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The human non-gastric H,K-ATPase has a different cation specificity than the rat enzyme

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Abstract

The primary sequence of non-gastric H,K-ATPase differs much more between species than that of Na,K-ATPase or gastric H,K-ATPase. To investigate whether this causes species-dependent differences in enzymatic properties, we co-expressed the catalytic subunit of human non-gastric H,K-ATPase in Sf9 cells with the β_1 subunit of rat Na,K-ATPase and compared its properties with those of the rat enzyme (Swarts et al., J. Biol. Chem. 280, 33115–33122, 2005). Maximal ATPase activity was obtained with NH_4^+ as activating cation. The enzyme was also stimulated by Na^+, but in contrast to the rat enzyme, hardly by K^+. SCH 28080 inhibited the NH_4^+-stimulated activity of the human enzyme much more potently than that of the rat enzyme. The steady-state phosphorylation level of the human enzyme decreased with increasing pH, [K^+], and [Na^+] and nearly doubled in the presence of oligomycin. Oligomycin increased the sensitivity of the phosphorylated intermediate to ADP, demonstrating that it inhibited the conversion of E_1P to E_2P. All three cations stimulated the dephosphorylation rate dose-dependently. Our studies support a role of the human enzyme in H^+/Na^+ and/or H^+/NH_4^+ transport but not in Na^+/K^+ transport.

Keywords: H,K-ATPase; Cation-specificity; Ouabain; SCH 28080; Oligomycin

1. Introduction

The non-gastric H,K-ATPase is a member of the small IIC subfamily of P-type ATPases, which furthermore includes Na,K-ATPase and gastric H,K-ATPase [1]. These enzymes are characterized by their complete dependence on a β-subunit and the fact that they transport K^+ into the cell in exchange for either Na^+ or H^+. The non-gastric H,K-ATPase is about equally distant to Na,K-ATPase and gastric H,K-ATPase. In contrast to its family members, non-gastric H,K-ATPase has no own β-subunit. However, immunoprecipitation and expression studies suggest that it uses the β_1-subunit of Na,K-ATPase [2–5]. Species differences in amino acid sequence are only very minor (1–2%) for Na,K-ATPase and gastric H,K-ATPase [6]. For non-gastric H,K-ATPase, however, these differences are much larger (10–15%). This leaves the possibility of species-dependent variations in kinetic properties of non-gastric H,K-ATPase that are of physiological relevance.

At present, the physiological role of the non-gastric H,K-ATPase is unknown. The enzyme is mainly localized in colon brush border membranes and in apical membranes of epithelial cells located in the cortical collecting duct. The non-gastric H,K-ATPase in distal kidney, but not colon, is upregulated in rats put on a low K^+-diet [3,7–9], suggesting its involvement in K^+-reabsorption. Its name suggests that the enzyme exchanges H^+ for K^+ ions. However, in a recent study we found that the maximal ATPase activity of the rat enzyme is 3-fold higher with NH_4^+ than with K^+ [5]. This might suggest that the enzyme is involved in the reabsorption of NH_4^+ rather than K^+. At high concentrations, even Na^+ was able to partially activate the rat enzyme, which would suggest a role in Na^+ reabsorption.

Like the gastric H,K-ATPase, the non-gastric H,K-ATPase is electroneutral [10]. However, ion flux measurements in rat

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indicate that inward transport of K^+ is much larger than outward transport of H^+ [11]. Evidence has been provided that the enzyme might pump, in addition to H^+, Na^+ out of the cell [12,13]. However, Na^+ did not affect the steady-state phosphorylation level, even not at a pH of 8.5, of rat non-gastric H,K-ATPase expressed in Sf9 cells [5]. Because the enzyme completely obeyed the Post-Albers reaction mechanism, this finding did not support a role in outward Na^+ transport. Moreover, the apical localization of this enzyme would mean a transport of Na^+ in the luminal direction.

Another aspect of non-gastric H,K-ATPase is its sensitivity towards the inhibitors ouabain, specific for Na,K-ATPase, and SCH 28080, specific for gastric H,K-ATPase. Most studies indicate that non-gastric H,K-ATPase has a very low, but significant, sensitivity for ouabain. This was confirmed for the rat enzyme following expression in Sf9 cells [5]. The degree of inhibition might be species dependent [14,15]. The inhibitory effect of SCH 28080 on the Sf9 expressed rat enzyme was even smaller [5]. The degree of inhibition by both compounds strongly depended on the cation used to activate the enzyme. Swarts et al. [5] also found that oligomycin inhibited the ATPase activity, but in this case in a cation-independent fashion. Since the latter drug is primarily known to inhibit mitochondrial ATPases it has been used to inhibit these ATPases in an expression study with non-gastric H,K-ATPase [16], which may explain the very low ATPase activity measured in that particular study.

In animal studies, it is possible to determine the properties of non-gastric H,K-ATPase in brush border membranes isolated from colon [17]. However, a major problem with such membranes is the presence of other transporters. In this respect, the use of Sf9 cells has clear advantages. Moreover, Sf9 cells allow the study of the human enzyme. This is most important because the properties of the rat and human enzymes may be different due to differences in primary structure. Thus results obtained with the rat enzyme may not suffice for proper understanding of the physiological role of non-gastric H,K-ATPase in man. The work described in this paper shows that there are indeed large differences in cation-dependency and inhibitor-sensitivity between the human and the rat enzyme. Our findings emphasize that findings obtained with the rat enzyme should be treated with caution when transferred to the human situation.

2. Materials and methods

2.1. Recombinant H,K-ATPase

The cDNA of the non-gastric human H,K-ATPase α_2-subunit, a gift of Dr. H. Binder [18], was cloned with *Bam*HI behind the polyhedrin promotor in the pFastbacDual (pFD) vector (Invitrogen, Breda, The Netherlands). The cDNA of the non-gastric rat Na,K-ATPase β_3-subunit (NaKβ_3, also called HKcβ), a gift of Dr. H. Binder [18], was cloned with *Kpn*I and *Bam*HI into the pFD vector (*Bbs*I and *Kpn*I site) behind the P10 promotor. The generation of pFD vectors containing the β_1-subunit of rat Na,K-ATPase (NaKβ_1) and the β-subunit of rat gastric H,K-ATPase (HKβ) has been reported before [19,20]. Finally, the pFD-vectors were combined to yield: pFD-HKα_2, pFD-HKα_2-NaKβ1, pFD-HKα_2-NaKβ_3, pFD-HKα_2-HKβ. All DNA manipulations were carried out according to standard molecular biology techniques described by Sambrook et al. [21], and the modifications were controlled by sequence analysis.

