of childhood (AHC), as well as the CAPOS (cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss) syndrome, early infantile epileptic encephalopathy (EIEE), and relapsing encephalopathy with cerebellar ataxia (RECA). The order of these distinct α3-phenotypes arranged according to severity/disability is as follows: EIEE > AHC > CAPOS > RECA > RDP. However, some of the patients present intermediate phenotypes (7–14). In the present work the focus is on the functional consequences for the Na\textsuperscript{+},K\textsuperscript{+}-ATPase of the mutation causing CAPOS syndrome. The CAPOS syndrome was first described in 1996 (15). Apart from pes cavus, the key signs of the CAPOS syndrome including the optic atrophy and sensorineural hearing loss show a high penetrance. The pes cavus deformity is not always present together with the other symptoms, suggesting the use of the acronym CAOS instead of CAPOS (10). The genetic basis of CAPOS, as well as CAOS, has been identified as a single recurrent missense mutation, E818K. So far, close to 50 CAPOS or CAOS patients with this mutation have been reported (16–22). Two of these patients presented a phenotype described as intermediate/overlapping between AHC and CAPOS (18, 22).

The Na\textsuperscript{+},K\textsuperscript{+}-ATPase α-subunit belongs to the P-type ATPase family of membrane transporters and is made up by three characteristic cytoplasmic domains (phosphorylation, nucleotide binding, and actuator), together with a transmembrane sector consisting of 10 helical segments (M1–M10), forming the binding sites and transport pathway for the three Na\textsuperscript{+} and two K\textsuperscript{+} ions that are moved across the membrane in opposite directions in each ATP hydrolysis cycle (2, 23, 24). The CAPOS mutation E818K is located in the cytoplasmic loop connecting M6 and M7. It is nearest to M6, which contains two aspartates (Asp-801 and Asp-805) participating in the binding of Na\textsuperscript{+} and K\textsuperscript{+}. Glu-818 is furthermore part of a hydrogen-
bonding/salt-bridge network involving M5, which contains other Na\(^+\) - and K\(^+\)-binding residues. Using biochemical and electrophysiological methods we have addressed the functional properties of the CAPOS mutant, as well as two other Glu-818 mutants, E818A and E818N, designed to test the importance of the positive charge introduced by the CAPOS mutation and the role of polarity and size of the substituent. We demonstrate the effects of the CAPOS mutation on the binding of Na\(^+\) and K\(^+\) from both sides of the membrane, as well as on the turnover rates for ATP hydrolysis and pump current and on conformational changes of the enzyme.

**Results**

**Expression and ATPase catalytic turnover rates of Glu-818 mutants**

The three mutations E818K, E818A, and E818Q were introduced in a ouabain-insensitive version of the human α3 isoform of the Na\(^+\),K\(^+\)-ATPase (25), and it was examined whether the mutants could be stably expressed in COS-1 cells under ouabain selection pressure. In this method, the ouabain-sensitive endogenous COS-1 cell Na\(^+\),K\(^+\)-ATPase is silenced by adding ouabain to the cell culture, and the survival of the cells therefore depends on the functional capability of the exogenous mutant enzyme. Like the α3 WT enzyme, all the three mutants were able to support cell growth, allowing isolation of stable transfectants. Hence, it could be concluded that these mutants are expressed in the plasma membrane and transport Na\(^+\) and K\(^+\).

Examination of the Na\(^+\),K\(^+\)-ATPase activity of isolated, leaky membranes in the presence of 130 mM Na\(^+\), 20 mM K\(^+\), and 3 mM ATP, i.e. conditions resulting in maximum activity of the α3 WT, showed turnover rates \(k_{cat}\) of 60, 68, and 62% for mutants E818K, E818A, and E818Q, respectively, as compared with the α3 WT (Fig. 1). In addition we determined the Na\(^+\),K\(^+\)-ATPase activity under ionic conditions (15 mM Na\(^+\) and 130 mM K\(^+\)) corresponding to the intracellular Na\(^+\) and K\(^+\) concentrations in mammalian cells ("physiological turnover”; Fig. 1). ATPase activity requires that Na\(^+\) binds to the E1 form of the enzyme, thereby activating the phosphorylation from ATP, followed by K\(^+\) binding to the E2P form, which activates the dephosphorylation (Scheme 1). K\(^+\) binding to E1 in competition with Na\(^+\) inhibits phosphorylation and thereby ATPase activity. Likewise, the ATPase activity is inhibited by Na\(^+\) binding to E2P in competition with K\(^+\). Because of the high affinity of the K\(^+\) sites on E2P, these sites are saturated with K\(^+\) in the leaky plasma membrane Na\(^+\),K\(^+\)-ATPase preparation at the high K\(^+\) concentration of 130 mM applied here. At the applied Na\(^+\) concentration of 15 mM, Na\(^+\) does not compete efficiently with K\(^+\) for binding at the sites on E2P. Therefore, the turnover rate is limited predominantly by the saturation of the internallly facing sites with Na\(^+\) and the inhibition by K\(^+\) competing with Na\(^+\) for binding at these sites, just as in the physiological situation in cells, the only difference being the absence of a membrane potential (the effect of membrane potential is described below). Under these conditions the pump operates only at a fraction of its maximal capacity, and an increase of intracellular Na\(^+\) will lead to enhancement of the activity (1). It is seen in Fig. 1 that under the ionic conditions mimicking the physiological situation, the mutational effects were larger than seen for the maximal activity conditions. Hence, mutations E818K, E818A, and E818Q resulted in a decrease of the physiological turnover rate to 29, 36, and 32% of the α3 WT, respectively.

**Na\(^+\) and K\(^+\) dependences of ATPase activity and phosphorylation**

The Na\(^+\) dependence of the Na\(^+\),K\(^+\)-ATPase activity of the isolated, leaky membranes examined at a K\(^+\) concentration of
For direct comparison, the dotted line reproduces the data for α3 WT from the upper left panel. The CAPOS mutant E818K together with E818Q showed a substantial reduction of the apparent Na⁺ affinity (3- and 4-fold, respectively), whereas E818A showed a more modest, 2-fold reduction of the apparent Na⁺ affinity, relative to the α3 WT.

Although the apparent Na⁺ affinity for activation of Na⁺,K⁺-ATPase activity reflects the binding of Na⁺ to the cytoplasmic-facing sites on E₁ (Scheme 1, step 2), promoting phosphorylation (Scheme 1, step 3), it is also affected by K⁺, which inhibits ATPase activity by binding to E₁ in competition with Na⁺ without promoting phosphorylation (Scheme 1, step 1). To examine the interaction with Na⁺ in the absence of K⁺, the Na⁺ dependence of phosphorylation was studied using [γ-³²P]ATP. Because of the absence of K⁺, the apparent affinity for Na⁺ determined in the phosphorylation assay is generally much higher than in the ATPase assay. Again, mutations E818K and E818Q caused a notable decrease of the apparent Na⁺ affinity (∼2- and ∼3-fold, respectively), relative to the α3 WT, whereas the effect of E818A was only a slight, 1.3-fold decrease of the apparent Na⁺ affinity (Fig. 3).

