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Relationship between Intracellular Na^+ Concentration and Reduced Na^+ Affinity in Na^+,K^+-ATPase Mutants Causing Neurological Disease*

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Background: Na^+,K^+-ATPase mutations extending the C terminus cause neurological disease.
Results: C-terminal extension reduces Na^+ affinity. Analysis of several mutants establishes a relationship between change in Na^+ affinity and change of intracellular Na^+ and K^+ concentrations.
Conclusion: The Na^+ affinity of the Na^+,K^+-ATPase is a major *in vivo* determinant of the intracellular Na^+ concentration.
Significance: Insight in pathophysiology and regulation of the Na^+,K^+-ATPase is obtained.

The neurological disorders familial hemiplegic migraine type 2 (FHM2), alternating hemiplegia of childhood (AHC), and rapid-onset dystonia parkinsonism (RDP) are caused by mutations of Na^+,K^+-ATPase α_2 and α_3 isoforms, expressed in glial and neuronal cells, respectively. Although these disorders are distinct, they overlap in phenotypical presentation. Two Na^+,K^+-ATPase mutations, extending the C terminus by either 28 residues ("+28" mutation) or an extra tyrosine ("+Y"), are associated with FHM2 and RDP, respectively. We describe here functional consequences of these and other neurological disease mutations as well as an extension of the C terminus only by a single alanine. The dependence of the mutational effects on the specific α isoform in which the mutation is introduced was furthermore studied. At the cellular level we have characterized the C-terminal extension mutants and other mutants, addressing the question to what extent they cause a change of the intracellular Na^+ and K^+ concentrations ($[Na^+]_i$ and $[K^+]_i$) in COS cells. C-terminal extension mutants generally showed dramatically reduced Na^+ affinity without disturbance of K^+ binding, as did other RDP mutants. No phosphorylation from ATP was observed for the +28 mutation of α_2 despite a high expression level. A significant rise of $[Na^+]_i$ and reduction of $[K^+]_i$ was detected in cells expressing mutants with reduced Na^+ affinity and did not require a concomitant reduction of the maximal catalytic turnover rate or expression level. Moreover, two mutations that increase Na^+ affinity were found to reduce $[Na^+]_i$. It is concluded that the Na^+ affinity of the Na^+,K^+-ATPase is an important determinant of $[Na^+]_i$.

Na^+,K^+-ATPase is a ubiquitous, membrane-bound enzyme responsible for creating vital gradients for Na^+ and K^+ across cell membranes. Mutations in the Na^+,K^+-ATPase have been found associated with neurological disorders. The catalytic α-subunit of the Na^+,K^+-ATPase $\alpha\beta\gamma$ complex occurs in four isoforms (α_1–α_4) that are non-uniformly expressed in a tissue- and development-dependent manner (1, 2). Rapid-onset dystonia parkinsonism (RDP)[3] is a movement disorder characterized by abrupt onset (often triggered by stressful events) of dystonia with signs of parkinsonism. RDP is caused by mutation of the Na^+,K^+-ATPase α_3-isoform, which in the CNS is expressed exclusively in neurons (3, 4). More recently, alternating hemiplegia of childhood (AHC) has also been associated with α_3 mutations, some involving the same residues as in RDP (5, 6). AHC generally has earlier onset than RDP and is characterized by transient episodes of hemiplegia often shifting from one side of the body to the other. Although AHC and RDP present as distinct entities, there are clearly overlapping clinical features such as dystonia with a bulbar preference. AHC and RDP may be part of a phenotypical continuum of one disease, with AHC at the severe end of the spectrum and RDP at the mild end (5, 6). Mutation of the α_2-isoform of Na^+,K^+-ATPase expressed in glial cells of the CNS causes familial hemiplegic migraine type 2 (FHM2), a severe subtype of migraine with aura and temporary hemiparesis (7). The clinical spectrum caused by mutations in α_2 and α_3 isoforms includes other paroxysmal manifestations such as episodes of abnormal ocular movements and various types of seizures as well as neurodevelopmental delay and in some families the phenotypes overlap (5, 8). Notably, one family case of AHC has been reported as being caused by mutation of α_2 (9). RDP, AHC, and FHM2 are autosomal dominant disorders. Little is known about the actual neuropathophysiological mechanisms responsible for the development of these disorders. For FHM2 it has been suggested that an impaired clear-

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[3] The abbreviations used are: RDP, rapid-onset dystonia parkinsonism; FHM2, familial hemiplegic migraine type 2; AHC, alternating hemiplegia of childhood; $K_{0.5}$, ligand concentration giving half-maximal effect.

ance of extracellular K^+ produces a wide cortical depolarization (10, 11). A reduced Na^+ affinity has been described for certain RDP mutants (12–14); however, no information is available about Na^+ or K^+ handling by the mutants at the cellular level. It has not been examined whether a change of the intracellular Na^+ and/or K^+ concentration ($[Na^+]_i$ and $[K^+]_i$) is part of the pathophysiological picture associated with the Na^+,K^+-ATPase spectrum of neurological disorders. Although changes to $[Na^+]_i$ and/or $[K^+]_i$ might be expected for complete-loss-of-function mutants, it is not clear whether and to what extent such changes in ionic concentrations would occur for mutants with residual function and whether a change of Na^+ affinity influences the ionic concentrations to the same extent as a change in the maximal pumping rate.

The crystal structures of the Na^+,K^+-ATPase have revealed a strategic location of the C terminus, docking in between transmembrane helices both in the K^+-bound E_2 form (15) and in the Na^+-bound E_1 form, where it appears to stabilize Na^+ binding at the third Na^+ site (site III) (16), and truncation mutagenesis has demonstrated that the C terminus is critical for optimal binding of Na^+ (15, 17–20). It is, therefore, intriguing that two neurological disease mutations of the Na^+,K^+-ATPase have been reported in which the C terminus is extended, in one case by a single tyrosine residue ("+Y" mutation) in a patient with RDP (13) and in the other case by a 28-amino acid residue segment consisting of RPHWKKNQAWKDGELWRCCGDGDGEGWK in a patient with FHM2 (21) (in this paper denoted "+28" mutation, originally "X1021R"). Here we have functionally characterized and compared these and other neurological disease mutants as well as a mutant where the C terminus only is extended by the smaller alanine. At the cellular level we have addressed the question to what extent these mutations cause a change of $[Na^+]_i$ and $[K^+]_i$. By taking advantage of the different properties of a series of mutant Na^+,K^+ pumps, we have been able to analyze the contributions of change of Na^+ affinity, maximal catalytic turnover rate, and expression level to observed changes of $[Na^+]_i$ and $[K^+]_i$.

EXPERIMENTAL PROCEDURES

C-terminal extension mutations were introduced into full-length cDNA encoding the ouabain resistant rat α_1-isoform of Na^+,K^+-ATPase or in cDNA encoding the human α_2- or α_3-isoform made ouabain resistant by mutations Q116R/N127D and Q108R/N119D, respectively (14, 22). Before stable transfection of COS-1 cells, the introduction of the desired mutation was confirmed by full-length sequencing. Mutants and wild type were expressed stably in COS-1 cells under ouabain selection pressure (22, 23). The presence of the correct mutation was confirmed also by sequencing genomic DNA from the isolated stable cell lines. Because of the lack of activity of mutant α_2+28, which precluded isolation of a stable cell line, this mutant as well as the corresponding α_2 wild type was expressed transiently in the presence of siRNA to knock down the endogenous Na^+,K^+-ATPase of the cells as previously described (24). The plasma membrane fraction was isolated and made leaky by treatment with deoxycholate or alamethicin to allow access of incubation media from both sides of the membrane (25), and ATPase activity was studied at 37 °C by following the liberation of P_i (23). The molecular ATPase activity was calculated by relating the specific ATPase activity to the active site concentration determined by phosphorylation from ATP in the presence of 150 mM Na^+ and oligomycin to obtain the maximum phosphorylation capacity (12, 23, 26). Measurements of phosphorylation and dephosphorylation to characterize the partial reaction steps of the enzyme cycle (Fig. 1) were performed at 0 °C as previously described (12, 14, 23, 26). The acid-precipitated ^{32}P-labeled phosphoenzyme was washed by centrifugation and subjected to SDS-polyacrylamide gel electrophoresis at pH 6.0, and the radioactivity associated with the separated Na^+,K^+-ATPase band was quantified by phosphorimaging using a cyclone storage phosphor system (PerkinElmer Life Sciences) (14). Ouabain was included in the media used for ATPase and phosphorylation measurements to eliminate the contribution of the endogenous Na^+,K^+-ATPase as detailed in the figure legends. Expression of α_2 wild type and mutant was detected by Western blotting after SDS-PAGE using rabbit polyclonal α_2 isoform-specific antibody (07–674 Millipore/Upstate, 1:2000 dilution) and HRP-conjugated goat anti-rabbit IgG secondary antibody.

