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.
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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

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 $2000 \times g$ 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 $10,000 \times g$ the supernatant was collected and recentrifuged for 60 min at $100,000 \times g$ 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 $10,000 \times g$. 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