The K⁺ concentration dependence of the Na⁺,K⁺-ATPase activity is shown in Fig. 4. These profiles comprise of an activation phase, reflecting stimulation of dephosphorylation through K⁺ binding to the E₆P state (Scheme 1, steps 5 and 6), followed at high K⁺ concentrations by an inhibition phase (very shallow for the α3 WT), which reflects the above-described binding of K⁺ to E₁, in competition with Na⁺. The activation of the CAPOS mutant occurred with a normal, WT-like apparent affinity for K⁺, and the other two Glu-818 mutants exhibited only a slight, ∼1.3-fold change to the apparent K⁺ affinity. However, a pronounced inhibition at high K⁺ concentrations is seen for the CAPOS and the glutamine mutants, whereas this inhibitory phase is much less prominent for the alanine mutant, resembling that of the α3 WT.
CAPOS mutation of Na⁺,K⁺-ATPase

**Figure 5. ATP dependence of Na⁺,K⁺-ATPase activity of the α3 WT and the E818K, E818A, and E818Q mutants.** ATPase activity was measured on isolated, leaky plasma membranes from transfected COS cells at 37 °C in the presence of 30 mM histidine (pH 7.4), 130 mM Na⁺, 20 mM K⁺, 3 mM Mg²⁺, 1 mM EGTA, 10 μM ouabain, and the indicated concentrations of ATP. Multiple sets (n = 4–7) of experiments, each covering the whole concentration range, were carried out, and each data point shown represents the mean ± S.D. The Kₜ₅ was determined for each set of experiments by fitting a Hill function (Equation 1 under “Experimental procedures”), and the mean values are indicated ± S.D. Each line represents the best fit of Equation 1 to all data points. For direct comparison, the dotted line reproduces the data for α3 WT from the upper left panel.

**Figure 6. Vanadate dependence of Na⁺,K⁺-ATPase activity of the α3 WT and the E818K, E818A, and E818Q mutants.** ATPase activity was measured on isolated, leaky plasma membranes from transfected COS cells at 37 °C in the presence of 30 mM histidine (pH 7.4), 130 mM Na⁺, 20 mM K⁺, 3 mM Mg²⁺, 1 mM EGTA, 3 mM ATP, 10 μM ouabain, and the indicated concentrations of vanadate. Multiple sets (n = 3–5) of experiments, each covering the whole concentration range, were carried out, and each data point shown represents the mean ± S.D. Each line represents the best fit of Equation 2 to all data points. For direct comparison, the dotted line reproduces the data for α3 WT from the upper left panel.

**E₁,E₂ distribution assays by ATP and vanadate concentration dependences**

Because the observed reduction of Na⁺ affinity for activation of phosphorylation might be due to a shift of the E₁–E₂ conformational equilibrium away from the Na⁺-binding E₁ form toward E₂, the E₁–E₂ distribution was examined by studying the ATP and vanadate dependences of the Na⁺,K⁺-ATPase activity. ATP binds with high affinity to the E₁ form and with low affinity to E₂, and vanadate binds exclusively to E₂. Hence, a shift of the conformational equilibrium toward E₂ should reduce the apparent affinity for ATP and increase the apparent affinity for vanadate. However, as seen in Figs. 5 and 6, the apparent affinities for ATP and vanadate were WT-like in the E818K and E818Q mutants, thus indicating that these mutations do not disturb the E₁–E₂ equilibrium. The E818A mutant showed a slight increase of the apparent ATP affinity, relative to the WT, i.e., a trend toward an E₁ shift, although not statistically significant (p = 0.064 in one-way analysis of variance test). It can be concluded that none of the mutants showed an E₂ shift that could explain the reduced apparent affinity for Na⁺.

**Electrophysiological characterization of the CAPOS mutant and the α3 WT**

The biochemical experiments described above allow assessment of the Na⁺ binding properties of the sites on the E₁ form that face the cytoplasm in intact cells. The Na⁺-binding properties of the sites in the externally facing conformation of the phosphoenzyme can be addressed in electrophysiological measurements of transient Na⁺ currents. Because the voltage-sensitive step is the Na⁺ ion movement associated with the E₁P–E₂P conformational transition, which occurs before K⁺ binding, the measurements must be carried out in the absence of external K⁺ (26). To this end, mutant E818K and the α3 WT were expressed in Xenopus oocytes, and two-electrode voltage-clamp assays were performed on intact oocytes. Transient currents associated with the exogenous expressed pump were obtained by subtracting records obtained in the presence of 10 mM ouabain from those in its absence for a given superfusion condition (see “Experimental procedures”). Fig. 7 shows the results of such pre–steady-state relaxation measurements in the absence of external K⁺. Transient charge movements, induced by steps in membrane voltage, are associated with release and binding of Na⁺ ions from the external medium (26–28). Voltage steps evoked robust ouabain-sensitive current relaxations, as shown for two representative oocytes expressing the α3 WT and mutant E818K superfused with a K⁺-free solution (Fig. 7A). For the WT, substantial charge was evoked by both depolarizing and hyperpolarizing steps from the holding potential of –40 mV, whereas for E818K, charge movements were more obvious for hyperpolarizing steps. This asymmetrical behavior was also seen for the off steps returning to –40 mV. These pump-related pre–steady-state relaxations comprised a fast and slow component. The resolution of the former was strongly dependent on the speed of voltage clamp and accuracy of the subtraction procedure, and the fast component was not further analyzed in this study. Charge balance between the on and off steps of the main relaxation was confirmed (data not shown), which indicated that the relaxations indeed arose from displacement charges associated with the expressed WT or mutant Na⁺,K⁺-ATPase. The slower relaxation of the ouabain-
sensitive current for the on steps (from −40 mV to the test potential) was fitted with a single exponential and extrapolated to the time of voltage step onset for numerical integration (see “Experimental procedures”). For the α3 WT, the relaxation time constant (τ_on) was strongly voltage-dependent, with a peak close to 0 mV (Fig. 7B). In contrast, for E818K, although its τ_on was similar to the α3 WT at hyperpolarizing potentials, τ_on showed little voltage dependence over the voltage range investigated. Hence, the relaxation reflected by τ_on was faster for E818K than for the α3 WT at all potentials down to −120 mV, suggesting that the Na⁺ ion movement associated with the E₁P–E₂P conformational transition is faster for the mutant. For the corresponding normalized charge–voltage (QV) relationship (Fig. 7C), E818K also deviated markedly from the α3 WT by a large hyperpolarizing shift and no saturation of the charge movement in the hyperpolarizing direction. Fitting the QV data with a Boltzmann function (see “Experimental procedures”) yielded the following parameters: α3 WT ($V_{0.5} = -39.6 \pm 1.2$ mV; $z = 0.74 \pm 0.02$) and E818K ($V_{0.5} = -162 \pm 1$ mV; $z = 0.58 \pm 0.01$) (n = 4, S.E. values as provided by the fitting program). The −120-mV shift of $V_{0.5}$ toward negative voltage caused by the CAPOS mutation corresponds to a ~30-fold reduction of the affinity for extracellular Na⁺, because a shift of 25 mV occurs for each 2-fold change of the external Na⁺ concentration (27).