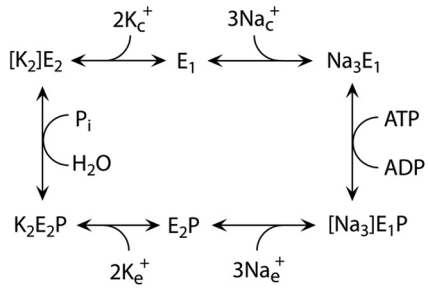

FIGURE 1. **Reaction cycle of the Na^+,K^+-ATPase.** E_1 and E_2 are the main conformational states of the enzyme. *P* indicates phosphorylation. Occluded ions are shown in *brackets*. Cytoplasmic and extracellular ions are indicated by *c* and *e*, respectively.

To determine the apparent affinity for extracellular K^+ in intact cells expressing mutants or wild type, uptake of the K^+ congener $^{86}Rb^+$ was measured in the presence of various concentrations of extracellular K^+ (27). Cells were grown to confluence in culture medium containing 5 μM ouabain and incubated for 10 min at 37 °C and 5% CO_2 in serum- and K^+-free medium (Na^+ concentration 154 mM), to which had been added $^{86}Rb^+$ (0.5 μCi/ml) with 0.5, 1, 3, 5, or 10 mM KCl and 5 μM ouabain (to inhibit the endogenous Na^+,K^+-ATPase) or 10 mM ouabain (to inhibit all Na^+,K^+-ATPase). $^{86}Rb^+$ uptake was terminated by washing the cells with an ice-cold solution containing 300 mM sucrose, 1.25 mM $CaCl_2$, 1.18 mM $MgSO_4$, and 10 mM Tris (pH 7.5). After incubation in 0.2 M NaOH for 2 h at 4 °C, the cells were scraped off, and the amount of $^{86}Rb^+$ in the suspension was determined by liquid scintillation counting. K^+/congener uptake was calculated by multiplying the relative $^{86}Rb^+$ uptake per mg protein by the K^+ concentration in the uptake medium (27).

To determine $[Na^+]_i$ and $[K^+]_i$, cells were grown to confluence in culture medium containing 154 mM Na^+ and 5.4 mM K^+ as well as 5 μM ouabain. A radiometer FLM3 flame photometer was used to measure Na^+ or K^+ content after extraction with

trichloroacetic acid (27, 28). $[Na^+]_i$ and $[K^+]_i$ were calculated by relating nmol of Na^+ or K^+/mg of total protein to μl of cell volume/mg of total protein determined by hematocrit centrifugation; trypsinized cells were washed and transferred to microcapillary tubes (75 × 0.575 mm) and centrifuged for 5 min at 15,000 × *g*. The relative volume fraction of the cells was determined using a microcapillary reader.

The results are reported as average values ± S.E. (shown by *error bars* in the figures, only visible when larger than the size of the symbols). The number of independent determinations (different days or different plasma membrane harvests/clonal isolates) is indicated by *n* in tables. Data were analyzed using SigmaPlot (SPSS, Inc.) for nonlinear regression (12, 14). The Na^+, K^+, ATP, and vanadate concentration dependences were fitted either by a single Hill function or by a function consisting of two Hill components (when both an activation phase and an inhibition phase were present).

RESULTS

Human α_2+28 Is Expressed but Is Inactive—The ouabain selection technique (22, 23) was used in an attempt to express human α_2 enzyme containing the +28 extension FHM2 mutation in mammalian cell culture (COS cells). However, no stable colonies appeared upon selection in growth media containing ouabain even though three different expression vectors (pcDNA3.1+, pCMV6-XL5, and pMT2) were tried, and the transfection was carried out several times. Because sustained growth of the transformed COS cells in the presence of ouabain, inhibiting the endogenous Na^+,K^+-ATPase, depends on the enzyme activity of exogenous, expressed enzyme, we concluded that the α_2+28 mutant is either not expressed or is nonfunctional. This was further examined in transient expression studies where the endogenous COS cell Na^+,K^+-ATPase was knocked down using a combination of ouabain and siRNA (24). The Western blot of the isolated plasma membrane fraction shown in Fig. 2*A* indicates that the α_2+28 mutant was well expressed to a level even higher than that of wild type α_2, irrespective of whether the pCMV6-XL5 or pMT2 vector was used (compare *lanes 4* and *5* with *lane 2*). In the wild type Na^+,K^+-ATPase, phosphorylation from ATP occurs upon binding of three Na^+ ions to the cytoplasmically facing sites of the E_1 form (see Fig. 1). Fig. 2*B* shows enzymatic studies carried out on the isolated plasma membrane fraction. Despite the high protein expression level, ^{32}P phosphorylation from [γ-^{32}P]ATP was negligible in the α_2+28 mutant relative to wild type α_2. Hence, at 100 mM Na^+ as well as 300 mM Na^+, the phosphorylation level of α_2+28 was similar to the background represented by the D371N mutant, in which the phosphorylate-able aspartate has been replaced. The incompetence of α_2+28 with respect to phosphorylation and, thus, ATP utilization explains why the mutant was unable to support cell growth.

Human α_3+Y Exhibits a Conformational Shift toward E_1 Despite Reduced Na^+ Affinity—In contrast to α_2+28, the RDP mutant α_3+Y could be expressed stably in COS cells using the ouabain selection technique, thus indicating retention of significant Na^+,K^+ pump function by α_3+Y (13). Our finding of a 41-fold reduction of the apparent Na^+ affinity of this mutant relative to wild type α_3 (Table 1) left open the question of whether this is a direct effect on the intrinsic Na^+ affinity of the E_1 form or an indirect effect caused by a shift of the E_1-E_2 conformational equilibrium in favor of E_2. Fig. 3 presents the results of further enzymatic studies carried out on isolated plasma membranes containing the α_3+Y mutant to resolve this question. Indeed, the reduced Na^+ affinity is a "true" effect on the intrinsic Na^+ affinity, because the α_3+Y mutation shifts the E_1-E_2 equilibrium in favor of E_1 rather than E_2, as evidenced by a higher apparent affinity of the mutant for ATP (binding preferentially to E_1) and lower apparent affinity for vanadate (binding exclusively to E_2) relative to the wild type (Fig. 3, *upper panels*, and Table 1).

Fig. 3, *lower panels*, shows the K^+ dependence of Na^+,K^+-ATPase activity measured on the leaky plasma membranes in the presence of 40 or 200 mM Na^+. The activating phase represents binding of K^+ to the E_2P form triggering dephosphorylation, thereby activating the whole Na^+,K^+-ATPase cycle (*cf.* Fig. 1). The extracted $K_{0.5}$ values for K^+ activation (listed in Table 1) indicate a 2–3-fold increase of the apparent K^+ affinity for α_3+Y as compared with the wild type. In the intact cell the activating sites on E_2P face the external side. At 40 mM Na^+, an inhibitory phase was seen for α_3+Y at K^+ concentrations >2 mM, which was much less pronounced for the wild type. The inhibitory effect of K^+ is actually more marked for the wild type if the Na^+ concentration is lowered, which is explained by competition between Na^+ and K^+ at the cytoplasmically facing E_1 sites (29). The pronounced inhibition seen for the mutant is,

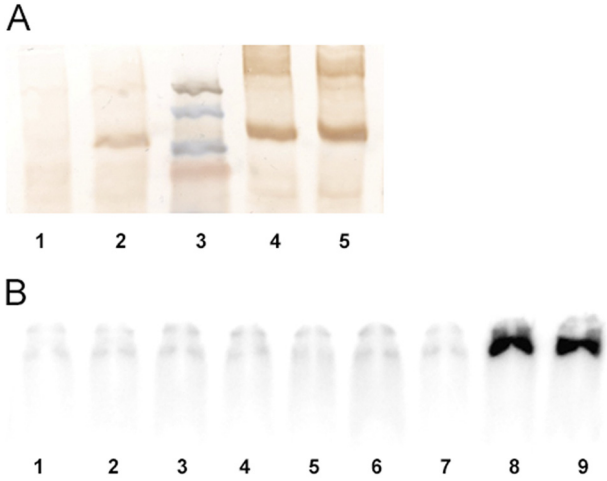

FIGURE 2. **Expression of α_2+28 and deficiency of phosphorylation.** *A*, representative Western blot of the plasma membrane fraction of COS cells transiently expressing wild type α_2 and α_2+28 probed with specific α_2 antibody. *Lane 1*, mock transfection (32 μg); *lane 2*, α_2 wild type (40 μg); *lane 3*, molecular size marker; *lane 4*, α_2+28 expressed using pCMV6-XL5 vector (25 μg); *lane 5*, α_2+28 expressed using pMT2 vector (25 μg). The indicated amount applied to each lane refers to total membrane protein. *B*, representative phosphor image autoradiograph showing ^{32}P incorporation in the separated Na^+,K^+-ATPase band after SDS-PAGE. Phosphorylation was carried out for 10 s at 0 °C in the presence of 20 mM Tris (pH 7.4), 3 mM Mg^{2+}, 1 mM EGTA, 2 μM [γ-^{32}P]ATP, 20 μg/ml oligomycin to block dephosphorylation, 100 μM ouabain to inhibit the endogenous COS cell enzyme, and the concentration of Na^+ indicated below. *Lane 1*, phosphorylation-inactive mutant α_1D371N at 100 mM Na^+; *lane 2*, phosphorylation-inactive mutant α_1D371N at 300 mM Na^+; *lanes 3* and *4*, α_2+28 at 100 mM Na^+; *lanes 5* and *6*, α_2+28 at 300 mM Na^+; *lane 7*, phosphorylation-inactive mutant α_1D371N at 300 mM Na^+; *lanes 8* and *9*, wild type α_2 at 100 mM Na^+. 10 μg of total membrane protein were applied to each lane. Wild type α_2 and α_2+28 mutant are the same membrane preparations as used for the Western blot in *A*, *lanes 2* and *4*.