2.2. Generation of recombinant viruses

The pFD vectors containing the different cDNAs were transformed to competent DH10bac *Escherichia coli* cells (Invitrogen) harboring the baculovirus genome (bacmid) and a transposition helper vector. Upon transposition between the Tn7 sites in the transfer vector and the bacmid, recombinant bacmids were selected and isolated [22]. Subsequently, insect Sf9 cells were transfected with recombinant bacmids using Cellfectin reagent (Invitrogen). After a three-day incubation period, recombinant baculoviruses were isolated and used to infect Sf9 cells at a multiplicity of infection of 0.1. Four days after infection, the amplified viruses were harvested.

2.3. Preparation of Sf9 membranes

Sf9 cells were grown at 27 °C in 100-ml spinner flask cultures as described by Klaassen et al. [19]. For production of H,K-ATPase, 1.5×10^6 cells ml^{-1} were infected at a multiplicity of infection of 1–3 in the presence of 1% (v/v) ethanol [23] and 0.1% (w/v) pluronic F-68 (ICN, Aurora, OH) in Xpress medium (Biowittaker, Walkersville, MD) as described before [24]. After 3 days, Sf9 cells were harvested by centrifugation at 2000×*g* for 5 min. The cells were washed once at 0 °C with 0.25 M sucrose, 2 mM EDTA, and 25 mM HEPES/Tris (pH 7.0), resuspended in sucrose/EDTA/Tris buffer and sonicated at 60 W (Branson Power Company, Denbury, CT) for 30 s at 0 °C. After centrifugation for 30 min at 10,000×*g* the supernatant was collected and recentrifuged for 60 min at 100,000×*g* at 4 °C. The pelleted membranes were resuspended in the above-mentioned buffer and stored at −20 °C.

2.4. Protein determination

The protein concentrations were quantified with the modified Lowry method according to Peterson [25] using bovine serum albumin as a standard.

2.5. Western blotting

Protein samples from the membrane fraction were solubilized in SDS-PAGE sample buffer and separated on SDS-gels containing 10% acrylamide according to Laemmli [26]. For immunoblotting, the separated proteins were transferred to Immobilon polyvinylidenefluoride membranes (Millipore Corporation, Bedford, MA). The expressed subunits were visualized with the antibodies Godiva, (HKα_2), 2G11 (HKβ), C385-M77 (NaKβ_1) and C386-M82 (NaKβ_3).

2.6. ATPase assay

The ATPase activity was determined using a radiochemical method [27]. For this purpose, 0.6–15 μg of Sf9 membranes were added to 100 μl of medium containing 10–2000 μM Mg-[γ-^{32}P]-ATP, 0.8 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM EDTA, 1 mM $TrisN_3$, 50 mM Tris–HCl (pH 7.0) and various concentrations of activating cations as indicated. Ionic strength was kept constant with choline chloride. After incubation for 30 min at 37 °C, the reaction was stopped by adding 500 μl 10% (w/v) charcoal in 6% (v/v) trichloroacetic acid and after 10 min at 0 °C the mixture was centrifuged for 10 s at 10,000×*g*. To 0.15 ml of the clear supernatant, containing the liberated inorganic phosphate ($^{32}P_i$), 3 ml OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added and the mixture was analyzed by liquid scintillation analysis. In general, blanks were prepared by incubating in the absence of membranes. ATPase activity is presented as the difference in activity between membranes of HKα_2-expressing cells and mock-infected cells.

2.7. ATP-dependent phosphorylation

ATP-dependent phosphorylation was determined as described before [23,24,28]. Sf9 membranes (1–6 μg) were incubated at 0 °C or 22 °C in 50 mM Tris–acetate (pH 6.0) containing 1.3 mM $MgCl_2$ and other ions and drugs as indicated in a volume of 80 μl. After 30–60 min, 20 μl of 0.5 μM [γ-^{32}P]ATP was added and the mixture was incubated for 10 s at the same temperature as before. The reaction was stopped by adding ice-cold 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid and the phosphorylated protein

was collected by filtration over a 0.8-μm membrane filter (Schleicher and Schuell, Dassel, Germany). After washing twice with 5 ml stop-solution, the filters were analyzed by liquid scintillation analysis. Data are corrected for the levels of phosphorylated protein obtained with mock-infected membranes.

2.8. Dephosphorylation studies

After ATP-dependent phosphorylation the reaction mixture was diluted from 100 μl to 500 μl with non-radioactive ATP (final concentration 1 mM; in order to prevent rephosphorylation with radioactive ATP) in 50 mM Tris–acetate (pH 6.0) and varying concentrations of cations or ADP as indicated. The mixture was incubated for another 3 s at 0 °C. The reaction was stopped as described above and the levels of phosphorylated protein were determined.

2.9. Potassium determinations

The K^+ concentrations of the enzyme containing assay media, to which no K^+ was added, were determined by flame photometry (FCM 6343, Eppendorf, Hamburg, Germany) and varied between 0.5 and 2.3 μM.

2.10. Gel electrophoresis

SDS gel electrophoresis of the phosphorylated proteins was carried out as described before [37].

2.11. Chemicals

Cellfectin, competent DH10bac *Escherichia coli* cells and all enzymes used for DNA cloning were purchased from Invitrogen. [γ-^{32}P]ATP (3000 Ci·mmol^{-1}) was obtained from Amersham Biosciences, Buckinghamshire, UK. Oligomycin was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). SCH 28080 was kindly provided by Dr. C.D. Strader (Schering-Plough, Kenilworth, NJ). The antibodies Godiva and 2G11 were gifts from Drs. M. Caplan (Yale) and J. Forte (Berkeley), respectively.

2.12. Analysis of data

All data are presented as mean values for two to five individual preparations with standard error of the mean. Differences were tested for significance by means of Student's *t*-test. IC_{50} and $K_{0.5}$ values were determined by analyzing the plots using the Non-Linear Curve Fitting program (Hill equation function) of Origin 6.1 (OriginLab Corporation Northampton, MA).

3. Results

3.1. Subunit expression in Sf9 cells

The α_2-subunit of human non-gastric H,K-ATPase ($HK\alpha_2$) was co-expressed with either the β_1-subunit of rat Na,K-ATPase ($NaK\beta_1$), the β_3-subunit of rat Na,K-ATPase ($NaK\beta_3$) or the β-subunit of rat gastric H,K-ATPase (HKβ) in Sf9 cells. After 72 h at 27 °C, total membranes were isolated and analyzed for proper expression of the relevant subunits. All β-subunits were present in non-glycosylated and core-glycosylated forms, as indicated by the mobilities of the various bands. (Fig. 1A). As usual in Sf9 cells, hardly any highly glycosylated β-subunit was found. A considerable, NH_4^+-stimulated, ATPase activity was obtained only with $NaK\beta_1$ (Fig. 1B, lane 5). With HKβ the activity was 50-times lower, whereas with $NaK\beta_3$ the activity was not significantly different from zero. Addition of tunicamycin during cell growth resulted in a dose-dependent reduction of the amount of glycosylated β-subunit accompanied by a similar reduction in NH_4^+-stimulated ATPase activity (Fig. 1, lanes 6 and 7).