The altered pre–steady-state kinetics for E818K was also reflected in the normalized ouabain-sensitive steady-state currents detected in the presence of 15 mm external K⁺ (Fig. 8, A and B). As expected, 15 mm K⁺ suppressed the pre–steady-state relaxations for both α3 WT and E818K (compare the initial part of the current traces in Fig. 8A with Fig. 7A), and the magnitude of the steady-state pump current ($I_{\text{pump}}$) induced by the same voltage step was significantly reduced for E818K. Normalization of $I_{\text{pump}}$ to take account of variations in absolute expression levels revealed that its voltage dependence was considerably weaker for E818K compared with the α3 WT (Fig. 8B) and deviated significantly from the WT behavior for hyperpolarizing potentials. We used the estimates of total charge movement ($Q_{\text{max}}$) obtained from Boltzmann fits to the Q–V data associated with the slow relaxation component and slope (z) to determine the number of active pumps for each oocyte. This allowed us to estimate the pump turnover rate, clearly revealing an obvious difference between the mutant and the α3 WT with respect to slope of the voltage dependence (Fig. 8C). At approximately −100 mV their turnover rates were similar (~3 s⁻¹); however, at less hyperpolarizing membrane potentials, E818K showed a markedly weaker voltage dependence with the turnover rate remaining below 5 s⁻¹ up to 0 mV, where it was 38% of the WT (compare this with the 29%
determined for the ATPase physiological turnover rate in the biochemical experiments, where the membrane potential is 0 mV (Fig. 1). At a membrane potential of approximately −70 mV characteristic of neurons under normal physiological conditions, the turnover rate of the mutant was 60% of WT.

In the absence of external Na⁺ and K⁺, the α3 WT displays an inward ouabain-sensitive proton leak current (Iₚᵣᵒᵗ) at strongly hyperpolarizing negative membrane potentials, when the external pH is reduced (29), as reported for other Na⁺,K⁺-ATPase isoforms (30–32). It has been suggested that changes to leak currents in P-type ATPases generally represent a gain-of-function mechanism that may account for dominant-negative effects leading to disease (33). Previously it was demonstrated that the AHC mutation E815K located close to the CAPOS mutation E818K diminishes the proton leak current, suggesting a disturbance of pH regulation and consequent intracellular alkalization as part of the pathophysiology (29). It was therefore of interest to examine the Iₚᵣᵒᵗ in the CAPOS mutant. As shown in Fig. 8D, the Iₚᵣᵒᵗ magnitudes per pump protein were markedly smaller for E818K compared with the α3 WT at hyperpolarizing voltage, although the voltage dependences were very similar as shown by the normalized curves (Fig. 8E). At the resting membrane potential of neurons of approximately −70 mV, there was little difference between Iₚᵣᵒᵗ per pump protein for the mutant and the WT, and at depolarizing voltages the proton leak current was slightly larger (more negative) for the mutant compared with the WT (Fig. 8D).
The CAPOS mutation promotes accumulation of the E₂⁻P form

On the basis of the finding that the proton leak current through α3 Na⁺,K⁺-ATPase WT occurs independently of inhibition of the pump activity with beryllium fluoride, which stabilizes the E₃P form (34), it was previously suggested that E₃P (or an E₃P substate) mediates the proton leak (31). The faster Na⁺ ion movement (E₁P to E₂P) for the CAPOS mutant was inferred from the electrophysiological measurements described above suggests that more E₂P accumulates during the enzyme cycle of the mutant, as compared with the WT. The accumulation of E₂P was examined directly by determining the distribution of the phosphoenzyme between E₃P and E₂P, taking advantage of the ADP sensitivity of E₃P (Fig. 9). Upon addition of ADP to the phosphoenzyme, two clearly distinguishable phases of the dephosphorylation reaction are observed. The rapid phase reflects E₁P, donating the phosphoryl group back to ADP, forming ATP (Scheme 1, step 3), whereas the slow phase reflects the hydrolysis of E₂P (Scheme 1, step 6), which occurs at a rather low rate in the absence of K⁺. The relative amplitude of the slow phase reflects the initial E₂P fraction present after phosphorylation and is seen to be enhanced by the CAPOS mutation (87% in E818K versus 71% in the α3 WT). This observation is in line with a faster release of Na⁺ from the extracellular-facing sites in the mutant and indicates that any reduction of proton leak cannot be due to dephosphorylation of the E₃P form.

Discussion

We have here characterized the functional defects of the CAPOS mutant E818K using a combination of biochemical and electrophysiological measurements, which allowed demonstration of a reduced Na⁺ affinity of the transport sites of the CAPOS mutant in internally as well as externally facing conformations. The Na⁺ dependence of Na⁺,K⁺-ATPase activity and phosphorylation of the CAPOS mutant indicated a 2–3-fold reduction of the affinity for Na⁺ binding from the cytoplasmic side (Figs. 2 and 3). The WT-like ATP and vanadate dependences show that the reduction of the apparent Na⁺ affinity on the cytoplasmic side is not caused by a shift of the E₁–E₂ conformational equilibrium away from the Na⁺-binding E₁ form (Figs. 5 and 6). Hence, it can be concluded that the CAPOS mutation reduces the intrinsic Na⁺ affinity of the E₁ form. A similar effect (3–4-fold reduction of Na⁺ affinity without shift of the E₁–E₂ equilibrium) was observed for the mutant E818Q, thus indicating that the positive charge of the lysine substituent in the CAPOS mutation is not mandatory for the effect, although a smaller reduction of Na⁺ affinity was observed for E818A. In the CAPOS mutant the voltage dependence of transient currents was shifted toward hyperpolarizing voltages (Fig. 7C), indicating a weaker binding of Na⁺ at the extracellular-facing sites of the phosphoenzyme compared with the WT. The magnitude of the shift corresponds to a ~30-fold reduction of the apparent affinity for extracellular Na⁺, i.e. considerably more than observed for the sites in the intracellular-facing conformation. That Na⁺ binding from the extracellular side is perturbed in the CAPOS mutant is also evident from the voltage dependence of the pump turnover rate, which is weaker for the mutant than for the WT (Fig. 8C). It has been proposed that the steep voltage dependence of the WT is due to a high-field access channel in the pump molecule, being part of the transport pathway connecting the Na⁺-binding site(s) with the extracellular medium (35). It is therefore likely that the CAPOS mutation causes a combined defect of a Na⁺-binding site and this release channel toward the extracellular medium, which at present is not well-defined, because crystal structures have not been solved for the various intermediates involved in the extracellular release of Na⁺.