TABLE 1

Functional effects of C-terminal extension mutations in α_3 and α_1 and α_3F780L/α_1F785L

All values are shown ± S.E. and with the number of independent experiments (*n*) indicated. Maximal molecular Na^+,K^+-ATPase activities are indicated in Table 2 except for that of α_1+A, which was 7231 ± 247 min^{-1}.

Mutant	$K_{0.5}(Na^+)$[a]	$K_{0.5}$(ATP)[b]	$K_{0.5}$(vanadate)[c]	$K_{0.5}(K^+)$ at 40 mM Na^+[d]	$K_{0.5}(K^+)$ at 200 mM Na^+[e]	$K_{0.5}(K^+)$ from $^{86}Rb^+$ uptake[f]	$K_{0.5}$(ouabain)[g]
	mM	μM	μM	mM	mM	mM	μM
$\alpha_3$wt	0.61 ± 0.03	137 ± 5	4.7 ± 0.2	0.55 ± 0.01	2.20 ± 0.04	ND[h]	352 ± 40
	n = 4	*n* = 7	*n* = 7	*n* = 4	*n* = 3		*n* = 3
α_3+Y	24.9 ± 1.0	26 ± 1	69.2 ± 6.9	0.19 ± 0.01	0.85 ± 0.07	ND	1471 ± 323
	n = 5	*n* = 4	*n* = 10	*n* = 4	*n* = 6		*n* = 3
α_3F780L	7.3 ± 0.4	ND	28.5 ± 9.8	0.19 ± 0.02	ND	ND	>10,000[i]
	n = 4		*n* = 4	*n* = 4			*n* = 7
$\alpha_1$wt	0.44 ± 0.01	502 ± 27	2.2 ± 0.1	0.67 ± 0.01	2.40 ± 0.04	2.24 ± 0.17	153 ± 14
	n = 16	*n* = 5	*n* = 7	*n* = 5	*n* = 4	*n* = 3	*n* = 3
α_1+Y	14.6 ± 0.5	102 ± 5	27.1 ± 1.0	0.61 ± 0.08	1.93 ± 0.09	0.94 ± 0.08	208 ± 22
	n = 10	*n* = 3	*n* = 4	*n* = 5	*n* = 3	*n* = 4	*n* = 4
α_1+A	10.6 ± 0.4	132 ± 6	4.0 ± 0.1	0.77 ± 0.02	2.62 ± 0.09	ND	161 ± 22
	n = 5	*n* = 3	*n* = 6	*n* = 4	*n* = 3		*n* = 3
α_1+28	25.4 ± 1.8	135 ± 5	3.7 ± 0.1	0.61 ± 0.03	2.08 ± 0.04	1.20 ± 0.13	193 ± 17
	n = 9	*n* = 7	*n* = 6	*n* = 7	*n* = 3	*n* = 3	*n* = 3
α_1F785L[j]	5.8 ± 0.4	462 ± 75	2.2 ± 0.3	0.33 ± 0.01	ND	ND	~8,000
	n = 4	*n* = 5	*n* = 4	*n* = 4			*n* = 3

[a] $K_{0.5}$ values for Na^+ activation of phosphorylation, from Fig. 4 and Blanco-Arias *et al.* (13) (for $\alpha_3$wt and α_3+Y), and Rodacker *et al.* (12) (α_1F785L).
[b] $K_{0.5}$ values for ATP activation of Na^+,K^+-ATPase activity, from Figs. 3 and Fig. 5 and Rodacker *et al.* (12) (α_1F785L).
[c] $K_{0.5}$ values for vanadate inhibition of Na^+,K^+-ATPase activity, from Figs. 3, 6, and 11 and Rodacker *et al.* (12) (α_1F785L).
[d] $K_{0.5}$ values for K^+ activation of Na^+,K^+-ATPase activity at 40 mM Na^+, from Figs. 3, 7*A*, and 11 and Rodacker *et al.* (12) (α_1F785L).
[e] $K_{0.5}$ values for K^+ activation of Na^+,K^+-ATPase activity at 200 mM Na^+, from Figs. 3 and 7*B*.
[f] $K_{0.5}$ values for K^+ activation of $^{86}Rb^+$ uptake in intact cells, from Fig. 7*C*.
[g] $K_{0.5}$ values for ouabain inhibition of Na^+,K^+-ATPase activity, determined as described in Rodacker *et al.* (12).
[h] ND, not determined.
[i] Ouabain affinity of this mutant was too low to determine accurately, as a consequence of the direct involvement of this residue in the ouabain binding site.
[j] Data taken from Rodacker *et al.* (12); this mutation is homologous to α_3F780L.

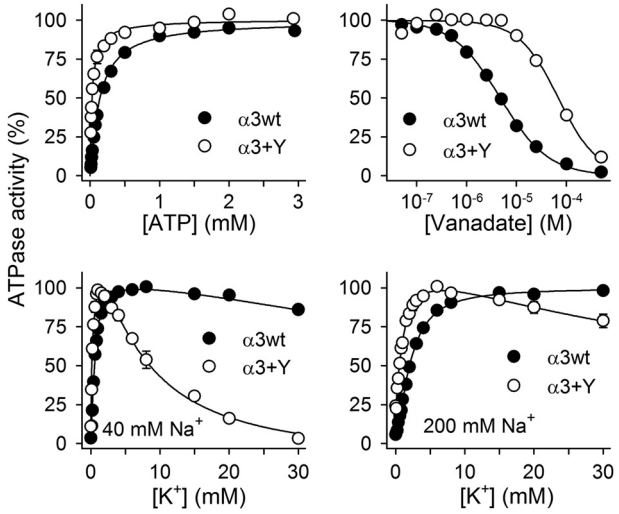

FIGURE 3. **Functional characterization of α_3+Y.** *Upper left panel*, ATP dependence of Na^+,K^+-ATPase activity. ATPase activity was measured on isolated, leaky plasma membranes from transfected COS cells at 37 °C in the presence of 30 mM histidine (pH 7.4), 130 mM Na^+, 20 mM K^+, 3 mM Mg^{2+}, 1 mM EGTA, 10 μM ouabain, and the indicated concentrations of ATP. *Upper right panel*, vanadate dependence of Na^+,K^+-ATPase activity. 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 Mg^{2+}, 1 mM EGTA, 3 mM ATP, 10 μM ouabain, and the indicated concentrations of vanadate. *Lower left* and *right panels*, K^+ dependence of Na^+,K^+-ATPase activity. ATPase activity was measured on isolated, leaky plasma membranes from transfected COS cells at 37 °C in the presence of 40 mM (*left*) or 200 mM Na^+ (*right*), 30 mM histidine (pH 7.4), 3 mM Mg^{2+}, 1 mM EGTA, 3 mM ATP, 10 μM ouabain, and the indicated concentrations of K^+. Average values are shown with *error bars* indicating S.E. (only visible when larger than the size of the symbols). $K_{0.5}$ values with statistics are listed in Table 1.

therefore, consistent with its strongly reduced Na^+ affinity, allowing K^+ to compete more efficiently for binding to E_1. Accordingly, the inhibitory phase was much less pronounced when the Na^+ concentration was increased to 200 mM (Fig. 3, *lower right panel*).

Functional Properties of C-terminal α_1 Mutants—To be able to compare the effects of the +Y and +28 mutations directly, both were introduced in the same isoform, α_1 ("α_1+Y" and "α_1+28," respectively). In addition, a mutation extending the C terminus by a single alanine ("α_1+A") was examined. The choice of α_1 furthermore allowed comparison of these mutants with other α_1 mutants in our library (among those, mutants described in Refs. 12, 17, 23, and 26, see below). The α_1+28 mutant, unlike α_2+28 described above, was able to support growth of the COS cells under ouabain selection pressure, as was α_1+Y and α_1+A. Hence, enzymatic studies were carried out on plasma membranes isolated from these cells. The apparent Na^+ affinity was determined by measuring the dependence of phosphorylation on Na^+ concentration in the absence of K^+. As seen in Fig. 4 and Table 1, all three extensions to the C terminus produced a dramatic reduction in apparent Na^+ affinity relative to that of the wild type, 24-, 34-, and 58-fold reduction for α_1+A, α_1+Y, and α_1+28, respectively. The α_1+Y mutant exhibited a 5-fold increase of ATP affinity and 12-fold decrease of vanadate affinity relative to wild type α_1, which is very similar to the observation described above for α_3+Y. Furthermore, α_1+A and α_1+28 likewise showed higher ATP affinity and lower vanadate affinity relative to wild type α_1 (Figs. 5 and 6 and Table 1) thus in all cases allowing the conclusions that the reduced Na^+ affinity is a true, direct effect on the Na^+ site(s) and not an indirect effect of displacement of the E_1-E_2 conformational equilibrium away from the Na^+ binding E_1 form.