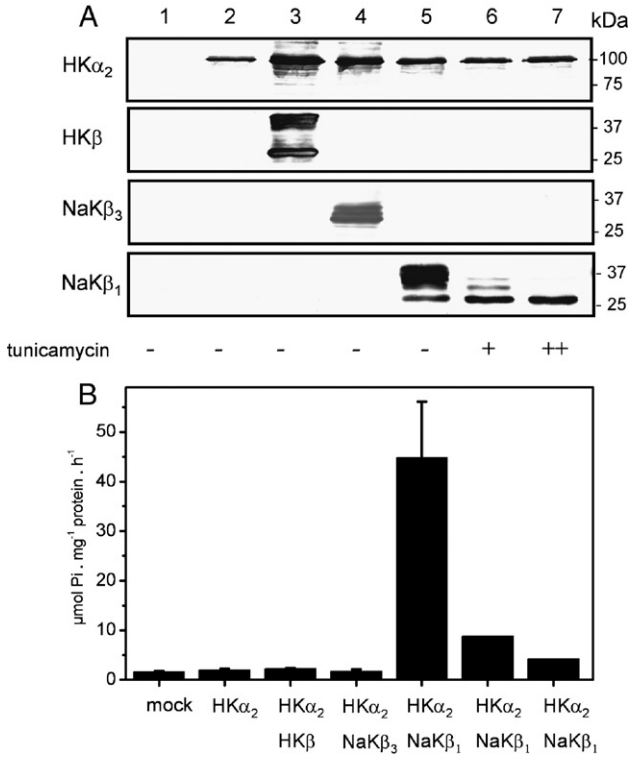

Fig. 1. Expression of non-gastric H,K-ATPase in Sf-9 cells. (A) Western blot (±10 μg membranes) of the different non-gastric H,K-ATPase preparations probed with anti-$HK\alpha_2$ (Godiva), anti-HKβ (2G11), anti-$NaK\beta_1$(C322-M77; [5]) and anti-$NaK\beta_3$ (C353-M82;[5]). In lanes 6 and 7 the $HK\alpha_2$-$NaK\beta_1$ preparations were expressed in the presence of 0.1 and 1 μg/ml tunicamycin, respectively. (B) ATPase activity of the membrane preparations measured in the presence of 2.0 mM MgATP, 0.1 mM EGTA, 0.2 mM EDTA, 0.8 mM $MgCl_2$, 1 mM $TrisN_3$, 50 mM Tris–Acetate (pH 7.0) and 10 mM NH_4Cl. Average with S.E. of 3–9 expression experiments.

3.2. Cation effects on the ATPase activity

In the presence of NH_4^+, the ATPase activity increased dose-dependently reaching a maximum of 41.1±2.2 μmol P_i mg^{-1} protein h^{-1} at 10 mM NH_4^+ (see Fig. 2A for a representative experiment). The activity was half-maximal at 2 mM NH_4^+. Surprisingly, the maximal activity obtained with Na^+ (100 mM) was very high (89.2±4.0% of that with 10 mM NH_4^+). However, compared to NH_4^+ about 10-times more Na^+ was required for half-maximal activation. In contrast, the maximal activity obtained with K^+ (10 mM) was only 27.2±3.2% of that with 10 mM NH_4^+ and decreased upon increasing the K^+ concentration to 100 mM. In the absence of added ions there was still a significant ATPase activity above the mock background. This activity was 11.8±1.8% of that with 10 mM NH_4^+. The results with human non-gastric H,K-ATPase markedly differ from those previously reported for the rat enzyme, where the maximum activities with K^+ and Na^+ were 60% and 20% of that with NH_4^+, respectively [5] (Table 1). The lower activity with K^+ as compared to NH_4^+ was not due to the presence of inside-out vesicles, since the activity did not increase upon

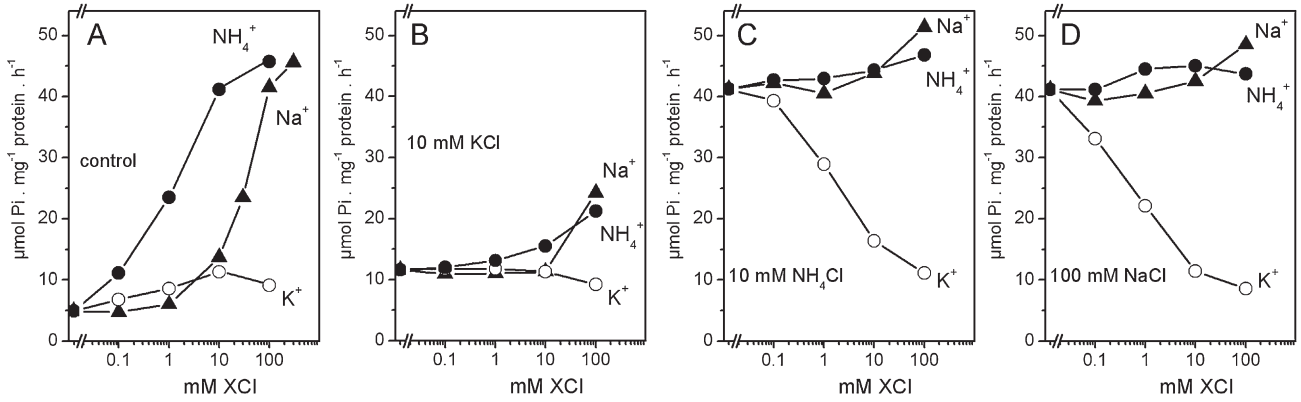

Fig. 2. Effect of various cations on the ATPase activity of HKα_2-NaKβ_1. The ATPase activity of the membrane preparations was measured in the presence of 2.0 mM MgATP, 0.1 mM EGTA, 0.2 mM EDTA, 0.8 mM $MgCl_2$, 1 mM $TrisN_3$, at pH 7.0 (50 mM Tris–acetate buffer) at the indicated KCl (○), NaCl (▲), and NH_4Cl (●) concentrations. In panel B 10 mM KCl, in panel C 10 mM NH_4Cl and in panel D 100 mM NaCl was additionally present. The ionic strength was kept constant with choline chloride. All ATPase activities were corrected for those of mock-infected membranes.

addition of low concentrations of nigericin or $C_{12}E_8$ (not shown).

In order to further understand the cation effects on the ATPase activity, we repeated the dose-activation curves in the presence of either 10 mM KCl (Fig. 2B), 10 mM NH_4Cl (Fig. 2C) or 100 mM NaCl (Fig. 2D). The figures show that the stimulatory effects of both NH_4^+ and Na^+ were blocked by relatively low concentrations of K^+. In addition, Fig. 2C and D show that the stimulatory effects of NH_4^+ and Na^+ were not additive, suggesting that these cations apparently act at the same binding site.

Despite the very low ATPase activity with HKβ we also studied this construct in more detail because this was reported in an earlier study [16]. Although the activities with HKβ were only about 2% of those with NaKβ_1 the relative cation dependencies were rather similar (not shown).