The binding of K⁺ that activates the ATPase activity was not disturbed by the CAPOS mutation and only slightly affected by the other two Glu-818 mutations studied (Fig. 4), indicating minimal effects of the mutation on the two K⁺ sites of the E₃P state that face the external side in intact cells. On the other hand, the inhibition by K⁺ binding in competition with Na⁺ at the cytoplasmic-facing E₁ site(s) was much more pronounced in the CAPOS mutant and E818Q than in the WT. Our data show that such inhibition is substantial under physiological conditions, where the intracellular K⁺ concentration is 120–150 mM and the Na⁺ concentration is rather low, in the range of 10–20 mM. Hence, under ionic conditions mimicking the physiological conditions (15 mM Na⁺ and 130 mM K⁺), the ATPase turnover rate of the CAPOS mutant was only 29% WT as determined for leaky membranes, i.e. in the absence of a membrane potential. From the current measurement in oocytes at zero membrane potential, a value of 38% was obtained, which is in reasonable accordance with the ATPase measurement, taking into consideration that the electrophysiological experiments were conducted at 18–20 °C and the ATPase measurements at 37 °C. In the presence of a membrane potential of approximately –70 mV as normally seen in neurons under resting conditions, the turnover rate of the CAPOS mutant was ~60% WT, because the steeper voltage dependence of the WT tends to reduce the difference between the turnover rates of mutant and WT at negative voltages. However, during action potentials neurons are depolarized, and Na⁺ influx occurs. Both the depolarization and the rise of intracellular Na⁺ stimulate the pump activity of the α3 WT Na⁺,K⁺-ATPase, but because of the weaker voltage dependence and the reduced affinity for cyto-
plasmic Na⁺ of the CAPOS mutant, it is expected to respond with weaker activation than the WT pump in this situation. The stronger K⁺ inhibition of the mutant compared with WT furthermore contributes to counteract the activation of the mutant. Thus, the CAPOS mutant may fail to clear the neuron properly of the accumulated Na⁺ sufficiently rapidly in relation to action potentials, and this defect might be part of the pathophysiological mechanism. Moreover, a rise of internal Ca²⁺ would occur because of slowing of the Na⁺/Ca²⁺ exchanger as a consequence of the rise of intracellular Na⁺. In this context it is important that a rise in intracellular Na⁺ indeed has been shown to occur in connection with mutations reducing the Na⁺ affinity and turnover rate of Na⁺,K⁺-ATPase (25).

A recent electrophysiological study reported an accelerated release of Na⁺ toward the extracellular side in the CAPOS mutant (17), which is in good agreement with the present electrophysiological findings and is further underscored by our finding of an increased E₃/P/E₁,P ratio in the biochemical experiments (Fig. 9). Such an acceleration of the rate-limiting E₁,P to E₃,P transition is unlikely to contribute to the pathophysiology. The reduced affinity for cytoplasmic Na⁺, the weaker voltage dependence, and the stronger K⁺ inhibition of the CAPOS mutant compared with WT, on the other hand, provide important clues to the pathophysiology of the mutant, as explained above. The previous study (17) moreover pointed to an observed increase of the inward proton leak through the CAPOS mutant as a probable disease-causing mechanism. The leak current is also increased by α2 Na⁺,K⁺-ATPase C-terminal mutations causing familial hemiplegic migraine (36), as well as by C-terminal deletions (37, 38), and certain mutations causing aldosterone producing adrenomas (39). An increased leak current might represent a gain of function with dominant-negative effect leading to disease (33), because the leak current in principle could cause depolarization of the neuron, although it has been questioned whether the magnitude of the measured current is sufficient to cause significant cell depolarization (40). In contrast to the reports on an enhanced leak current being associated with disease, the AHC mutation E815K has been found to diminish the proton leak current, and a disturbance of pH regulation and consequent intracellular alkalization might therefore be part of the pathophysiology caused by E815K (29). Hence we were curious about the effect of the nearby E818K CAPOS mutation on leak current. The magnitude of the proton leak current per pump protein was markedly smaller for the CAPOS mutant than for the WT at hyperpolarizing voltage (Fig. 8D). However, because there was no marked difference between the proton leak currents of mutant and WT within the physiological voltage range, the pathophysiology of CAPOS cannot be accounted for by a mutation-induced change of proton leak current.

The functional characteristics of the CAPOS mutant are suggestive of a defect that specifically involves the third, Na⁺-specific Na⁺-binding site (Na⁺ site III) rather than the two other ion transport sites that are able to bind either Na⁺ (in E₁ conformation) or K⁺ (in E₂ conformation), as judged from the WT-like K⁺ activation profile of the mutant (Fig. 4). Hence, because it is the same 10 residues (Val-319, Ala-320, Val-322, Glu-324, Thr-769, Ser-772, Asn-773, Glu-776, Asp-801, and Asp-805; α3 numbering; cf. Fig. 6 of Ref. 14) that make up Na⁺ sites I and II as those making up K⁺ sites I and II, although with difference in the distances to Na⁺ and K⁺ and in the placement of an intervening water molecule, it seems reasonable to assume that no major disturbance of Na⁺ sites I and II has occurred, as long as the apparent K⁺ affinity is WT-like. The CAPOS residue Glu-818 is located in the cytoplasmic loop (L6–7) connecting the transmembrane helices M6 and M7 and cannot be directly involved in Na⁺ coordination or in shaping the extracellular-facing access/release channel. The crystal structure of the α1 enzyme with bound Na⁺ (Fig. 10) provides a useful basis for identifying indirect ways in which mutation of Glu-818 can perturb Na⁺ site III, because the relevant residues are conserved between α1 and α3. First of all, Glu-818 is connected via L6–7 to M6, which contains Asp-805, one of the Na⁺ coordinating residues contributing to Na⁺ site III. Glu-818 is moreover part of a hydrogen-bonding/salt-bridge network involving
M5 and M8, which contain other residues that according to the α1 crystal structure coordinate Na⁺ at site III: Tyr-768, Thr-771, Ser-772, Gln-920, and Asp-923 (numbering here according to α3). Tyr-768 donates not only the aromatic side-chain π-electrons to Na⁺ coordination but also the main-chain carbonyl oxygen (Fig. 10, red arrow). The side-chain oxygens of Glu-818 form bonds to the side-chain nitrogen of Lys-764 of M5 and the two main-chain nitrogens of Arg-930 and Arg-931, which are part of the loop (L8–9) connecting M8 and M9. The Arg-930 side chain interacts with Tyr-1012, which is again connected to M5 through a salt bridge between the C-terminal carboxyl group (Tyr-1013) and Lys-763 of M5 (41). The fact that the disturbance of Na⁺ affinity was more pronounced for the mutants E818K and E818Q than for E818A can be explained by the introduction of a positive charge leading to repulsion, particularly of Lys-764, and the hydrogen-binding potential of glutamine, which will allow the formation of alternative bonds. Taken together, these structural relations provide a reasonable explanation of the observed reduction of the affinity for cytoplasmic Na⁺, whereas a full understanding of the dramatic effect of the CAPOS mutation on the binding of Na⁺ from the extracellular side must await advances in the structural information on the extracellular-facing access/release channel mentioned above.