Moreover, the K^+ dependence of Na^+,K^+-ATPase activity supported the conclusion that Na^+ affinity is markedly reduced in α_1+Y, α_1+A, and α_1+28 by showing a conspicuous inhibi-

tory phase at K^+ concentrations >5 mM in the presence of 40 mM Na^+, which was much less pronounced or absent when the Na^+ concentration was increased to 200 mM (Fig. 7, *A* and *B*). The $K_{0.5}$ values for K^+ activation were close to that of the wild type for these mutants at both Na^+ concentrations (Table 1).

For the wild type, α_1+Y, and α_1+28, the affinity for K^+ was studied in intact COS cells as well by measuring ouabain-suppressible cellular $^{86}Rb^+$ uptake at various extracellular K^+ concentrations in the presence of 154 mM Na^+ (Fig. 7*C*). Under these conditions the apparent K^+ affinity of α_1+Y and α_1+28 was found slightly increased relative to wild type, thus again demonstrating that the K^+ sites are intact. The increase of apparent K^+ affinity may be a consequence of a lack of saturation of the extracellularly facing sites on E_2P with Na^+, allowing K^+ to compete more efficiently also here as further substantiated below.

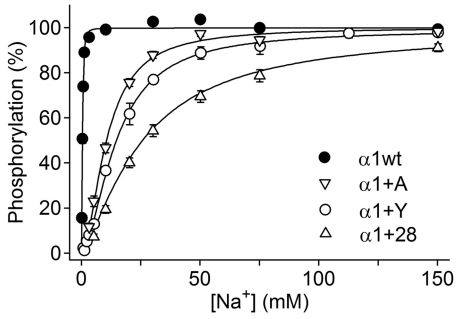

FIGURE 4. **Na^+ dependence of phosphorylation from ATP.** Phosphorylation was carried out on isolated, leaky plasma membranes from transfected COS cells for 10 s at 0 °C in the presence of 20 mM Tris (pH 7.4), 3 mM Mg^{2+}, 1 mM EGTA, 2 μM [γ-^{32}P]ATP, 20 μg/ml oligomycin to block dephosphorylation, 10 μM ouabain to inhibit the endogenous COS cell enzyme, and the indicated concentrations of Na^+ with various concentrations of *N*-methyl-D-glucamine added to maintain a constant ionic strength. Average values are shown with *error bars* indicating S.E. (only visible when larger than the size of the symbols). $K_{0.5}$ values for Na^+ activation of phosphorylation with statistics are listed in Table 1.

To examine Na^+ interaction at the sites on E_2P that in the intact cell face the extracellular side, the ATPase activity of the isolated, leaky plasma membranes was determined at various Na^+ concentrations in the absence of K^+ ("Na^+-ATPase activity"). Under these conditions the enzyme can still hydrolyze ATP, although only slowly as a consequence of Na^+ substituting for K^+ to promote E_2P dephosphorylation (30, 31). However, at high Na^+ concentrations the Na^+-ATPase activity decreases, because Na^+ binding at one or more of the Na^+ sites on E_2P drives the $E_1P \rightarrow E_2P$ transition backwards to E_1P, thereby blocking the enzyme cycle (*cf.* the reaction cycle in Fig. 1). As seen in Fig. 8, Na^+ at concentrations up to 250 mM stimulates the Na^+-ATPase activity of the wild type, whereas Na^+ at concentrations >350 mM inhibit the Na^+-ATPase activity with close to 100% inhibition at 1000 mM Na^+. In the mutants α_1+Y, α_1+A, and α_1+28, little or no inhibition was observed up to 1000 mM Na^+, indicating a reduced ability of Na^+ to drive the $E_1P \rightarrow E_2P$ transition backwards to E_1P by interaction with the site(s) on E_2P. This conclusion was further strengthened by following the dephosphorylation upon the addition of a high Na^+ concentration of 600 mM with and without ADP (Fig. 9). In the wild type, the dephosphorylation is relatively rapid in the presence of both high Na^+ and ADP, because E_1P (accumulated by back conversion of E_2P to E_1P) reacts with ADP to form ATP (*cf.* Fig. 1), whereas in the absence of ADP the dephosphorylation is slow because the accumulated E_1P is unable to dephosphorylate by hydrolysis. In α_1+Y, α_1+A, and α_1+28, the rate of dephosphorylation in the presence of ADP was lower than that of the wild type, and the dephosphorylation rates with and without ADP were very similar (Fig. 9), thus supporting the notion that the high Na^+ concentration is ineffective in promoting the back conversion of E_2P to E_1P in these mutants due to defective binding of Na^+ to one or more of the sites on E_2P that in the intact cell face the extracellular side.

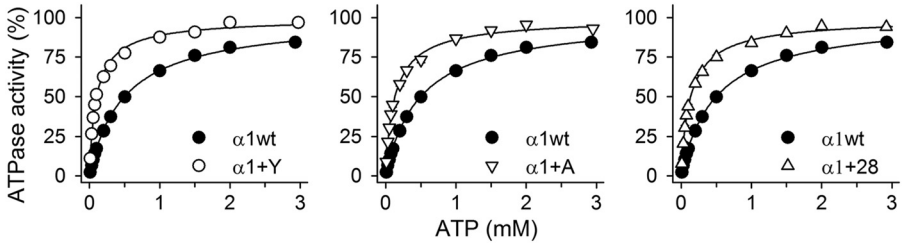

FIGURE 5. **ATP dependence of Na^+,K^+-ATPase activity.** ATPase activity was measured on isolated, leaky plasma membranes from transfected COS cells at 37 °C in the presence of 30 mM histidine (pH 7.4), 130 mM Na^+, 20 mM K^+, 3 mM Mg^{2+}, 1 mM EGTA, 10 μM ouabain, and the indicated concentrations of ATP. Average values are shown with *error bars* indicating S.E. (only visible when larger than the size of the symbols). $K_{0.5}$ values for ATP activation with statistics are listed in Table 1.

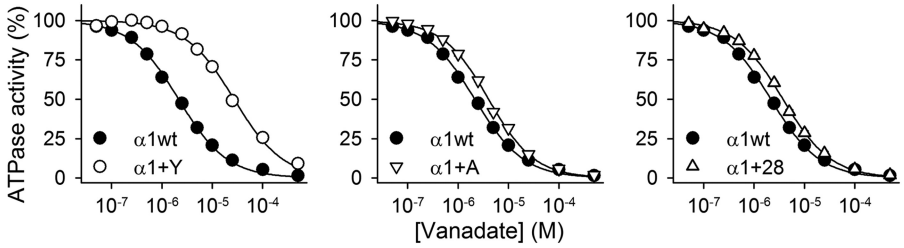

FIGURE 6. **Vanadate dependence of Na^+,K^+-ATPase activity.** ATPase activity was measured on isolated, leaky plasma membranes from transfected COS cells at 37 °C in the presence of 30 mM histidine (pH 7.4), 130 mM Na^+, 20 mM K^+, 3 mM Mg^{2+}, 1 mM EGTA, 3 mM ATP, 10 μM ouabain, and the indicated concentrations of vanadate. Average values are shown with *error bars* indicating S.E. (only visible when larger than the size of the symbols). $K_{0.5}$ values for vanadate inhibition with statistics are listed in Table 1.

