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Pharmacological profiles of the murine gastric and colonic H,K-ATPases

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ABSTRACT

Background: The H,K-ATPase, consisting of α and β subunits, belongs to the P-type ATPase family. There are two isoforms of the α subunit, HKα$_1$ and HKα$_2$ encoded by different genes. The ouabain-resistant gastric HKα$_1$-H,K-ATPase is Sch28080-sensitive. However, the colonic HKα$_2$-H,K-ATPase from different species shows poor primary structure conservation of the HKα$_2$ subunit between species and diverse pharmacological sensitivity to ouabain and Sch28080. This study sought to determine the contribution of each gene to functional activity and its pharmacological profile using mouse models with targeted disruption of HKα$_1$, HKα$_2$, or HKβ genes.
Methods: Membrane vesicles from gastric mucosa and distal colon in wild-type (WT), HKα$_1$, HKα$_2$, or HKβ knockout (KO) mice were extracted. K-ATPase activity and pharmacological profiles were examined.
Results: The colonic H,K-ATPase demonstrated slightly greater affinity for K^+ than the gastric H,K-ATPase. This K-ATPase activity was not detected in the colon of HKα$_2$ KO but was observed in HKβ KO with properties indistinguishable from WT. Neither ouabain nor Sch28080 had a significant effect on the WT colonic K-ATPase activity, but orthovanadate abolished this activity. Amiloride and its analogs benzamil and 5-*N*-ethyl-*N*-isopropylamiloride inhibited K-ATPase activity of HKα$_1$-containing H,K-ATPase; the dose dependence of inhibition was similar for all three inhibitors. In contrast, the colonic HKα$_2$-H,K-ATPase was not inhibited by these compounds.
Conclusions: These data demonstrate that the mouse colonic H,K-ATPase exhibits a ouabain- and Sch28080-insensitive, orthovanadate-sensitive K-ATPase activity. Interestingly, pharmacological studies suggested that the mouse gastric H,K-ATPase is sensitive to amiloride.
General Significance: Characterization of the pharmacological profiles of the H,K-ATPases is important for understanding the relevant knockout animals and for considering the specificity of the inhibitors.
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1. Introduction

The H,K-ATPase is an integral membrane protein that actively transports protons (H^+) and potassium (K^+) ions across the plasma membrane and is important for acid–base balance and potassium homeostasis [1]. The H,K-ATPase belongs to the P-type ATPase family and consists of two essential subunits, α and β. The α subunit contains ATP and cation binding sites responsible for ATP hydrolysis and electroneutral exchange of H^+ and K^+. The highly glycosylated β subunit is involved in the correct assembly, plasma membrane localization, and stabilization of the holoenzyme [2]. There are two isoforms of the α subunit (HKα$_1$ and HKα$_2$) and each exhibits a unique tissue distribution. The HKα$_1$ isoform is expressed in the stomach, kidney, and other tissues [3,4]. The HKα$_1$ isoform in the gastric parietal cells is the motor of the ion transport system responsible for acid secretion. The gastric H,K-ATPase is sensitive to the P-type ATPase inhibitor orthovanadate, and to the more specific inhibitors Sch28080 and omeprazole, but is insensitive to ouabain, an Na,K-ATPase inhibitor [5]. The HKα$_2$ isoform is expressed in the distal colon, skin, kidney, prostate, and uterus [6], principally expressed in distal colon epithelial cells, and renal collecting duct. Colonic H,K-ATPase was initially characterized as a ouabain-insensitive activity in the rabbit descending colon epithelium membranes [7]. Biochemical studies found that the apical membranes from rat distal colon epithelial cells contain both ouabain-sensitive and ouabain-insensitive K-ATPase activities [8,9].