Cation activation of the enzyme using NaKβ_1 as accompanying subunit strongly depended on the concentration of ATP. Half-maximal activities were obtained at 0.1 mM ATP (Na^+), 0.4 mM ATP (NH_4^+) and 1 mM ATP (K^+), respectively (Fig. 3A). The ion-independent ATPase activity did not depend on the ATP concentration.

Vanadate is a potent inhibitor of P-type ATPases provided, however, that the enzyme is in the E_2 conformation [29]. Thus, the less vanadate is needed for inhibition of the enzyme, the more enzyme molecules are in the E_2 conformation under the given set of conditions. To determine the sensitivity of human non-gastric H,K-ATPase for this inhibitor, vanadate-inhibition curves were made at various cation concentrations. Fig. 3B shows that the IC_{50} values for vanadate inhibition decreased with increasing concentration of each of the three cations, indicating an increase in sensitivity for this inhibitor. Vanadate inhibited the ATPase activity at maximally stimulatory concentrations of K^+ (10 mM) and NH_4^+ (10 mM) equally potently and much more potently than that of Na^+ (100 mM). These findings indicate that at higher cation concentrations considerably more enzyme is in the E_2-form. When vanadate inhibition was measured at pH 6.0 instead of pH 7.0, more vanadate was needed to inhibit the ATPase activity (not shown). This is in agreement with an E_1-promoting effect of H^+ (see also, Fig. 10).

3.3. Effects of Inhibitors on the ATPase activity

Fig. 4 shows the effects of ouabain (A), SCH 28080 (B) and oligomycin (C) on the enzyme activity with either 10 mM NH_4^+, 10 mM K^+ or 100 mM Na^+. Ouabain inhibited the Na^+-activated enzyme much more potently than the NH_4^+-activated enzyme (apparent IC_{50} values of 56±10 µM and 380±50 µM,

Table 1
Kinetic parameters of ATPase activity of rat and human non-gastric H,K-ATPase

	Rat [5]			Human (this study)		
ATPase activity	NH_4^+ (10 mM)	K^+ (10 mM)	Na^+ (100 mM)	NH_4^+ (10 mM)	K^+ (10 mM)	Na^+ (100 mM)
Relative activity	100%[1]	**64.3%**	**18.4%**	100%[2]	**27.2%**	**89.2%**
$K_{0.5}$ cation	1 mM	1 mM	>30 mM	2 mM	1 mM	20 mM
$K_{0.5}$ ATP	0.3 mM	0.5 mM	0.1 mM	0.4 mM	1.2 mM	0.06 mM
IC_{50} vanadate	5 µM	1.5 µM	**1.0 mM**	1.3 µM	1.6 µM	**8 µM**
IC_{50} ouabain	0.2 mM	4 mM	0.05 mM	0.4 mM	>8 mM	0.06 mM
IC_{50} SCH 28080	**0.4 mM**	>5 mM	0.4 mM	**0.03 mM**	>4 mM	0.02 mM
IC_{50} oligomycin	3 µM	3 µM	3 µM	3 µM	3 µM	3 µM

$K_{0.5}$: The concentration at which 50% of the maximal activity is obtained. IC_{50}: the concentration of the inhibitor in which the activity is reduced to 50% of the maximal activity. (1) absolute activity=24.0±4.0 µmol Pi mg^{-1} protein. h^{-1}. (2) absolute activity=41.1±2.2 µmol Pi mg^{-1} protein h^{-1}.
In case that the differences between the rat and human enzyme are very large the numbers are indicated in bold.

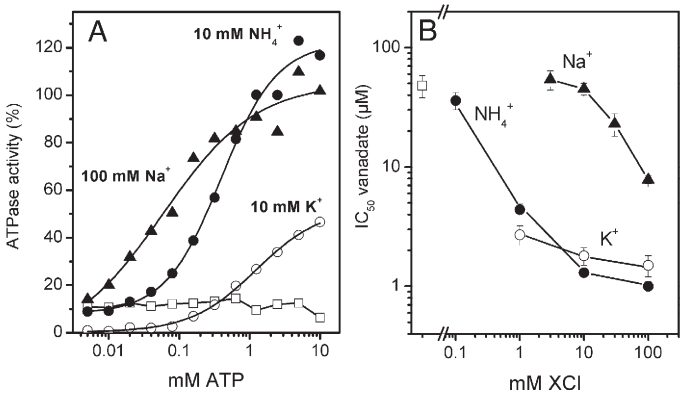

Fig. 3. The dependence of the cation-activated ATPase activity of HKα_2-NaKβ_1 on ATP and vanadate. (A) ATPase activity was measured in the presence of different MgATP concentrations as indicated, 0.1 mM EGTA, 0.2 mM EDTA, 0.8 mM $MgCl_2$, 1 mM $TrisN_3$, 50 mM Tris–Acetate pH 7.0 and either no added cations (□), or 100 mM NaCl (▲), or 10 mM KCl (○) or 10 mM NH_4Cl (●). Ionic strength was kept constant with choline chloride. The activity in the presence of 2.5 mM ATP and 10 mM NH_4^+ (= 32.1 μmol Pi. mg^{-1} protein h^{-1}) was set at 100%. (B) ATPase activity was measured with various vanadate and cation concentrations with 2 mM ATP and other additions as in panel A. At each cation concentration the IC_{50} value for vanadate was calculated as described under Materials and methods and plotted as function of the cation concentration used. All ATPase activities were corrected for those of mock-infected membranes.

respectively). In contrast, SCH 28080 inhibited the Na^+- and NH_4^+-activated enzyme almost equally potently (apparent IC_{50} values of 19±5 μM and 30±5 μM, respectively). Both the K^+-stimulated and the ion-independent activity were inhibited only at very high inhibitor concentrations. Fig. 4C shows that all activities were equally sensitive to inhibition by oligomycin (apparent IC_{50} values of 3±1 μM).

3.4. ATP-phosphorylation

P-type ATPases are called as such, because of the formation of a phosphorylated intermediate during the catalytic cycle. Fig. 5A depicts an SDS-PAGE electrophoretogram of the K^+-sensitive phosphorylated intermediate of human non-gastric H,K-ATPase (lanes 2 and 3 without and with K^+, respectively). The presence of either SCH 28080 (lane 4) or oligomycin (lane 5) apparently increased the level of phosphorylated intermediate. Ouabain (lane 6) had no effect, whereas vanadate (lane 7) decreased the amount of phosphorylated intermediate but to a lesser extent than K^+.