Mutations to some of the above-mentioned residues that are part of the bonding network of Glu-818 have previously been studied in α1 (41) and found to reduce the apparent affinity for Na⁺ binding from the cytoplasmic side to an extent comparable with that seen for the CAPOS mutation and E818Q. Moreover, these mutations increased K⁺ inhibition to a similar extent as seen for the CAPOS mutation and E818Q, thus underscoring the importance of the interaction network for both Na⁺ binding and K⁺ inhibition. The K⁺ inhibition could be due to a reduced Na⁺ selectivity of site III, allowing K⁺ to bind at this site, which would prevent phosphorylation from ATP and ATPase activity and turn the enzyme into the K⁺-occluded [K₂]E₂ form (Scheme 1). Such a loss of Na⁺ selectivity would expectedly occur if the inclination of M5 is changed by as little as 10°, which would move the main-chain carbonyl oxygen of Tyr-768 (red arrow in Fig. 10) sufficiently away from the ion-coordination sphere to allow for binding of the larger K⁺ ion (24). This change might well occur as a consequence of the perturbation of the bonding network of M5 caused by replacement of Glu-818.

In conclusion, we have shown that the CAPOS mutation selectively reduces the apparent Na⁺ affinity at both the internally and externally accessible cation-binding sites, with the most pronounced effect occurring at the externally facing sites. The reduced Na⁺ affinity at the internal sites together with a weaker voltage dependence and a stronger K⁺ inhibition of the CAPOS mutant compared with WT cause a reduced pump turnover rate under physiological conditions and failure to rapidly regain the resting membrane potential following action potentials, which seems to contribute to the pathophysiology. The symptoms and signs characterizing the CAPOS syndrome are compatible with the abundant expression of the α3-isofrom in the optic nerve, various parts of the cochlea, afferent and efferent nerve fibers innervating the muscle spindles, and cerebellar cortex. Hence, the defect leading to sensorineural hearing loss in CAPOS syndrome has been located to the synapses between the type I afferent terminals and the inner hair cells (20, 42). Our results, moreover, exclude an increase of the proton leak current as contributing to the pathophysiology. However, although the revealed properties of the CAPOS mutant represent a progress in understanding the pathophysiology and amplify our knowledge about the structure–function relationship of the Na⁺,K⁺-ATPase, they do not expose the underlying reason for the different clinical presentation of the CAPOS syndrome compared with other α3 neurological disorders. Among the many known α3 disease mutations leading to low activity of the enzyme, only E818K consistently causes defective hearing. This enigma illustrates the need to identify an additional player on the scene, such as, for example, a cytoplasmic regulatory protein that interacts differently with the E818K mutant than with the WT and other α3 mutants, possibly because of alteration to the L6–7 loop.

**Experimental procedures**

**Biochemical experiments**

Point mutations E818K, E818A, and E818Q were introduced by PCR into full-length cDNA encoding a ouabain-resistant form of the human α3-isoform (ATP1A3 gene) with mutations Q108R and N119D present in the ouabain-binding region (25, 43). The resulting constructs were full-length sequenced to verify the correct point mutation, both before transfection into COS-1 cells and after stable cell lines had been obtained under ouabain-selection pressure (25, 43, 44). No exogenous β-subunit was introduced, because the exogenous α3-subunit associates with the endogenous COS-1 cell β-subunit. The plasma membrane fractions containing the expressed WT or mutants were isolated from the COS-1 cells by differential centrifugation and made leaky by incubation with either sodium deoxycholate or alamethicin to allow access of ATP and ions from both sides of the membrane (44, 45).

The ATPase activity of the enzyme in the isolated leaky membrane was determined at 37 °C by following the liberation of P_i using the method of Baginski et al. (46). The medium composition was 30 mM histidine (pH 7.4), 1 mM EGTA, 3 mM MgCl₂, 10 μM ouabain (inhibiting the endogenous COS-1 cell Na⁺,K⁺-ATPase), and various concentrations of NaCl, KCl, ATP, and vanadate allowing determination of the concentration dependences of these ligands (see figure legends for a detailed description). For background subtraction, similar measurements were carried out in the presence of 10 mM ouabain (inhibiting all Na⁺,K⁺-ATPase activity).

The Na⁺ dependence of phosphorylation was determined by incubating the membranes for 10 s at 0 °C with 2 μM [γ-³²P]ATP in the presence of 20 mM Tris (pH 7.5), 3 mM MgCl₂, 1 mM EGTA, 20 μM oligomycin/ml (to prevent dephosphorylation), 10 μM ouabain, and various concentrations of NaCl (together with N-methyl-D-glucamine maintaining the ionic strength) as indicated in the figure legend. The phosphorylation was quenched with ice-cold 1 M phosphoric acid (pH 2.4) followed by washing of the acid precipitate by centrifugation.
**CAPOS mutation of Na\(^{+}\), K\(^{+}\)-ATPase**

The E\(_P\)–E\(_P\) distribution of the phoshoenzyme was determined by adding 2.5 mm ADP together with 1 mm ATP (non-radioactive) to the [\(\gamma\)-\(^{32}\)P]ATP–phosphorylated enzyme, followed at varying time intervals by quenching. The ADP-sensitive fraction (E\(_P\)) is thereby dephosphorylated rapidly, and the ADP-insensitive fraction (E\(_P\)) disappears slowly by hydrolysis. The phosphorylation with 2 \(\mu\)M [\(\gamma\)-\(^{32}\)P]ATP was in this case performed in medium containing 20 mm Tris (pH 7.5), 20 mm NaCl, 3 mm MgCl\(_2\), 1 mm EGTA, and 10 \(\mu\)M ouabain, i.e. without the oligomycin preventing dephosphorylation. In all phosphorylation experiments, the acid-stable \(^{32}\)P-labeled phoshoenzyme was isolated by SDS-PAGE at pH 6.0, and quantification of the associated radioactivity was obtained by phosphorimaging using a cyclone storage system (PerkinElmer Life Sciences).

The active-site concentration was determined by phosphorylation from [\(\gamma\)-\(^{32}\)P]ATP under stoichiometric conditions, i.e. in the presence of 150 mm NaCl with 20 \(\mu\)g oligomycin/ml to prevent dephosphorylation. For calculation of the catalytic turnover rate, the ATPase activity determined as described above was related to the active site concentration (47).