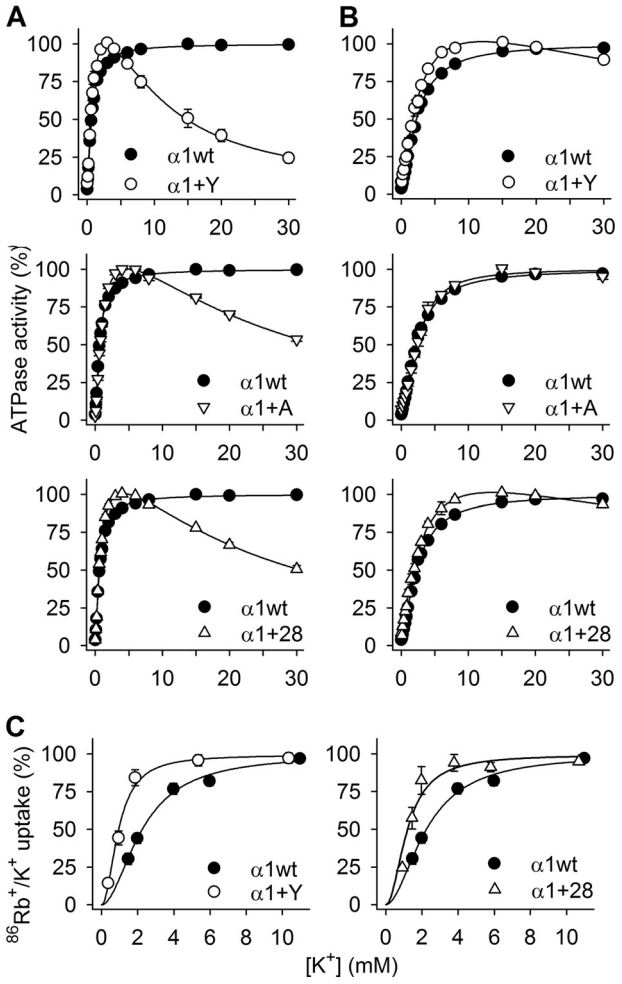

FIGURE 7. **K^+ dependence of Na^+,K^+-ATPase activity and $^{86}Rb^+$ uptake.** ATPase activity was measured on isolated, leaky plasma membranes from transfected COS cells at 37 °C in the presence of 40 mM (*A*) or 200 mM Na^+ (*B*), 30 mM histidine (pH 7.4), 3 mM Mg^{2+}, 1 mM EGTA, 3 mM ATP, 10 μM ouabain, and the indicated concentrations of K^+. *C*, $^{86}Rb^+$ uptake in intact COS cells was determined at the indicated K^+ concentrations in the presence of 154 mM Na^+. Ouabain-suppressible $^{86}Rb^+$ uptake mediated by expressed mutant or wild type was calculated by subtracting data obtained at 10 mM ouabain from those obtained at 5 μM ouabain. Average values are shown with *error bars* indicating S.E. (only visible when larger than the size of the symbols). $K_{0.5}$ values for K^+ activation with statistics are listed in Table 1.

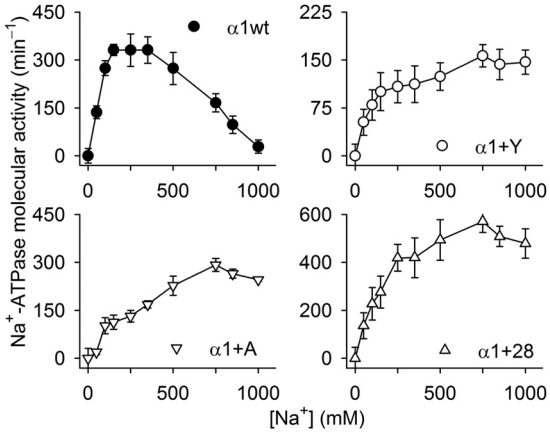

FIGURE 8. **Na^+ dependence of ATPase activity in the absence of K^+.** Na^+-ATPase activity was measured on isolated, leaky plasma membranes from transfected COS cells at 37 °C in 30 mM histidine (pH 7.4), 3 mM ATP, 3 mM Mg^{2+}, 1 mM EGTA, 10 μM ouabain, and the indicated concentrations of Na^+. The molecular ATPase activity was calculated as the ratio between the specific ATPase activity and the active site concentration determined as the maximum capacity for phosphorylation from ATP. Average values are shown with *error bars* indicating S.E. (only visible when larger than the size of the symbols). Each data point is the average of $n = 4–8$ independent determinations.

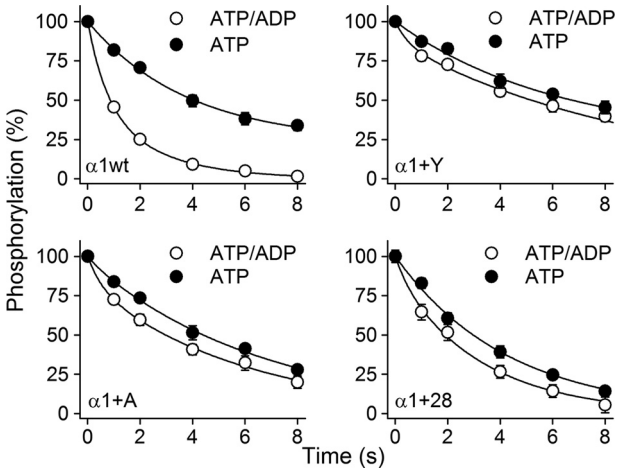

FIGURE 9. **Dephosphorylation at 600 mM Na^+ with or without ADP.** Phosphorylation was carried out on isolated, leaky plasma membranes from transfected COS cells at 0 °C for 10 s in 20 mM Tris (pH 7.5), 150 mM Na^+, 3 mM Mg^{2+}, 1 mM EGTA, 2 μM [γ-^{32}P]ATP, and 10 μM ouabain. Dephosphorylation was followed at 0 °C upon the addition of 1 mM non-labeled ATP (*filled symbols*) or 1 mM non-labeled ATP plus 2.5 mM ADP (*open symbols*), together with NaCl to give a total Na^+ concentration of 600 mM. Average values are shown with *error bars* indicating S.E. (only visible when larger than the size of the symbols). Each data point is the average of $n = 3–8$ independent determinations.

Intracellular Na^+ and K^+ Concentrations—The ability of α_1+Y and α_1+28 to support cell growth due to their retention of partial function made it feasible to determine the effects of the mutations on cellular Na^+ and K^+ contents in the stable cell lines expressing these mutants using flame photometry (27, 28). The $[Na^+]_i$ and $[K^+]_i$ were calculated by relating the Na^+ and K^+ contents to the cell volume, and for comparison measurements were made also on stable cell lines expressing exogenous α_1 wild type. The cells had been grown in the presence of 5 μM ouabain to selectively silence the endogenous COS cell Na^+,K^+-ATPase (note in Table 1 that the ouabain affinities of wild type and mutant exogenous enzymes are >100-fold lower than that of the endogenous enzyme, exhibiting a $K_{0.5}$ for ouabain inhibition of <1 μM). For both α_1+Y and α_1+28, $[Na^+]_i$ was considerably higher (2.1- and 1.6-fold, respectively), whereas $[K^+]_i$ was lower (1.5- and 1.4-fold, respectively) than observed for the wild type α_1 (Fig. 10 and Table 2). The $[Na^+]_i$ and $[K^+]_i$ were also determined for the +Y insertion in α_3 ("α_3+Y"), and a 2.6-fold increase in $[Na^+]_i$ and 2.2-fold decrease in $[K^+]_i$ were seen relative to wild type α_3 (Fig. 10 and Table 2). This is in accordance with our findings that the difference in enzymatic properties between α_3 and α_3+Y is similar to the difference between α_1 and α_1+Y (Table 1 and Fig. 3). In addition, several other mutants previously shown to display altered Na^+ affinity were examined for their ability to induce changes in $[Na^+]_i$ and $[K^+]_i$. RDP mutation α_3D923N of the aspartate in transmembrane segment M8 reduces the apparent Na^+ affinity for activation of phosphorylation by >200-fold (14). As shown in Fig. 10 and Table 2, we found a 3-fold increase in $[Na^+]_i$ and 2-fold decrease in $[K^+]_i$ for α_3D923N. Likewise, cells expressing α_1F785L showed a marked increase of $[Na^+]_i$

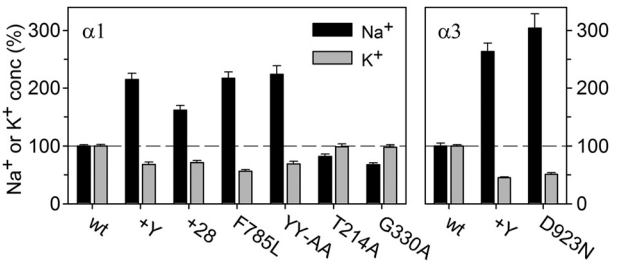

FIGURE 10. **Relative intracellular Na^+ and K^+ concentrations in COS cell lines stably expressing mutants or wild type.** The cellular Na^+ and K^+ contents were determined by flame photometry and related to cell volume. The data are presented relative to the levels in cells expressing the corresponding wild type. Absolute values for concentrations and cell volumes with statistics are listed in Table 2.

and reduction of $[K^+]_i$. This mutant is also characterized by a conspicuously reduced Na^+ affinity (Ref. 12 and Table 1). The corresponding mutation, F780L, in human α_3 has been found to cause RDP (3). Fig. 11 and Table 1 show results indicating that α_3F780L lowers Na^+ affinity to an extent comparable with that of α_1F785L with the characteristic inhibition by K^+ at high concentration and without reducing K^+ affinity for activation. The reduced vanadate affinity of α_3F780L also depicted in Fig. 11 indicates a shift of the conformational equilibrium in favor of E_1, thus once again attesting that the reduced Na^+ affinity is a true effect on the intrinsic affinity and not an indirect effect of a conformational shift toward E_2.