The steady-state level of the phosphorylated intermediate that is reached after 10 s in the absence of any added cation and at pH 6.0 and 0 °C, increased with the ATP concentration up to an apparently maximal value (Fig. 5B, open symbols). Scatchard plot analysis of these mock-corrected phosphorylation values revealed a high apparent affinity for ATP (K_d=40 nM), whereas extrapolation to infinite ATP concentrations indicated a maximal phosphorylation level of 19.6±4.0 pmol P mg^{-1} protein (Fig. 5C). These phosphorylation experiments were carried out at the lowest possible protein concentrations so that the endogenous K^+ concentration was minimal (≤1 μM). When the phosphorylation experiment was carried out in the presence of 100 μM oligomycin the maximal phosphorylation level markedly increased (31.2±6.4 pmol P mg^{-1} protein), whereas the apparent affinity increased about 4 times to 10±1 nM (see Fig. 5B and C, closed symbols).

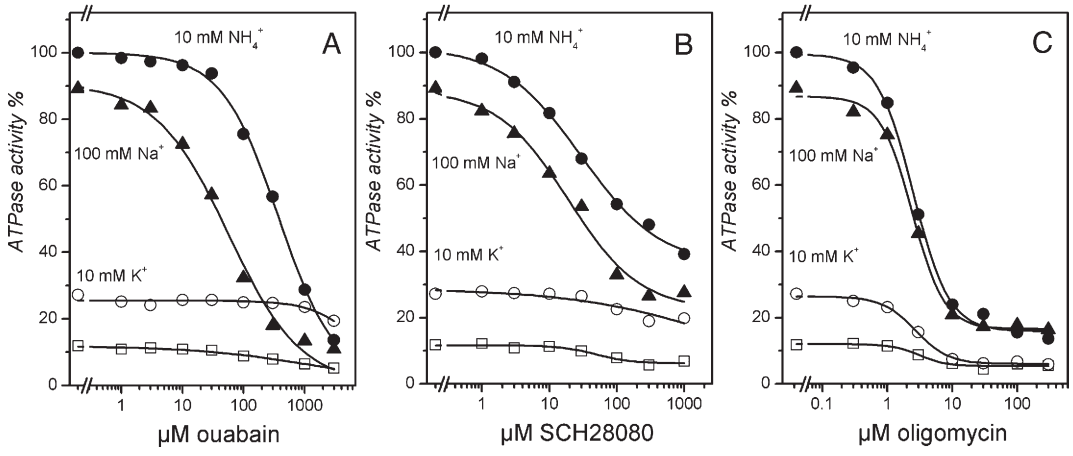

Fig. 4. The effects of ouabain (A), SCH 28080 (B) and oligomycin (C) on the ATPase activity of HKα_2-NaKβ_1. ATPase activity of the membrane preparations measured in the presence of 2.0 mM MgATP, 0.1 mM EGTA, 0.2 mM EDTA, 0.8 mM $MgCl_2$, 1 mM $TrisN_3$ and 50 mM Tris–acetate pH 7.0 in the absence of cations (□), with either 10 mM KCl (○), or 100 mM NaCl (▲), or 10 mM NH_4Cl (●) and the indicated inhibitor concentrations. The ionic strength was kept constant with choline chloride. All ATPase activities were corrected for those of mock-infected membranes. The activity in the presence of 10 mM NH_4Cl (42.2±9.0 μmol Pi. mg^{-1}. h^{-1}) without inhibitor was set at 100%.

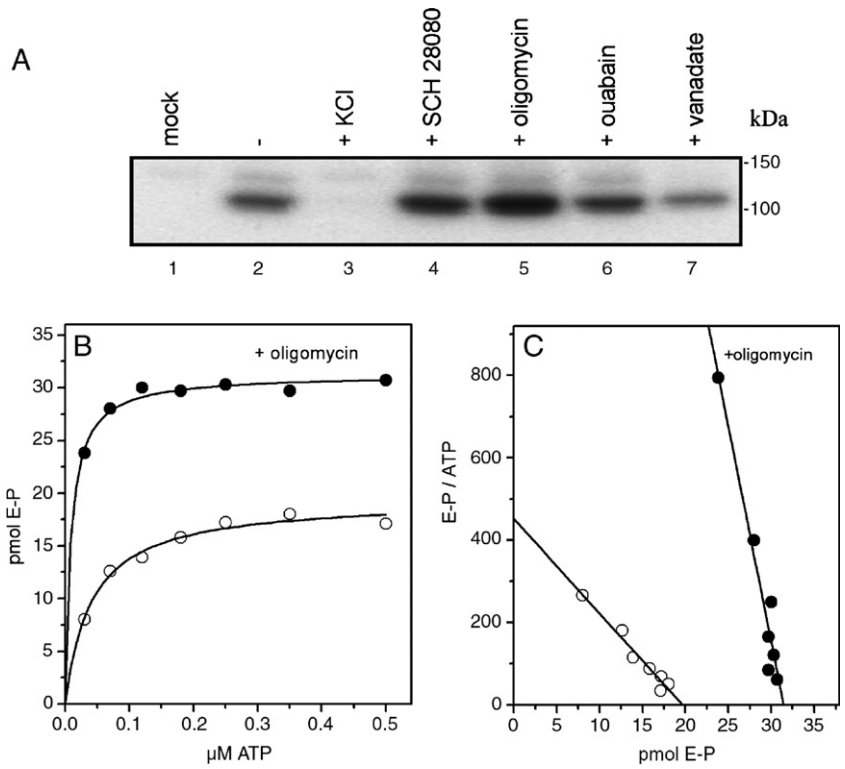

Fig. 5. The ATP dependence of the ATP phosphorylation level of $HK\alpha_2$-$NaK\beta_1$. (A) SDS-PAGE electrophoretogram of phosphorylated membranes of mock-infected cells (lane 1), and non-gastric H,K-ATPase (lanes 2 to 7) Phosphorylation was carried in the presence of either 10 mM KCl, 0.1 mM SCH 28080, 0.1 mM oligomycin, 1.0 mM ouabain and 1.0 mM vanadate. SDS gel electrophoresis was carried out as described before [37]. (B) Steady-state phosphorylation levels of $HK\alpha_2$-$NaK\beta_1$. Membranes of $HK\alpha_2$-$NaK\beta_1$ were treated with (●) and without (○) 100 μM oligomycin for 30 min at 0 °C after which they were incubated in the presence of the indicated ATP concentrations and 0.1 mM EGTA, 0.2 mM EDTA, 1.3 mM $MgCl_2$, 1 mM $TrisN_3$ and 50 mM Tris–acetate pH 6.0 for another 10 s. The phosphorylation level was determined as described under Materials and methods and corrected for the activity of mock-infected cells. (C) Scatchard plot of the data from panel B. Control: K_d apparent = 0.04 ± 0.004 μM, $E\text{-}P_{max}$ = 19.6 ± 4.0 pmol P. mg^{-1} protein. With oligomycin: K_d apparent = 0.01 ± 0.001 μM, $E\text{-}P_{max}$ = 31.2 ± 6.4 pmol P. mg^{-1} protein.