The SigmaPlot program (SPSS, Inc.) was used to fit the relevant equations to data points using nonlinear regression (48). The Na\(^{+}\) and ATP dependences of the ATPase activity or phosphorylation were fitted by the following Hill function.

\[
A = A_{max} \cdot \left( \frac{[L]^n}{K_{0.5}^n + [L]^n} \right) \quad \text{(Eq. 1)}
\]

The vanadate dependence of the ATPase activity was fitted by representing the inhibitory vanadate binding by a similar Hill function.

\[
A = A_{max} \cdot \left( 1 - \frac{[L]^n}{K_{0.5}^n + [L]^n} \right) \quad \text{(Eq. 2)}
\]

In these equations, \(A\) represents the actual ATPase activity or phosphorylation level at the given ligand (L) concentration, \(A_{max}\) is the maximum value, \(K_{0.5}\) is the ligand concentration giving half-maximum activation or inhibition (apparent affinity), and \(n\) is the Hill coefficient.

The determination of the E\(_1\)P–E\(_2\)P distribution of the phoshoenzyme was based on fitting a double exponential decay function to the dephosphorylation time course.

\[
EP = E_{1P} \cdot \exp(-k_{1t}) + E_{2P} \cdot \exp(-k_{2t}) \quad \text{(Eq. 3)}
\]

EP is the total amount of phoshoenzyme. E\(_{1P}\) and E\(_{2P}\) are the two phoshoenzyme intermediates that are ADP-sensitive and -insensitive, respectively. \(k_{1}\) and \(k_{2}\) are the decay constants for the E\(_1\)P and E\(_{2}\)P phases, respectively.

Data points in the figures are shown as mean \(\pm\) S.D. (\(n = 3–8\)). Note that error bars are only visible when larger than the size of the symbols. The constants determined by fitting are indicated in the figures \(\pm\) S.D.

**Electrophysiology**

The above-described cDNA encoding the ouabain-resistant version of the human WT \(\alpha\)3 and the E818K mutant were used together with the human ATP1B3 cDNA encoding the \(\beta\)3-subunit synthesized by GenScript (Piscataway, NJ). All cDNAs were subcloned into the pGEMHE-mcs vector and were transcribed into capped cDNA *in vitro* (mMessage mMachine, Ambion, TX). cRNA integrity was assessed spectrophotometrically (Nanodrop) and by denaturing gel electrophoresis.

Oocytes were incubated in modified Barth’s solution that contained 88 mm NaCl, 1 mm KCl, 0.41 mm CaCl\(_2\), 0.82 mm MgSO\(_4\), 2.5 mm NaHCO\(_3\), 2 mm Ca(NO\(_3\))\(_2\), 7.5 mm HEPES, adjusted to pH 7.5 with Tris and supplemented with antibiotics doxycycline and gentamicin (5 mg/l). Recording solutions contained 115 mm NaOH, 110 mm sulfamic acid, 10 mm HEPES, 5 mm BaCl\(_2\), 1 mm MgCl\(_2\) and 0.5 mm CaCl\(_2\) (pH 7.5), and 1 \(\mu\)M ouabain (to inhibit endogenous oocyte pump activity). Steady-state pump current was activated by the addition of 15 mm potassium sulfamate. For measuring proton currents, requiring the absence of both Na\(^{+}\) and K\(^{+}\) on the external side, NaOH was replaced by equimolar tetramethylammonium hydroxide, and the pH was adjusted to 6.5. To inhibit both exogenous and endogenous pumps, 10 mm ouabain was added. The oocytes were preincubated for 1 h prior to recording in solution that contained 95 mm NaOH, 90 mm sulfamic acid, 5 mm HEPES, 10 mm TEA-Cl, and 0.1 mm EGTA (pH 7.5).

Mature oocytes (stage V or VI) were obtained from *Xenopus laevis* frogs and prepared as previously described (49). Each oocyte was injected with 15 ng of cRNA encoding the human \(\alpha\)3 subunit and 10 ng of cRNA encoding the human \(\beta\)3 subunit. Total injection volume was 50 nl. The oocytes were stored in modified Barth’s solution at 18 °C for 3 days before recording.

Two electrode voltage-clamp recording was performed using a TEC-10X voltage clamp (NPI, Tamm, Germany). Voltage-clamp control, data acquisition and superfusion valve switching was under software control using pClamp version 8–10 software (Molecular Devices), and data were acquired using a Digidata 1332a (Molecular Devices) acquisition unit. Sampling frequency was 20 kHz, and the recording temperature was 18–20 °C. The oocytes were impaled with electrodes that contained 3 m KCl with a resistance of \(-0.2–0.4\) M\(\Omega\). Voltage dependence was determined by measuring currents during a series of 80-mv steps from \(-40\) mV to test potentials in the range \(-180\) to \(+40\) mV in 20-mV increments every 2 s. The data points are shown as means \(\pm\) S.D. (\(n = 4–5\)).

To estimate the charge moved (Q) for a step from the holding potential to the test potential, the fitted exponential corresponding to the slower component was numerically integrated after the step onset. The Q–V data were fitted with the following Boltzmann function,

\[
Q = Q_{hyp} + Q_{max}/(1 + \exp(ze(V_{0.5} - V)/kT)) \quad \text{(Eq. 4)}
\]