Because some of the mutants for which $[Na^+]_i$ could be examined exhibited a reduced catalytic turnover rate (maximal molecular Na^+,K^+-ATPase activity) in addition to the reduced Na^+ affinity (Table 2), the question arose of whether both parameters need to be reduced to cause the observed rise of $[Na^+]_i$. However, for α_1+28 a marked increase of $[Na^+]_i$ and lowering of $[K^+]_i$ were seen even though the maximal molecular Na^+,K^+-ATPase activity was little changed relative to wild type α_1 (6862 ± 270 min^{-1} *versus* 8474 ± 165 min^{-1} for wild type; Table 2). This question was further addressed by studying the mutant α_1YY-AA, in which the two C-terminal tyrosines are replaced by alanine, resulting in a 32-fold reduction of Na^+ affinity without any reduction of the maximal molecular Na^+,K^+-ATPase activity (compare 9341 ± 584 min^{-1} with 8474 ± 165 min^{-1} for wild type; Table 2). As seen in Fig. 10 and Table 2, cells expressing this mutant also displayed a marked 2.2-fold increase in $[Na^+]_i$ and 1.5-fold decrease in $[K^+]_i$, thus clearly indicating that a reduction of Na^+ affinity *per se* is sufficient to affect $[Na^+]_i$ and $[K^+]_i$ markedly.

It should furthermore be noted that the observed increase of $[Na^+]_i$ caused by the mutations reducing Na^+ affinity is not due to poor expression of the mutant Na^+,K^+-ATPases, as all these mutants exhibited an expression level close to or significantly higher than that of the wild type (range 69–188%; see Table 2). Hence, α_1+Y and α_3D923N were expressed at higher levels than the wild type, which *per se* would be expected to reduce $[Na^+]_i$ and increase $[K^+]_i$, whereas the opposite was in fact observed.

For further examination of the dependence of $[Na^+]_i$ and $[K^+]_i$ on mutant properties we selected two mutants, α_1T214A and α_1G330A, previously shown to exhibit an increased apparent affinity for Na^+ of 1.5- and 5-fold, respectively, as compared with wild type (23, 26). As shown in Fig. 10 and Table 2, α_1T214A and α_1G330A reduced $[Na^+]_i$ 1.2- and 1.5-fold, respectively, whereas $[K^+]_i$ remained wild type-like. Both of these mutants showed in addition to the increased Na^+ affinity also a higher expression level relative to wild type, which might contribute to reduce $[Na^+]_i$. However, because the 2.6-fold increase in expression level of α_1T214A is compensated by a matching 2.6-fold reduction of the maximal molecular Na^+,K^+-ATPase activity in this mutant (Table 2), the increased Na^+ affinity indeed seems to play a major role in reducing $[Na^+]_i$.

To properly evaluate the combined contributions of changes of Na^+ affinity, maximal molecular Na^+,K^+-ATPase activity, and expression level to changes of $[Na^+]_i$ and $[K^+]_i$, we determined the molecular Na^+,K^+-ATPase activity at 15 mM Na^+ and 130 mM K^+, which mimic the normal intracellular Na^+ and K^+ concentrations in mammalian cells (see Physiological molecular Na^+,K^+-ATPase activity in Table 2). Because of the high affinity of the externally facing K^+ sites on E_2P, the latter are saturated in the leaky plasma membrane Na^+,K^+-ATPase preparation at the high K^+ concentration of 130 mM applied here. By multiplying the physiological molecular Na^+,K^+-ATPase activity with the relative expression level, we have calculated the relative Na^+,K^+-ATPase activity under conditions mimicking the intact cells under normal physiological ionic conditions (see "Physiological cellular Na^+,K^+-ATPase activity" in Table 2). The mutants giving rise to a marked increase of $[Na^+]_i$ relative to wild type all showed a physiological cellular Na^+,K^+-ATPase activity ranging between 159 and 471 min^{-1}, *i.e.* 8–23% that of the wild type (2032 min^{-1}). It is of note that for α_1+28 and α_1YY-AA it is the reduced Na^+ affinity and not a reduced maximal molecular Na^+,K^+-ATPase activity or a reduced expression level that is the major cause of the reduced physiological cellular Na^+,K^+-ATPase activity. By contrast, the two mutants causing reduced $[Na^+]_i$ (α_1T214A and α_1G330A) displayed higher physiological cellular Na^+,K^+-ATPase activity than the wild type. Mutant α_1G330A retains particularly high activity because it is not inhibited by high K^+. Hence, the results in Table 2 show an inverse relationship between $[Na^+]_i$ and the physiological cellular Na^+,K^+-ATPase activity.

DISCUSSION

The neurological disorders FHM2, AHC, and RDP are caused by mutations in the Na^+,K^+-ATPase. Here we describe the functional consequences of RDP mutation +Y, extending the C terminus by an extra tyrosine residue (13), and FHM2 mutation +28, extending the C terminus by 28 residues (21). In its natural α_2 protein environment the +28 mutation was found to inactivate function but not protein expression. Hence, no Na^+-activated phosphorylation from ATP could be detected with α_2+28 even at a Na^+ concentration as high as 300 mM, which is far beyond the Na^+ concentration where phosphorylation of the wild type α_2 is maximal (Fig. 2, $K_{0.5}$ for Na^+ is 0.5 mM in wild type α_2; Ref. 32). The lack of Na^+-activated phosphorylation of α_2+28 would be in accordance with defective Na^+ binding in this mutant. Indeed, a deficiency in Na^+-activated phosphorylation would explain electrophysiological observations with mutant X1021R (here denoted

TABLE 2

Mutational effects on Na^+ affinity, intracellular Na^+ and K^+ concentrations, and molecular and cellular Na^+,K^+-ATPase activities

All values are shown ± S.E. and with the number of independent experiments (*n*) indicated.

Mutant	Relative $K_{0.5}(Na^+)$	$[Na^+]_i$	$[K^+]_i$	Cell volume	Relative expression[a]	Maximal molecular Na^+,K^+-ATPase activity[b]	Physiological molecular Na^+,K^+-ATPase activity[c]	Physiological cellular Na^+,K^+-ATPase activity[d]
		mM	*mM*	*μl (mg protein)*$^{-1}$	*%*	*min*$^{-1}$	*min*$^{-1}$	*min*$^{-1}$
$\alpha_1$wt	1 ± 0.02[e]	20.3 ± 0.5	99.9 ± 2.7	13.0 ± 0.2	100 ± 1	8474 ± 165	2032 ± 46	2032 ± 48
	n = 16	*n* = 43	*n* = 43	*n* = 6	*n* = 44	*n* = 11	*n* = 8	
α_1+Y	34 ± 1.4[e]	43.6 ± 2.0	68.3 ± 3.4	13.0 ± 0.2	188 ± 9	3096 ± 101	85 ± 6	159 ± 14
	n = 10	*n* = 15	*n* = 15	*n* = 15	*n* = 4	*n* = 6	*n* = 6	
α_1+28	58 ± 4.2[e]	32.8 ± 1.4	71.4 ± 2.8	14.0 ± 0.4	69 ± 1	6862 ± 270	420 ± 48	292 ± 34
	n = 9	*n* = 7	*n* = 7	*n* = 4	*n* = 7	*n* = 11	*n* = 16	
α_1YY-AA	32 ± 1.6[f]	45.4 ± 2.7	68.7 ± 3.9	14.1 ± 0.6	107 ± 4	9341 ± 584[g]	442 ± 40	471 ± 46
	n = 10	*n* = 11	*n* = 11	*n* = 3	*n* = 4	*n* = 8	*n* = 8	
α_1F785L	13 ± 1.0[f]	44.0 ± 1.9	56.5 ± 1.9	15.2 ± 0.3	92 ± 2	2247 ± 119[g]	177 ± 16	162 ± 15
	n = 4	*n* = 17	*n* = 17	*n* = 4	*n* = 12	*n* = 6	*n* = 12	
α_1T214A	0.63 ± 0.04[f]	16.6 ± 0.7	98.8 ± 3.9	13.9 ± 0.5	260 ± 55	3225 ± 262[g]	1250 ± 105	3253 ± 690
	n = 4	*n* = 32	*n* = 32	*n* = 6	*n* = 30	*n* = 6	*n* = 12	
α_1G330A	0.20 ± 0.04[f]	13.7 ± 0.5	97.8 ± 3.2	13.7 ± 0.4	285 ± 7	6796 ± 414[g]	5256 ± 325	15003 ± 1006
	n = 7	*n* = 17	*n* = 17	*n* = 6	*n* = 12	*n* = 11	*n* = 8	
$\alpha_3$wt	1 ± 0.06[f]	23.3 ± 1.2	93.6 ± 2.3	13.2 ± 0.2	89 ± 3	8199 ± 209	ND[h]	ND
	n = 4	*n* = 18	*n* = 18	*n* = 18	*n* = 10	*n* = 10		
α_3+Y	41 ± 2.8[f]	61.4 ± 1.4	41.9 ± 1.4	11.5 ± 0.2	184 ± 5	1134 ± 91	ND	ND
	n = 4	*n* = 9	*n* = 9	*n* = 9	*n* = 8	*n* = 8		
α_3D923N	216 ± 33[f]	70.9 ± 4.5	47.5 ± 2.4	12.7 ± 0.5	168 ± 9	1185 ± 37[g]	ND	ND
	n = 7	*n* = 13	*n* = 13	*n* = 7	*n* = 4	*n* = 10		

[a] Expression level relative to wild type was calculated from the active site concentration (phosphorylation capacity at 0 °C with 2 μM [γ-^{32}P]ATP in the presence of a saturating concentration of Na^+ and oligomycin (20 μg/ml) and absence of K^+, *cf.* Vilsen (23).