In the absence of oligomycin, the steady-state phosphorylation level decreased with increasing pH value (Fig. 6). A 10-fold decrease was observed when going from pH 6.0 to pH 8.0. This is in agreement with a stimulatory effect of H^+ on the phosphorylation reaction. At pH 6.0, Na^+ dose-dependently decreased the steady-state phosphorylation level to about half the initial value. At pH 7.0, however, this cation decreased the steady-state phosphorylation level down to zero. The very low level at pH 8.0 was only slightly further decreased at Na^+ concentrations beyond 100 mM. Na^+ never enhanced the steady-state phosphorylation level even not at pH 8.0, demonstrating that this cation does not stimulate the phosphorylation reaction. This was neither the case when the steady-state phosphorylation level was determined in the presence of oligomycin both at pH 6.0 and pH 8.0 (not shown).

When measured at pH 6.0 and 22 °C, the steady-state phosphorylation level was dose-dependently increased by SCH 28080 (Fig. 7A). This effect of the inhibitor was considerably less when the reaction was carried out at 0 °C (not shown). The observed increase can be explained by the fact that the phosphorylation level obtained under these conditions was significantly lower than the maximum attainable level. Ouabain did not increase the steady-state phosphorylation level at either 0 °C (not shown) or 22 °C (Fig. 7B). Fig. 7C shows that ouabain did also not alter the phosphorylation level when SCH 28080 was already present at its optimal concentration (100 μM).

The properties of the phosphorylated intermediate(s) generated in the absence (Fig. 8A) or presence of oligomycin (Fig. 8B) were tested by first phosphorylating the enzyme with or without this inhibitor, followed by a 3 s chase in the absence or presence of either 2 mM ADP or 1.0 mM K^+. Fig. 8A shows that in the control situation only 15 ± 2% of the phosphorylated intermediate was not hydrolyzed within 3 s of incubation in the absence of any added cation. In the presence of oligomycin, this value was slightly reduced to 28 ± 2% (Fig. 8B). ADP somewhat increased the dephosphorylation rate in the control situation, but dramatically increased this rate in the oligomycin condition. Conversely, K^+ did not alter the dephosphorylation rate in the oligomycin condition, whereas it stimulated the dephosphorylation step in the control situation. These findings indicate that the phosphorylated intermediate generated in the presence of oligomycin is nearly completely in the E_1-P conformation, whereas the intermediate generated in its absence is partly in the E_1-P and partly in the E_2-P form.

The cation specificity of the dephosphorylation reaction was further studied in the absence of oligomycin (Fig. 9). All three cations used in this study dose-dependently increased the dephosphorylation rate but the apparent affinity for Na^+ (Fig. 9C) was much lower that that for K^+ (Fig. 9A) and NH_4^+ (Fig. 9B).

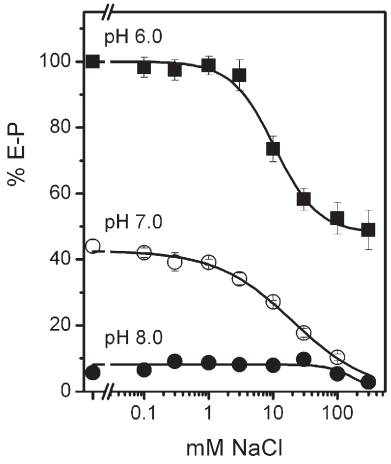

Fig. 6. The effect of NaCl on the steady-state ATP phosphorylation level of HKα_2-NaKβ_1. The membranes of HKα_2-NaKβ_1 were incubated with 0.1 μM ATP, 0.1 mM EGTA, 0.2 mM EDTA, 1.3 mM $MgCl_2$, 1 mM $TrisN_3$ and 50 mM Tris–Acetate pH 6.0 (■), pH 7.0 (○) or pH 8.0 (●) and the indicated NaCl concentrations for 10 s at 0 °C. Data were corrected for those of mock-infected cell membranes. and the phosphorylation level in the absence of added cation at pH 6.0 (19.7±3.1 pmol mg^{-1} protein) was set at 100%.

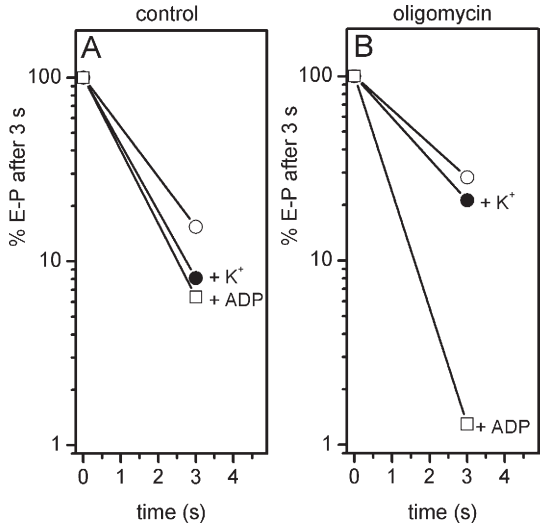

Fig. 8. The effect of K^+ and ADP on the apparent dephosphorylation rates of phosphorylated intermediates of HKα_2-NaKβ_1 generated in the absence (panel A) and presence of oligomycin (panel B). The membranes of HKα_2-NaKβ_1 were incubated with 0.1 μM ATP, 0.1 mM EGTA, 0.2 mM EDTA, 1.3 mM $MgCl_2$, 1 mM $TrisN_3$ and 50 mM Tris–Acetate pH 6.0 without further addition (panel A), or 100 μM oligomycin (panel B) for 10 s at 0 °C. Next, the mixture was 8.3 times diluted with 1 mM non-radioactive ATP without (○) and with either 1 mM KCl (●) or 2 mM ADP (□) after which the incubation was continued for another 3 s. The residual phosphorylation level corrected for that of mock-infected cell membranes was expressed as percentage of the level at $t=0$. The 100% values were: no addition: 15.6±2.1 pmol. mg^{-1} protein; oligomycin: 37.6±1.1 pmol. mg^{-1} protein.

In this study the β_1-subunit of rat Na,K-ATPase was used as accompanying subunit. In order to exclude the possibility that the difference in cation sensitivities between the human and the rat non-gastric H,K-ATPase could be attributed to the use of two different species in the human study, we carried out some experiments with the human β_1-subunit. The cation dependence of the latter preparation was similar to that of the preparation with the rat β_1-subunit as shown in Fig 2A. The sensitivity for inhibitors, like vanadate, oligomycin, ouabain and SCH 28080 was also hardly changed (not shown).

4. Discussion

In this study, we investigated the enzymatic properties of the human non-gastric H,K-ATPase following functional expression together with the β_1 subunit of Na,K-ATPase in Sf9 cells. The presence of this particular β subunit was absolutely required because a 50 times lower activity was obtained in combination with the β-subunit of gastric H,K-ATPase, whereas the combination with the β_3-subunit of Na,K-ATPase showed no activity at all. Therefore, the latter two β-subunits were not included in the remainder of this study.

This study was carried out with the rat β_1-subunit, which excludes the possibility that kinetic differences between human and rat can be attributed to the species of the β_1-subunit.