where \(V_{0.5}\) is the voltage at which the charge is distributed equally between two hypothetical states, \(z\) is the apparent valency of an equivalent charge that moves through the whole of the membrane field, \(Q_{max}\) is the total mobile charge available, \(Q_{hyp}\) is the displaced charge at the hyperpolarizing limit, and \(e\), \(k\), and \(T\) have their usual meanings. Curve fitting and data analysis were performed using ClampFit V10 (Molecular Devices).
References
1. Skou, J. C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerve. Biochim. Biophys. Acta 23, 394–401 CrossRef Medline
2. Kaplan, J. H. (2002) Biochemistry of Na,K-ATPase. Annu. Rev. Biochem. 71, 511–535 CrossRef Medline
3. Shull, G. E., Greeb, J., and Lingrel, J. B. (1986) Molecular cloning of three distinct forms of the Na+,K+-ATPase α-subunit from rat brain. Biochemistry 25, 8125–8132 CrossRef Medline
4. Blanco, G., and Mercer, R. W. (1998) Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. Am. J. Physiol. 275, F633–F650 Medline
5. De Fusco, M., Marconi, R., Silvestri, L., Atorino, L., Rampoldi, L., Morante, S., Fiori, S., Ganelin-Cohen, S. E., as provided by the fitting program.
6. de Carvalho Aguiar, P., Swedner, K. J., Penniston, J. T., Zaremba, J., Liu, L., Caton, M., Linazasoro, G., Borg, M., Tijssen, M. A., Bressman, S. B., Dobyns, W. B., Brashear, A., and Ozuelus, L. J. (2004) Mutations in the Na/K-ATPase α3 gene ATP1A3 are associated with familial hemiplegic migraine type 2. Nat. Genet. 33, 192–196 CrossRef Medline
7. Heinzen, E. L., Swooboda, K. J., Hitomi, Y., Gurrieri, F., Nicole, S., de Vries, B., Tiziano, F. D., Fontaine, W., Walley, N. M., Heavins, S., Panagiotakakis, E., European Alternating Hemiplegia of Childhood (AHC) Genetics Consortium, Biobanca e Registro Clinico per l’Emiplegia Alternante (IBAHC) Consortium, European Network for Research on Alternating Hemiplegia (ENRAH) for Small and Medium-sized Enterprises (SMES) Consortium, Fiori, S., et al. (2012) De novo mutations in ATP1A3 cause alternating hemiplegia of childhood. Nat. Genet. 44, 1030–1034 CrossRef Medline
8. Rosewich, H., Thiele, H., Ohlenbusch, A., Maschke, U., Altmüller, J., Frommolt, P., Zinn, B., Ebinger, F., Siehms, H., Nürnberg, P., Brockmann, K., and Gärtringer, J. (2012) Heterozygous de-novo mutations in ATP1A3 in patients with alternating hemiplegia of childhood: a whole-exome sequencing gene-identification study. Lancet Neurol. 11, 764–773 CrossRef Medline
9. Demos, M. K., van Karnebeek, C. D., Ross, C. I., Adam, S., Shen, Y., Zhan, S. H., Shyr, C., Horvath, G., Suri, M., Fryer, A., Jones, S. J., and Friedman, J. M., and FORGE Canada Consortium (2014) A novel recurrent mutation in ATP1A3 causes CAPOS syndrome. Orphanet J. Rare Dis. 9, 15 CrossRef Medline
10. Heimer, G., Sadaka, Y., Israelian, L., Feiglin, A., Ruggieri, A., Marshall, C. R., Scherer, S. W., Ganelin-Cohen, E., Marek-Yagel, D., Tzadok, M., Nissenkorn, A., Anikster, Y., Minassian, B. A., and Zeev, B. B. (2015) CAOS-episodic cerebellar ataxia, areflexia, optic atrophy, and sensorineural hearing loss: A third allelic disorder of the ATP1A3 gene. J. Child. Neurol. 30, 1749–1756 CrossRef Medline
11. Paciorkowski, A. R., McDaniel, S. S., Jansen, L. A., Tully, H., Tuttle, E., Ghoneim, D. H., Tupal, S., Gunter, S. A., Vasta, V., Zhang, Q., Tran, T., Liu, Y. B., Ozelius, L. J., Brashear, A., Swedner, K. J., et al. (2015) Novel mutations in ATP1A3 associated with catastrophic early life epilepsy, episodic prolonged apnea, and postnatal microcephaly. Epilepsia 56, 422–430 CrossRef Medline
12. Dard, R., Mignot, C., Durr, A., Lesca, G., Sanlaville, D., Roze, E., and Mohel, F. (2015) Relapsing encephalopathy with cerebellar ataxia related to an ATP1A3 mutation. Dev. Med. Child Neurol. 57, 1183–1186 CrossRef Medline
13. Panagiotakaki, E., De Grandis, E., Stagnaro, M., Heinen, E. L., Fons, C., Sisodiya, S. D., de Vries, B., Goubau, C., Weckhuysens, S., Kelmink, D., Scheffer, I., Lesca, G., Ribboud, M., Klich, A., Ramirez-Camacho, A., et al. (2015) Clinical profile of patients with ATP1A3 mutations in alternating hemiplegia of childhood—a study of 155 patients. Orphanet J. Rare Dis. 10, 123 CrossRef Medline
14. Holm, R., Toustrup-Jensen, M. S., Einholm, A. P., Schack, V. R., Andersen, J. P., and Vilsen, B. (2016) Neurological disease mutations of α3 Na+,K+-ATPase: Structural and functional perspectives and rescue of compromised function. Biochim. Biophys. Acta 1857, 1807–1828 CrossRef Medline
15. Nicolaidis, P., Appleton, R. E., and Fryer, A. (1996) Cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss (CAPOS): a new syndrome. J. Med. Genet. 33, 419–421 CrossRef Medline
16. Maas, R. P., Schieving, J. H., Schouten, M., Kamsteeg, E. J., and van de Warrenburg, B. P. (2016) The genetic heterogeneity of CAPOS syndrome: Four new patients with the c.2452G>A (p.Glu818Lys) mutation in the ATP1A3 gene. Pediatr. Neurol. 59, 71–75 CrossRef Medline
17. Tanebjerj, L., Strenzke, N., Lindholm, S., Rendtorff, N. D., Poulsen, H., Khandelia, H., Kopeck, W., A. J., Hamel, C., Delettre, C., Bocquet, B., Bille, M., Owen, H. H., Bek, T., Jensen, H., et al. (2018) The CAPOS mutation in ATP1A3 alters Na/K-ATPase function and results in auditory neuropathy which has implications for management. Hum. Genet. 137, 111–127 CrossRef Medline
18. Rosewich, H., Weise, D., Ohlenbusch, A., Gärtner, J., and Brockmann, K. (2014) Phenotypic overlap of alternating hemiplegia of childhood and CAPOS syndrome. Neurology 83, 861–863 CrossRef Medline
19. Duet Rodriguez, A., Prochazkova, M., Santos Santos, S., Rubio Cabetos, O., Cantarín Extevera, V., and Gonzalez-Gutierrez-Solana, L. (2017) Early diagnosis of CAPOS syndrome before acute-onset ataxia: review of the literature and a new family. Pediatr. Neurol. 71, 60–64 CrossRef Medline