HKα$_2$ has been cloned from several species [10–13]. The mammalian interspecies difference in amino acid composition of the

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α_2 subunit of the colonic H,K-ATPase is about 15%, in contrast to only 1–2% difference in amino acid sequence of gastric H,K-ATPase and Na,K-ATPase from different species. The properties of the colonic H,K-ATPase have been studied in several functional expression systems including *Xenopus* oocytes [13–18], HEK 293 cells [19–21], and baculovirus expression system [22–24]. These studies yield rather diverse results, especially regarding the pharmacological sensitivity to ouabain and Sch28080. This might be due to the different species and functional expression systems used. Most functional expression studies have been performed with rat colonic H,K-ATPase [15,16,21–23]. Expression of rat HKα_2 in *Xenopus* oocytes resulted in Sch28080-insensitive and poorly ouabain-sensitive K^+-activated rubidium uptake and K-ATPase activity, with IC50 values at 0.4–0.6 mM or 1 mM, respectively [15,16]. Rat HKα_2 expressed in HEK293 cells resulted in a ouabain- and Sch28080-insensitive, orthovanadate-sensitive K-ATPase activity and rubidium uptake [21]. Similar results were observed with rat HKα_2 expressed in baculovirus expression system [23]. However, rat HKα_2 without pairing β subunit expressed in insect Sf9 cells displayed a ouabain-resistant, Sch28080-sensitive K-ATPase activity with 18% inhibition at 0.1 mM Sch28080 [22]. Overall, the functionally expressed rat colonic H,K-ATPase is rather insensitive to the inhibition by Sch28080 and ouabain. *Bufo marinus* bladder H,K-ATPase expressed in *Xenopus* oocytes displays intermediate sensitivity to ouabain and Sch28080 with Ki of 25 μM and 230 μM, respectively [13]. Expression of human HKα_2 in *Xenopus* oocytes, HEK 293 cells, and baculovirus expression system resulted in similar Sch28080- and ouabain-sensitive rubidium uptake and ATPase activity [18,20,24]. However, guinea pig HKα_2 expressed in HEK 293 cells shows intermediate ouabain sensitivity (IC50 value of 52 μM) but resistance to Sch28080 [19]. Thus, there is a need to characterize the pharmacological profile of the colonic H,K-ATPase in mouse, given the ability to examine the effect of single gene disruption in the intact animal.

In recent years, mouse models with targeted disruption of the HKα_1, HKα_2, or HKβ genes [25–27] have been generated, allowing the contribution of the specific gene to functional activity to be unambiguously deciphered. In the present study, we measured K-ATPase activity in the native tissues of wild-type mice compared to the HKα_1, HKα_2, or HKβ knockout (KO) mice. Use of the KO mice allows the wild-type enzymatic activities to be specifically linked to each gene in question. Results from our study of HKα_1 knockout mice were consistent with the reported properties of the enzyme, thereby validating the assay conditions. Comparison of the K-ATPase activity between wild-type and HKα_2 knockout mice demonstrated that the colonic H,K-ATPase exhibits a ouabain- and Sch28080-insensitive, orthovanadate-sensitive K-ATPase activity. Interestingly, our pharmacological studies suggested that the gastric H,K-ATPase is sensitive to amiloride.

2. Materials and methods

2.1. Animals

All animal studies were approved by and performed in accordance with the VAMC IACUC. All mice were housed and bred in the VAMC animal facility with free access to standard laboratory chow (Harlan) and water. Mice between 10 and 20 weeks were used for the experiments. For HKα_1 knockout mice [27], heterozygous 129 Black Swiss mice were bred for wild type and knockout, and siblings were used for each experiment. The genotypes of the experimental mice were confirmed by polymerase chain reaction (PCR) analysis of genomic DNA extracted from the mouse tail. PCRs used two gene-specific primers and one neomycin resistance gene-specific primer. Primers a1.1 (5′-GCC TGT CAC TGA CAG CAA AGA GG-3′) and a1.2 (5′-GGT CTT CTG TGG TGT CCG CC-3′) amplified a 175-bp fragment from the HKα_1 wild-type allele. Primer a1.1 and neo-specific primer a1.3 (5′-CTG ACT AGG GGA GGA GTA GAA GG-3′) amplified a 310-bp fragment from the mutant allele. For HKα_2 C57BL/6 knockout mice [25], primers a2.1 (5′-CTG GAA TGG ACA GGC TCA ACG-3′) and a2.2 (5′-GTA CCT GAA GAG CCC CTG CTG-3′) amplified a 154-bp fragment of exon 20 from wild-type allele. Primer a2.1 and neo-specific primer a1.3 amplified a 298-bp fragment from mutant allele containing portions of exon 20 and the neomycin resistance gene. For H,K-ATPase β BALB/c knockout mice [26], primers b.1 (5′-CCT CAC ACA GAG GAG ACT A-3′) and b.2 (5′-TGC CCA GTG TCC GGG TTC CA-3′) amplified a 134-bp fragment from wild-type allele. Primer b.2 and the neo-specific primer b.3 (5′-ATA TTG CTG AAG AGC TTG GCG GC-3′) amplified a 650-bp fragment from the mutant allele that contains portions of exon 1 and neomycin resistance gene.