[b] The maximal molecular Na^+,K^+-ATPase activity (maximal catalytic turnover rate) was calculated as the ratio between the maximal Na^+,K^+-ATPase activity (determined at 3 mM ATP and optimal concentrations of Na^+ and K^+) and the corresponding active site concentration.

[c] The physiological molecular Na^+,K^+-ATPase activity is the ratio between Na^+,K^+-ATPase activity at physiological intracellular Na^+ and K^+ concentrations (15 mM Na^+ and 130 mM K^+) and the active site concentration.

[d] Physiological cellular Na^+,K^+-ATPase activity is the physiological molecular Na^+,K^+-ATPase activity multiplied by relative expression level.

[e] $K_{0.5}$ values for Na^+ activation of phosphorylation relative to wild type α_1 calculated from data in Table I.

[f] $K_{0.5}$ values for Na^+ activation of phosphorylation relative to the respective wild type α_1 or α_3 values, calculated from data in Refs. 12–14, 17, 23, and 26.

[g] Based on data in Refs. 12, 14, 17, 23, and 26.

[h] ND, not determined.

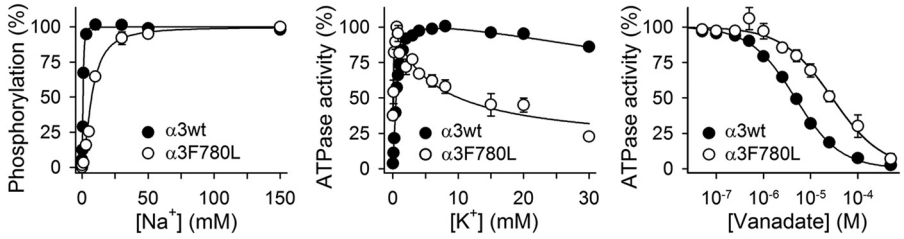

FIGURE 11. **Functional characterization of α_3F780L.** Na^+ dependence of phosphorylation (*left panel*) was determined by 10 s incubation with 2 μM [γ-^{32}P]ATP at 0 °C in the presence of 20 mM Tris (pH 7.4), 3 mM Mg^{2+}, 1 mM EGTA, 20 μg/ml oligomycin to block dephosphorylation, 10 μM ouabain to inhibit the endogenous COS cell enzyme, and the indicated concentrations of Na^+ with various concentrations of *N*-methyl-D-glucamine added to maintain a constant ionic strength. K^+ dependence of Na^+,K^+-ATPase activity (*middle panel*) was determined at 37 °C in the presence of 30 mM histidine (pH 7.4), 40 mM Na^+, 3 mM Mg^{2+}, 1 mM EGTA, 3 mM ATP, 10 μM ouabain, and the indicated concentrations of K^+. Vanadate dependence of Na^+,K^+-ATPase activity (*right panel*) was determined at 37 °C in the presence of 30 mM histidine (pH 7.4), 130 mM Na^+, 20 mM K^+, 3 mM Mg^{2+}, 1 mM EGTA, 3 mM ATP, 10 μM ouabain, and the indicated concentrations of vanadate. All experiments were carried out on isolated, leaky plasma membranes from transfected COS cells. Average values are shown with *error bars* indicating S.E. (only visible when larger than the size of the symbols). $K_{0.5}$ values with statistics are listed in Table 1.

α_2+28) in which the voltage dependence of transient currents indicated that a step accelerated by positive membrane potential is rate-limiting in this mutant (33). Our data obtained with α_1+28 provide direct evidence of defective Na^+ binding, because α_1+28 retained the ability to be phosphorylated from ATP, thus allowing the Na^+ affinity to be determined. This revealed a conspicuous 58-fold reduction of Na^+ affinity relative to wild type α_1. Hence, it may not be too far-fetched to speculate that Na^+ interaction is even more destabilized in α_2+28, thereby preventing accumulation of phosphoenzyme in α_2+28, even at 300 mM Na^+. A comparison of the amino acid sequences of α_1 and α_2 does not reveal any obvious reason for the difference between α_1+28 and α_2+28. Most amino acid differences occur in extracellular loops or in cytoplasmic regions such as the N domain or near the N terminus (2) where the distance from the C terminus is too large to allow direct interaction with the 28-residues extension. Although it is known that minor kinetic differences exist between the α_1 and α_2 isoforms, the structural basis for this is not clear. One possibility is that it is the outermost lysine of the 28-residues extension that disturbs Na^+ binding due to local conformational differences between α_1 and α_2 that particularly in α_2 allow access of this positively charged residue to the cation binding region.

The α_3+Y mutation reduces Na^+ affinity 41-fold (Tables 1 and 2). The present findings exclude that the observed effect on Na^+ affinity is indirect, caused by displacement of the E_1-E_2 conformational equilibrium in favor of E_2 because the apparent affinities for ATP and vanadate indicate that the +Y extension stabilizes the Na^+ binding E_1 form relative to E_2 (Fig. 3). The K^+ dependence showed a pronounced inhibition phase at high K^+ concentrations indicative of an enhanced ability of K^+ to compete with Na^+ at the E_1 site(s) resulting from the reduced Na^+ affinity. The activating phase of the K^+ dependence of α_3+Y furthermore indicated that the apparent K^+ affinity at the

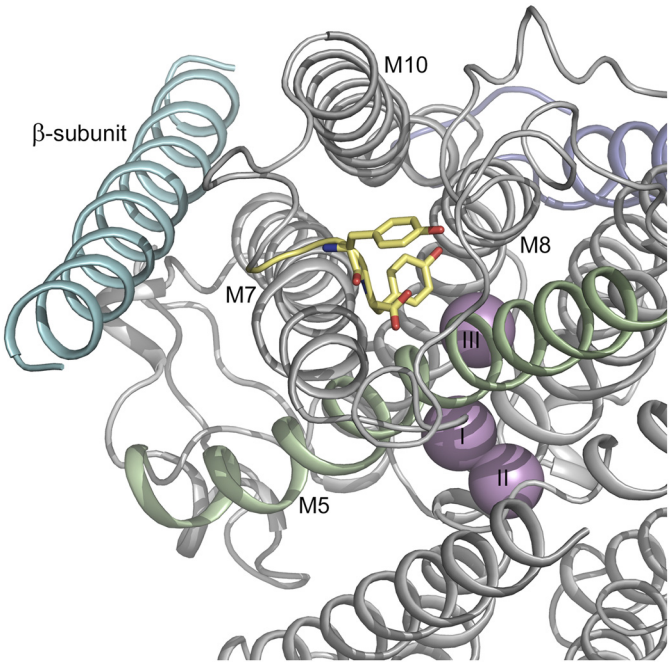

FIGURE 12. **Structural relations of the C terminus of Na^+,K^+-ATPase in Na^+-bound E_1 form.** View of the C-terminal region from the cytoplasmic side. The C-terminal five residues KETYY are indicated in *yellow* with the two terminal tyrosines highlighted by *stick representation*. The transmembrane helices M5 (*green*), M7, M8, and M10 of the α-subunit as well as the membrane helix of the β-subunit (*blue*) are labeled. The three Na^+ ions are represented by violet spheres numbered *I*, *II*, and *III*. Site III is the Na^+-specific third site, not involved in K^+ binding, which is located close to the M5 helix and is stabilized by the interaction of the C terminus with M5 (16).

externally facing sites of E_2P is higher than that of the wild type α_3 (Fig. 3). Our studies of +Y introduced in α_1 showed a 34-fold reduction of Na^+ affinity without reduction of K^+ affinity, and other functional properties were also similar to those of +Y in α_3, thus indicating that the isoform harboring the mutation is of minor importance in this case (Figs. 4–7, Tables 1 and 2). Likewise, another RDP mutation, α_3F780L, showed significantly reduced Na^+ affinity and no reduction of K^+ affinity, similar to the corresponding α_1 mutation, α_1F785L (Fig. 11 and Table 1, compare Ref. 12).