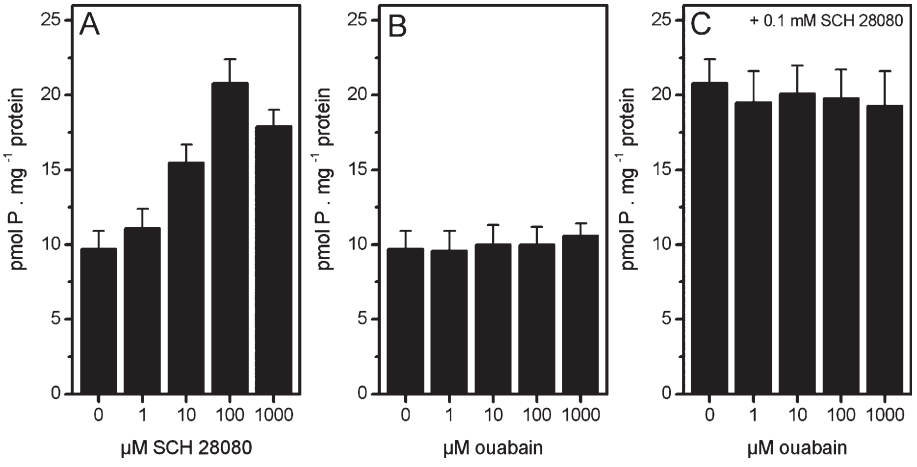

Fig. 7. Effects of SCH 28080 and ouabain on the steady-state phosphorylation levels of HKα_2-NaKβ_1. The membranes of HKα_2-NaKβ_1 were incubated with 0.1 μM ATP, 0.1 mM EGTA, 0.2 mM EDTA, 1.3 mM $MgCl_2$, 1 mM $TrisN_3$ and 50 mM Tris–Acetate pH 6.0 and the indicated SCH 28080 (A and C) and ouabain (B and C) concentrations for 10 s at 22 °C. Data given as means with SEM for 3 experiments were corrected for those of mock-infected cells.

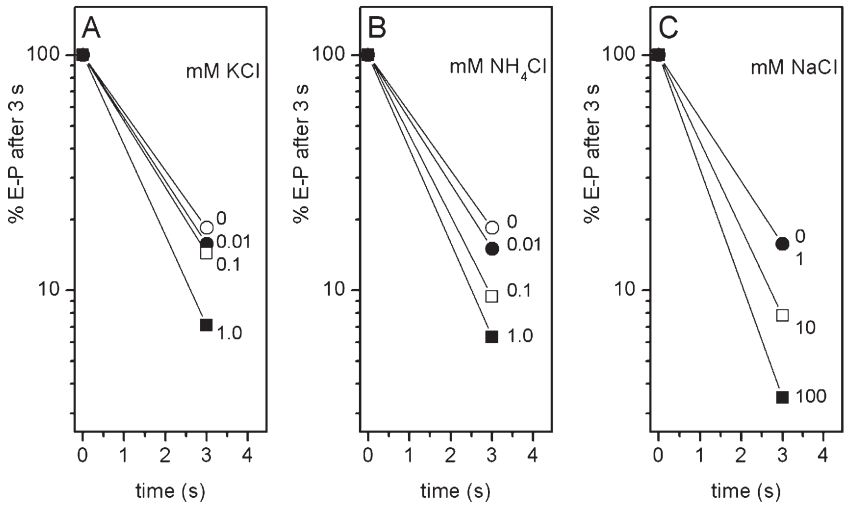

Fig. 9. The effect of cations on the dephosphorylation of phosphorylated intermediates of HKα_2-NaKβ_1. The membranes of HKα_2-NaKβ_1 were incubated at 0 °C for 10 s with 0.1 μM ATP, 0.1 mM EGTA, 0.2 mM EDTA, 1.3 mM $MgCl_2$, 1 mM $TrisN_3$ and 50 mM Tris–acetate pH 6.0. Next, the mixture was 8.3 times diluted with 1 mM non-radioactive ATP and the indicated KCl (A), NH_4Cl (B) or NaCl (C) concentrations and the incubation was continued for 3 s. The residual phosphorylation level corrected for that of mock-infected cell membranes was expressed as percentage of the level at t=0 (100%=23.8±6.4 pmol. mg^{-1} protein).

However, the experiments carried out with the human β_1-subunit suggest that the species of the β_1-subunit is not important for the findings of this study.

The majority of the rat β_1-subunit was glycosylated and inhibition of the glycosylation process with tunicamycin revealed that, as with the gastric H,K-ATPase [32], glycosylation is essential for cation activation of human non-gastric H,K-ATPase. This is in sharp contrast with the situation in Na,K-ATPase, the normal partner of the β_1-subunit, where prevention of glycosylation by mutation of all three asparagines into glutamine did not impede the catalytic activity of the enzyme [33].

The present study yielded several surprising results. In the first place, we found that the activity of human non-gastric H,K-ATPase could well be stimulated by NH_4^+ but hardly by K^+ (Fig. 2A). Because the enzyme fully obeys the Post-Albers reaction scheme and both ions stimulated the dephosphorylation reaction with similar affinity (Fig. 9), this finding most likely indicates that K^+ has a much stronger E_2-promoting effect than NH_4^+. This idea was substantiated by the observation that considerably more ATP was needed for activation of the enzyme by K^+ than for its activation by NH_4^+ (Fig. 3A). ATP promotes the E_2 to E_1 conversion (step 1 in Fig. 10) and this reaction is counteracted by cations that stimulate step 5 in the opposite direction. In addition, we found that high Na^+ concentrations could increase the activity of human non-gastric H,K-ATPase to a level that approached the maximal level obtained with NH_4^+. In agreement with this result, Na^+ was found to stimulate the dephosphorylation reaction of this enzyme and to have a strong E_1-promoting effect. Thus, in contrast to K^+, Na^+ does not slow down the E_2 to E_1 conversion (Fig. 10). Compared to the rat enzyme, the stimulatory effects of Na^+ and K^+ on the activity of the human enzyme are much larger and smaller, respectively [5].

A second surprising result was that Na^+ could not increase the steady-state phosphorylation level, even not at pH 8.0 where this level is very low in the absence of this cation. This finding indicates that Na^+ is an agonist for K^+ and NH_4^+ and not an agonist for H^+. A similar conclusion was reached before with the non-gastric [5] and gastric H,K-ATPase [27] of the rat. Thus, our findings do not support the idea that non-gastric H,K-ATPase transports Na^+ along with H^+ out of the cell [12,13].

Thirdly, this study shows that the human non-gastric H,K-ATPase is moderately sensitive to ouabain. Sensitivity was highest for the Na^+-activated enzyme, less high for the NH_4^+-activated enzyme and absent for the K^+-stimulated enzyme. This is in agreement with the well-known antagonistic effect of K^+ on ouabain binding to Na,K-ATPase.