20. Han, K. H., Oh, D. Y., Lee, S., Lee, C., Han, J. H., Kim, M. Y., Park, H. R., Park, M. K., Kim, N. K. D., Lee, J., Yi, E., Kim, J. M., Kim, J. W., Chae, J. H., Oh, S. H., et al. (2017) ATP1A3 mutations can cause progressive auditory neuropathy: a new gene of auditory synaptopathy. Sci. Rep. 7, 16504 CrossRef Medline
21. Chang, I. J., Adam, M. P., Jayadev, S., Bird, T. D., Natarajan, N., and Glass, I. A. (2018) Novel pregnancy-triggered episodes of CAPOS syndrome. Am. J. Med. Genet. A 176, 235–240 CrossRef Medline
22. Hayashida, T., Saito, Y., Ishii, A., Hirose, S., Hiraiwa, R., Maegaki, Y., and Ohno, K. (2018) Further characterization of CAPOS/CAOS syndrome with the Glu818Lys mutation in the ATP1A3 gene: A case report. Brain Dev. 40, 576–581 CrossRef Medline
23. Morth, J. P., Pedersen, B. P., Toustrup-Jensen, M. S., Sørensen, T. L., Petersen, J. J., Andersen, J. P., Vilsen, B., and Nissen, P. (2007) Crystal structure of the sodium-potassium pump. Nature 450, 1043–1049 CrossRef Medline
24. Kanai, R., Ogawa, H., Vilsen, B., Cornelius, F., and Toyoshima, C. (2013) Crystal structure of a Na-bound Na,K-ATPase preceding the E1P state. Nature 502, 201–206 CrossRef Medline
25. Toustrup-Jensen, M. S., Einholm, A. P., Schack, V. R., Nielsen, H. N., Holm, R., Sobrido, M. J., Andersen, J. P., Clausen, T., and Vilsen, B. (2014) Relationship between intracellular Na+ concentration and reduced Na+...
affinity in Na\(^+\),K\(^+\)-ATPase mutants causing neurological disease. *J. Biol. Chem.* [289](10.1074/jbc.M109.082857), 3186–3197 CrossRef Medline
26. Nakao, M., and Gadsby, D. C. (1986) Voltage-dependence of Na translocation by the Na/K pump. *Nature* 323, 628–630 CrossRef Medline
27. Rakowski, R. F. (1993) Charge movement by the Na/K pump in Xenopus oocytes. *J. Gen. Physiol.* [110](10.1083/jgp.110.1.17), 117–144 CrossRef Medline
28. Li, C., Capendeguy, O., Geering, K., and Horisberger, J. D. (2005) A third Na\(^+\)-binding site in the sodium pump. *Proc. Natl. Acad. Sci. U.S.A.* [102](10.1073/pnas.1250677102), 12706–12711 CrossRef Medline
29. Li, M., Jazayeri, D., Corry, B., McSweeney, K. M., Heinzen, E. L., Goldstein, D. B., and Petrou, S. (2015) A functional correlate of severity in alternating hemiplegia of childhood. *Nature* [526](10.1038/nature15886), 99–102 CrossRef Medline
30. Wang, X., and Horisberger, J. D. (1995) A conformation of Na\(^+\)-ATPase. *J. Biol. Chem.* [270](10.1074/jbc.270.37.37186), 37186–37197 CrossRef Medline
31. Vedovato, N., and Gadsby, D. C. (2014) Route, mechanism, and implications of proton import during Na\(^+\)/K\(^+\) exchange by native Na\(^+\)/K\(^+\)-ATPase pumps. *J. Gen. Physiol.* [143](10.1083/jgp.201310137), 449–464 CrossRef Medline
32. Mitchell, T. J., Zugarramurdi, C., Olivera, J. F., Gatto, C., and Artigas, P. (2014) Sodium and proton effects on inward proton transport through Na/K pumps. *Biophys. J.* [106](10.1016/j.bpj.2014.02.018), 2555–2565 CrossRef Medline
33. Kaneko, M., Desai, B. S., and Cook, B. (2014) Ionic leakage underlies a gain-of-function effect of dominant disease mutations affecting diverse P-type ATPases. *Nat. Genet.* [46](10.1038/ng.2935), 144–151 CrossRef Medline
34. Cornelius, F., Mahmoud, Y. A., and Toyoshima, C. (2011) Metal fluoride complexes of Na,K-ATPase: characterization of fluoride-stabilized phosphoenzyme analogues and their interaction with cardiotonic steroids. *J. Biol. Chem.* [286](10.1074/jbc.M111.258483), 29882–29892 CrossRef Medline
35. Sagar, A., and Rakowski, R. F. (1994) Access channel model for the voltage dependence of the forward-running Na\(^+\)/K\(^+\) pump. *J. Gen. Physiol.* [103](10.1083/jgp.103.6.869), 869–893 CrossRef Medline
36. Poulsen, H., Khandelia, H., Morth, J. P., Bublitz, M., Mouritsen, O. G., Egebjerg, J., and Nissen, P. (2015) Sodium effect on inward Na\(^+\)-permeability in Xenopus oocytes. *J. Biol. Chem.* [290](10.1074/jbc.M114.572593), 18715–18725 CrossRef Medline
37. McLean, W. J., Smith, K. A., Glowatzki, E., and Pyott, S. J. (2009) Distribution of the Na,K-ATPase \(\alpha\) subunit in the rat spiral ganglion and organ of corti. *J. Assoc. Res. Otolaryngol.* [10](10.1007/s10865-009-0136-6), 37–49 CrossRef Medline
38. Price, E. M., and Lingrel, J. B. (1988) Structure–function relationships in the Na,K-ATPase \(\alpha\) subunit: site-directed mutagenesis of glutamine-111 to arginine and asparagine-122 to aspartic acid generates a ouabain-resistant enzyme. *Biochemistry* [27](10.1021/bi00326a032), 8400–8408 CrossRef Medline
39. Vilsen, B. (1992) Functional consequences of alterations to Pro\(^{328}\) and Leu\(^{332}\) located in the 4th transmembrane segment of the \(\alpha\)-subunit of the rat kidney Na\(^+\),K\(^+\)-ATPase. *FEBS Lett.* [314](10.1016/0014-5793(92)81398-M), 301–307 CrossRef Medline
40. Toustrup-Jensen, M., and Vilsen, B. (2002) Importance of Glu\(^{282}\) in transmembrane segment M3 of the Na\(^+\),K\(^+\)-ATPase for control of cation interaction and conformational changes. *J. Biol. Chem.* [277](10.1074/jbc.200080200), 38607–38617 CrossRef Medline
41. Baginski, E. S., Foa, P. P., and Zak, B. (1967) Microdetermination of inorganic phosphate, phospholipids, and total phosphate in biologic materials. *Clin. Chem.* [13](10.4049/cjcn1967.13.6.326), 326–332 Medline
42. Vilsen, B. (1997) Leucine 332 at the boundary between the fourth transmembrane segment and the cytoplasmic domain of Na\(^+\),K\(^+\)-ATPase plays a pivotal role in the ion translocating conformational changes. *Biochemistry* [36](10.1021/bi970293k), 13312–13324 CrossRef Medline
43. Holm, R., Einholm, A. P., Andersen, J. P., and Vilsen, B. (2015) Rescue of Na\(^+\) affinity in aspartate 928 mutants of Na\(^+\),K\(^+\)-ATPase by secondary mutation of glutamate 314. *J. Biol. Chem.* [290](10.1074/jbc.M115.664569), 9801–9811 CrossRef Medline
44. Petrou, S., Ugur, M., Drummond, R. M., Singer, J. I., and Walsh, J. V. Jr. (1997) P2X7 purinoceptor expression in Xenopus oocytes is not sufficient to produce a pore-forming P2Z-like phenotype. *FEBS Lett.* [411](10.1016/S0014-5793(97)00119-8), 339–345 CrossRef Medline