2.2. Isolation of gastric and distal colon membrane protein

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (200 mg/kg body weight) then euthanized by cervical dislocation. Epithelial cells from the stomach were removed by scraping with a glass slide then homogenized in buffer (250 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA·Tris, 25 mM Tris·HCl, pH 7.2) containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.5 μM phenylmethylsulfonyl fluoride) [28]. The homogenate was centrifuged at $1000\times g$ for 10 min to remove fragmented cells and debris. The supernatant was centrifuged 15 min at $13{,}000\times g$ to pellet organelles. The resulting supernatant was further centrifuged at $100{,}000\times g$ for 1 h to pellet the plasma membrane fraction. The final pellet was homogenized in suspension buffer (2 mM $MgCl_2$, 1 mM EGTA·Tris, 25 mM Tris·HCl, pH 7.2) containing protease inhibitors. Resuspended gastric membrane vesicles were quickly frozen by dry ice/acetone and stored at −70 °C. The concentration of isolated membrane protein was measured using the BCA Protein Assay Kit from Pierce Biotechnology (Rockford, IL) using bovine serum albumin as a standard.

For collection of distal colonic membrane protein, the epithelial cells of the distal colon were scraped and the membrane vesicles were isolated by differential centrifugation as described above. The resuspended membrane vesicles were then permeabilized according to Forbush [29], mixed with 100 μl treatment solution (1.0 mg/ml lauryl sulfate Tris, 1% BSA, in suspension solution), incubated at 22 °C for 10 min, mixed with 500 μl ice-cold suspension solution with 0.3% BSA, then stored on ice until assays were performed.

2.3. K-ATPase activity assays

K-ATPase activity was determined by measuring inorganic phosphate hydrolyzed from (γ-^{32}P) ATP according to the protocol of Codina et al. [9] with some modifications. Briefly, in a total reaction volume of 100 μl, 0.5–1 μg membrane protein was used in the reaction solution containing 30 mM Tris·HCl, pH 7.2, 1 mM EDTA·Tris, 0.1 mM EGTA·Tris, 4 mM $MgCl_2$, 3 mM ATP·Tris, 4.0 μg/ml oligomycin without or with 2.5 mM KCl. Gastric K-ATPase activity was measured in the presence of 1.0 mM ouabain. The reaction was initiated by the addition of 3.0 mM ATP with $1–10\times10^6$ cpm of (γ-^{32}P) ATP, incubated at 37 °C for 30 min, then stopped by 500 μl ice-cold 15% (W/V) charcoal. The supernatant was filtered (0.45 μM, Whatman) then analyzed on a Beckman scintillation counter. K-ATPase activity was defined as the difference between ATPase activity measured in the presence and absence of 2.5 mM KCl and expressed as micromoles of inorganic phosphate librated per milligram protein per hour.

2.4. Statistical analysis

Results were expressed as mean values ± se (standard error) from the indicated number of animals (n). Comparison of the K-ATPase activities between groups was performed by Student's t-test. A P value <0.05 was considered statistically significant.