In the fourth place, the human enzyme appeared moderately sensitive to SCH 28080, whereas the rat enzyme was shown by us before to be virtually insensitive to this inhibitor (Table 1).

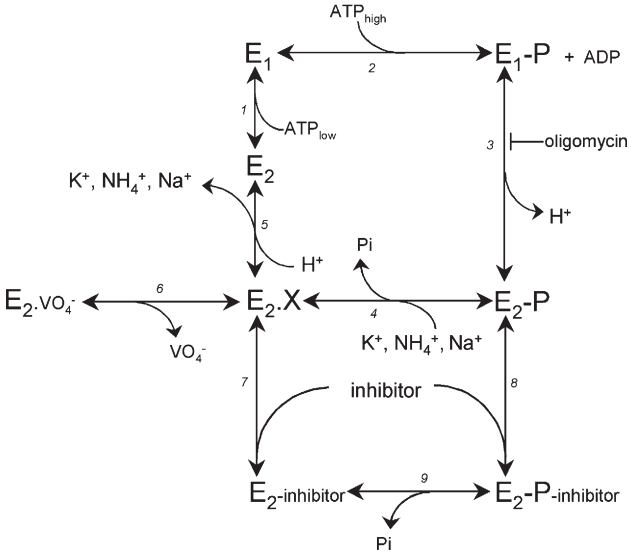

Fig. 10. Post-Albers reaction scheme for H,K-type ATPases. The simplified scheme includes the site of vanadate and oligomycin inhibition.

Like with ouabain, the sensitivity was highest with Na^+, slightly less with NH_4^+ and almost absent with K^+. Also in this case, the antagonism between K^+ and SCH 28080 binding might explain this difference.

Interestingly, we found that SCH 28080 could increase the steady-state phosphorylation level of human non-gastric H,K-ATPase (Fig. 7A). A similar finding was previously made with rat gastric H,K-ATPase and explained by a stabilizing effect of SCH 28080 on the phosphorylated intermediate [30]. Most likely this explanation holds also for human non-gastric H,K-ATPase. SCH 28080 did not increase the phosphorylation level of rat non-gastric H,K-ATPase, which is in agreement with its lower affinity for this inhibitor (not shown). The present finding that the stabilizing effect of SCH 28080 was less pregnant at 0 °C, probably points to differences in temperature sensitivity of the partial reactions. Surprisingly, no increase in phosphorylation level was found with ouabain (Fig. 7B). We previously reported that ouabain readily increased the phosphorylation level of a chimera between rat gastric H,K-ATPase and Na,K-ATPase [31]. Therefore, we assume that ouabain, in contrast to SCH 28080, is unable to stabilize the phosphorylated intermediate of the human non-gastric H,K-ATPase.

In some other aspects the human non gastric H,K-ATPase behaved rather similar to the rat enzyme. Oligomycin approximately doubled the phosphorylation level and the phosphorylated intermediate in the presence of this drug became ADP-sensitive and lost its K^+-sensitivity. This indicates that as in Na,K-ATPase and rat non-gastric H,K-ATPase oligomycin inhibits the E_1P to E_2P conversion (reaction 3 in Fig. 10).From the maximal NH_4^+-stimulated ATPase activity and the phosphorylation level measured in the presence of oligomycin a turnover number of $21{,}900 \pm 4600\ min^{-1}$ could be calculated. This value was not significantly different from that calculated for rat non-gastric H,K-ATPase [5], despite the fact that the absolute ATPase activity of the expressed human enzyme was about twice as high. As far as we know, it is the highest P-type ATPase activity ever measured in an expression system.

Table 1 summarizes the differences and similarities between the expressed human and rat enzymes as carried out in our laboratory. It is clear that the differences are mainly in the sensitivities for cations and drugs. The differences cannot be attributed to the β-subunit since in both preparations the β_1 subunit of rat Na,K-ATPase was used. Why are the properties of both enzymes so different? At the amino acid level the similarity between the human and rat enzyme is 86%, which is relatively low as compared to the species differences observed for Na,K- and gastric H,K-ATPase. The lowest similarity (50%) is present in the region encompassing the first 60 N-terminal amino acids. In agreement with the nature of the differences between human and rat non-gastric H,K-ATPase, this region was found to play an important role in regulating the E_1/E_2 equilibrium in Na,K-ATPase [34]. Transmembrane segments play an important role in both cation and drug binding. Within the ten putative transmembrane segments, 27 out of 200 amino acids are different, which is quite normal for subunits of this subfamily of ATPases. Strikingly, there are only two and three conservative differences in M4 and M5, respectively, whereas the amino acids in M6 are completely similar. None of the residues found to be crucial for cation binding in Na,K-ATPase and gastric H,K-ATPase differs between the human and rat non-gastric H,K-ATPase. This means that the observed differences in ion sensitivities between these two species must be due to subtle differences in amino acid composition that play a role in the hydrogen bond network around the ion binding sites and/or in residues involved in the access pathways to these binding sites.

Vagin et al. [35] postulated a model for the binding site of SCH 28080 on gastric H,K-ATPase. In their model the surface between Leu^{809} in the M5-M6 loop and Cys^{813} in the luminal end of M6 are implicated in SCH 28080 binding. In addition, residues on M8 play a role in SCH 28080 binding. As stated before, none of the residues in M6 are different between human and rat non-gastric H,K-ATPase. On the other hand, there are four differences between human and rat in M8. However, all four are rather far away from the postulated SCH 28080 binding site. There are also indications that Met^{334} and Val^{337} (corresponding to Ile^{325} an Ile^{328} in the rat non gastric H,K-ATPase) in M4 are involved in SCH 28080 binding [36]. It is very well possible that the Gly/Ser difference between human and rat in residue 326 causes the lower SCH 28080 affinity of the rat enzyme. Taken together, on basis of the present knowledge it is impossible to attribute the differences in cation and drug sensitivities between human and rat to specific residues.

What do these studies tell us about the physiological role of non-gastric H,K-ATPase in man? In rat it has been shown that after a hypokalemic diet this enzyme is upregulated in the kidney [3,8,9]. This has led to the conclusion that the primary role of this enzyme is in K^+ reuptake by the kidney. For the rat enzyme, we have reported that the maximum activity with NH_4^+ is much higher than with K^+ [5]. In human this difference is even higher. Studies with hypokalemia are nearly impossible to carry out with humans. It is therefore not sure whether also in man this condition results in an upregulation of non-gastric H,K-ATPase. The apical localization of this enzyme together with the present finding that Na^+ cannot replace H^+ as activating cation, suggests a role of this enzyme in apical H^+/ NH_4^+ exchange. Such an electroneutral transport would not contribute to pH regulation, but only to prevention of a too large loss of NH_4^+. It is also possible that under certain circumstances the enzyme might function as a H^+/Na^+ exchanger given the high ATPase activity that can be obtained with high Na^+ concentrations.

Acknowledgements

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