The introduction of the +28, +Y, and +A mutations in α_1 allowed us to compare the effects of these differently sized C-terminal extensions directly, independently of isoform variation. The magnitude of the reduction of Na^+ affinity (58-, 34-, and 24-fold, respectively, Fig. 4) appears to correlate with the size of the extension. Because neither the Na^+,K^+-ATPase in the isolated leaky membranes nor the sided system in intact cells showed any indication of a reduced K^+ affinity (Fig. 7), it may be concluded that only the Na^+-specific third site, not involved in K^+ binding, is disturbed, whereas the two sites thought to bind Na^+ and K^+ alternatively are intact. The importance of the C terminus in relation to the third Na^+ site is likely a consequence of the docking of the C-terminal tyrosines between transmembrane segments M5, M7, M8, and M10 (15, 16, 17). The recently published high resolution structure of the Na^+,K^+-ATPase with Na^+ bound at the three transport sites (Fig. 12) suggests that the C terminus works as a brake or damper that prevents excessive movements of the M5 helix, thereby stabilizing the Na^+ occluded state (16). Such a role is supported by the present finding that Na^+ binding from either side of the membrane is affected by the C-terminal extension mutations (Figs. 4, 8, and 9) because Na^+ can enter the binding pocket and become occluded from both membrane sides. The C-terminal extension likely interferes with the docking of the C terminus in the membrane, hence, with Na^+ occlusion.

We have taken advantage of the properties of a series of mutant Na^+,K^+ pumps to examine the relationship between change of Na^+ affinity and change of $[Na^+]_i$ and $[K^+]_i$ in intact cells expressing the mutants. For +Y, the changes of $[Na^+]_i$ and $[K^+]_i$ were detected independently of the isoform harboring the mutation, as seen from comparison of α_1+Y and α_3+Y. Our analysis of $[Na^+]_i$ and $[K^+]_i$ included in addition to +Y (in α_1 and α_3) and α_1+28 (α_2+28 could not be stably expressed) also RDP mutant α_3D923N and α_1F785L (homolog to RDP mutant α_3F780L). For each of these mutants a marked rise of $[Na^+]_i$ together with a decrease of $[K^+]_i$, relative to wild type, was observed. Low physiological cellular Na^+,K^+-ATPase activities amounting to 8–23% that of the wild type were calculated for the mutants with reduced Na^+ affinity as shown in Table 2, thus accounting for the observed rise of $[Na^+]_i$ and decrease of $[K^+]_i$ in the COS cells.

It has been proposed that reduced Na^+,K^+-ATPase protein expression is the pathophysiological mechanism leading to disease in case of +Y and certain other RDP mutations (6). However, for none of the mutants analyzed in the present study the low physiological cellular Na^+,K^+-ATPase activity owes to a markedly reduced expression level (Fig. 2*A* and Table 2). We find that specific functional changes, including a reduced Na^+ affinity, play major roles in the observed rise of $[Na^+]_i$ and decrease of $[K^+]_i$. The reduced Na^+ affinity leads to low occupation of the cytoplasmically facing Na^+ site(s) in the E_1 form, allowing K^+ to compete efficiently with Na^+ at the high physiological K^+ concentration of 130 mM, thereby leading to conversion of E_1 back to the E_2 state and, thus, to inhibition of activity (the inhibitory phase seen at high K^+ concentrations). In addition, slowing of a rate-limiting conformational change, as revealed by a reduced maximal molecular Na^+,K^+-ATPase activity, seems to contribute to reduce the physiological cellular Na^+,K^+-ATPase activity in some of the mutants (Table 2), thereby increasing $[Na^+]_i$. However, the results with α_1+28 and α_1YY-AA show clearly that a reduction of Na^+ affinity *per se*, with little or no reduction of the maximal molecular Na^+,K^+-ATPase activity, can lead to a marked rise of $[Na^+]_i$. It is also instructive to see that α_1T214A and α_1G330A cause a decrease of $[Na^+]_i$, *i.e.* increased pumping under physiological conditions despite the reduced maximal molecular Na^+,K^+-ATPase activity of these mutants. Relative to wild type, α_1T214A and α_1G330A exhibit an increased affinity for Na^+, which together with the high expression levels of these mutants leads to higher physiological cellular Na^+,K^+-ATPase activities compared with wild type, resulting in a low Na^+ concentration in the cytoplasm. Particularly for α_1G330A, K^+ seems unable to compete efficiently with Na^+ at the cytoplasmically facing sites of E_1, and the physiological molecular Na^+,K^+-ATPase activity is close to the maximal molecular Na^+,K^+-ATPase activity (Table 2, compare 5256 min^{-1} with 6796 min^{-1} for wild type).

Changes in $[Na^+]_i$ and $[K^+]_i$ may be part of the pathophysiological mechanisms underlying RDP and FHM2. A specific decrease of Na^+ affinity without impairment of K^+ binding appears to be a recurrent theme in RDP. Thus, in addition to α_3+Y and α_3F780L, also α_3D923N as well as two additional RDP mutants have been found to display this characteristic (12–14, 25). Several FHM2 mutations have been found to slow phosphorylation from ATP (32), thereby reducing the maximal molecular Na^+,K^+-ATPase activity. The deficiency of α_2+28 with respect to phosphorylation is in line with these observations, although for α_2+28 the effect is more pronounced than for the other FHM2 mutants characterized so far and possibly caused by defective Na^+ binding. In neurons as well as glial cells, an increase of $[Na^+]_i$ is expected to lead to a rise of $[Ca^{2+}]_i$ via the Na^+/Ca^{2+} exchanger, with secondary effects on Ca^{2+} signaling, and may also impair neurotransmitter reuptake by Na^+ gradient-dependent mechanisms. Epileptic seizures are common in FHM2 patients (34), whereas they have been described more rarely in patients with RDP (35). Although the pathophysiology of FHM2 may involve an impaired clearance of extracellular K^+, a reduced K^+ affinity is not a general finding in FHM2 (32). Although the K^+ affinity could not be addressed in α_2+28 due to the lack of phosphorylation, this parameter was wild type-like for α_1+28. Indeed, the lack of phosphorylation and, thus, ATPase activity seen for the α_2+28 mutant is likely to reduce the clearance of extracellular K^+ irrespective of any change of K^+ affinity.

The presently observed rise of $[Na^+]_i$ and decrease of $[K^+]_i$ caused by the mutations affecting Na^+ affinity are rather large, in the α_3 mutants amounting to as much as 40–50 mM. For comparison it may be mentioned that repetitive activation of excitatory synapses in the hippocampus is associated with 20–40 mM increases of $[Na^+]_i$ (36). Such an increase of $[Na^+]_i$ is similar to the recently reported rise of $[Na^+]_i$ in hippocampal neurons perfused with a large excess of the Na^+,K^+-ATPase inhibitor ouabain (37). Likewise, 20–30 mM changes to $[Na^+]_i$ and $[K^+]_i$ have been reported for skeletal muscle upon K^+ deprivation, resulting in a large reduction of the expression of α_2 Na^+,K^+-ATPase (38, 39). No measurements of $[Na^+]_i$ have been carried out in brain cells from RDP, AHC, or FHM2 patients (for obvious reasons) or in animal models of these disorders (40–42). It is important to stress that the patients are heterozygous, possessing a normal allele in addition to the mutant allele. The presence of some normally functioning Na^+,K^+-ATPase enzyme will of course limit the effect of the mutation in the patients, and the rise of $[Na^+]_i$ and decrease in $[K^+]_i$ are thus expected to be smaller than observed in our cell culture, where the normally functioning endogenous enzyme is silenced with ouabain. The studies of mouse models have, however, demonstrated that neurological/neurobehavioral changes are seen for animals with only 16–42% reduction of total brain Na^+,K^+-ATPase activity due to heterozygous α_3-silencing mutations (40–42). Recently, it has also been shown that a rise in $[Na^+]_i$ of only 2 mM is sufficient to alter Ca^{2+} transients in cardiac myocytes if caused by partial inhibition of the α_2-isoform with ouabain (43).

It has not been clear whether a change of Na^+ affinity of the Na^+,K^+-ATPase caused by mutation influences the ionic concentrations to the same extent as a change of the maximal pumping rate. The present finding that $[Na^+]_i$ rises when the Na^+ affinity is reduced and decreases when the Na^+ affinity is increased clearly indicates that, besides expression level and maximal molecular Na^+,K^+-ATPase activity (both being determinants of the maximal pumping rate), the Na^+ affinity *per se* is a major determinant of $[Na^+]_i$. This is an important concept because it also confirms that physiological regulation of the Na^+ affinity of the Na^+,K^+-ATPase, *e.g.* in response to hormones and excitation (44), as effectuated via change of interaction with various FXYD proteins (45) or PKA-mediated phosphorylation of the Na^+,K^+-ATPase α-subunit (37), can lead to a change of the intracellular Na^+ concentration. The PKA phosphorylation site is located in the C-terminal region of the protein, and transfer of a negatively charged phosphate group to this site likely disturbs the stabilizing effect of the C terminus on the third Na^+ site (17, 37). Because the Na^+,K^+-ATPase normally operates below its saturation level with respect to $[Na^+]_i$, exhibiting only a fraction of its maximal pump activity, regulation of pump activity can be achieved either by a change of $[Na^+]_i$ or by a change of Na^+ affinity. Hence, the normal low occupancy of the Na^+ sites under physiological conditions allows the cells to adjust to a lowering of Na^+ affinity induced by mutation through a compensatory rise of $[Na^+]_i$ that rescues pump activity to some extent. However, this rise of $[Na^+]_i$ may eventually lead to disease because of its effects on other cellular functions.

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