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Purification and characterization of the ouabain-sensitive H^+/K^+-ATPase from guinea-pig distal colon

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ABSTRACT

Distal colon absorbs K^+ through a Na^+-independent, ouabain-sensitive H^+/K^+-exchange, associated to an apical ouabain-sensitive H^+/K^+-ATPase. Expression of HKα2, gene associated with this ATPase, induces K^+-transport mechanisms, whose ouabain susceptibility is inconsistent. Both ouabain-sensitive and ouabain-insensitive K^+-ATPase activities have been described in colonocytes. However, native H^+/K^+-ATPases have not been identified as unique biochemical entities. Herein, a procedure to purify ouabain-sensitive H^+/K^+-ATPase from guinea-pig distal colon is described. H^+/K^+-ATPase is Mg^{2+}-dependent and activated by K^+, Cs^+ and NH_4^+ but not by Na^+ or Li^+, independently of K^+-accompanying anion. H^+/K^+-ATPase was inhibited by ouabain and vanadate but insensitive to SCH-28080 and bafilomycin-A. Enzyme was phosphorylated from [^{32}P]-γ-ATP, forming an acyl-phosphate bond, in an Mg^{2+}-dependent, vanadate-sensitive process. K^+ inhibited phosphorylation, effect blocked by ouabain. H^+/K^+-ATPase is an α/β-heterodimer, whose subunits, identified by Tandem-mass spectrometry, seems to correspond to HKα2 and Na^+/K^+-ATPase β1-subunit, respectively. Thus, colonic ouabain-sensitive H^+/K^+-ATPase is a distinctive P-type ATPase.

1. Introduction

In mammal distal colon, active K^+ absorption is a two step process in which K^+ enters the epithelial cell across the apical membrane, against its electrochemical gradient, and exits across the basolateral plasma membrane through K^+ conductive pathways or KCl cotransport [1,2]. Colonic K^+ absorption is inhibited by dinitrophenol and low temperature [3–5], indicating the need of metabolic supplies. In addition, this process is also inhibited by mucosal ouabain [6]. The active K^+ influx into the colonic epithelial cells has been associated to H^+ extrusion to the lumen, in a Na^+-independent, ouabain-sensitive process mediated by an apical Na^+-independent, ouabain-sensitive H^+/K^+-exchange pump [7]. Furthermore, isolated surface colonocytes have an active Na^+-independent, ouabain-sensitive K^+-transport mechanism, compatible with the H^+/K^+ pump [8].

These data suggest that the active K^+ absorption is related with an H^+/K^+-ATPase located at the apical membrane of colonocytes. In this sense, Gustin and Goodman [9] described an ouabain-insensitive K^+-ATPase in rat distal colon. Later, Watanabe et al. [10] demonstrated an ouabain-sensitive K^+-ATPase in homogenate of guinea pig colonic mucosa and del Castillo et al. [11] showed both ouabain-sensitive and ouabain-insensitive K^+-ATPase activities in apical membranes of rat colonocyte. Afterward, Rajendran et al. [12] recognized that the apical H^+/K^+-ATPase could work as a Na^+/K^+-ATPase. Additionally, Haragsim and Bastani [13] and Fejes-Toth and Naray-Fejes-Toth [14], using immuno-histochemistry, identified the H^+/K^+-ATPase at the luminal membrane of colonic epithelial cells.

A putative colonic H^+/K^+-ATPase gene (HKα2; GI:203034) has been cloned [15,16] and functionally co-expressed with different ATPase β-subunits in heterologous systems [17–22]. The HKα2 has been expressed in Sf9 cells, without β-subunit, inducing an ouabain-insensitive K^+-ATPase activity [21]. It has been also co-expressed in Xenopus leavis [18–20] or HEK-293 cells [17] with Na^+/K^+-ATPase β1-subunit or gastric H^+/K^+-ATPase βg-subunit inducing, in the absence of Na^+, ouabain-sensitive active ^{86}Rb transport. In contrast, the co-expression of HKα2 gene with a putative colonic β-subunit, identical to the Na^+/K^+-ATPase β3-subunit (GI:1944501), induced H^+/K^+-ATPase and ^{86}Rb uptake, both insensitive to ouabain [22]. Thus, the nature of this functional diversity and the quaternary structure of the native enzyme are still uncertain.

Although the existence of H^+/K^+-ATPases has been confirmed in the apical membrane of colonocytes, the evaluation of its biochemical, functional, pharmacological and structural characteristics have been limited by the lack of a purified preparation of the native enzyme. In the present study, we described the isolation, purifica-

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tion, preservation and biochemical characterization of the Na^+-independent, ouabain-sensitive H^+/K^+-ATPase from apical membranes of guinea pig colonocytes, identifying it as a unique biochemical entity.

2. Materials and methods

2.1. Animals

Male guinea pigs (300–350 g) and rats (180–200 g), maintained on a regular laboratory diet, were anaesthetized with pentobarbital sodium (10 mg/kg, intra-peritoneal) and distal colon, small intestine, stomach and kidneys were removed.

2.2. Materials

General laboratory reagents were from Sigma–Aldrich Co. (St. Louis, MO, USA), and Merck Co. (Rahway, NJ, USA). Molecular biology reagents were supplied by Invitrogen (Carlsbad, CA, USA) and manufacturer instructions were followed. [^{32}P]-γ-ATP (3000 Ci/mmol) was purchased from Amersham (Westborough, MA, USA). Solutions were prepared with 18 MΩ/cm de-ionized water.

2.3. Methods

2.3.1. Isolation of colonic apical plasma membranes

Apical and basolateral plasma membranes were obtained following the method described by Meyer zu Düttingdorf et al. [23] with modifications. Briefly, the distal colon was excised from the colonic flexure to the rectum and then rinsed with ice-cold 0.9% NaCl containing 1 mM dithiothreitol (DTT)[1] and 0.2 mM phenyl-methyl-sulfonyl fluoride (PMSF). The intestinal segment was open longitudinally and the epithelium was removed by gentle scraping. The scraping was suspended in 75 ml of Solution A (5 mM EDTA, adjusted to pH 7.4 with solid tris base, 1 mM DTT and 0.2 mM PMSF) at 4 °C and homogenized in a Omni-Mixer homogenizer, model 17105 (Omni International, Kennesaw, GA, USA) with 5 strokes of 5 s at the highest speed. The suspension was filtered through gauze. The filtrated was incubated at 4 °C for 30 min and centrifuged at 450*g* for 10 min at 4 °C. The pellet (P1), containing mainly apical membranes, was resuspended in 5 ml of Solution B (5 mM EDTA and 0.2 mM PMSF pH 7.4), re-homogenized with a Dounce-type homogenizer (glass–glass, loose pestle, Wheaton 33) and centrifuged at 450*g* at 4 °C for 10 min. The pellet (P2) was resuspended in 5 ml of Solution C (90 mM NaCl, 0.8 mM EDTA, 1 mM DTT and 0.2 mM PMSF, pH 7.4), re-homogenized with the Dounce-type homogenizer and incubated at 4 °C for 45 min. The suspension was diluted with 6.5 ml of Solution B and mixed with 2.5 ml of Percoll® (15% final concentration) and centrifuged at 35,000*g* at 4 °C for 60 min. The Percoll gradient was recollected in 1 ml fractions from bottom to top. Fractions 3, 4 and 5 were mixed and diluted with Solution D (2.5 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, pH 7.4) to 25 ml and centrifuged at 100,000*g* at 4 °C for 30 min. The pellet, containing apical membranes, was resuspended in the desired volume of Solution D, containing 10% DMSO and stored at −70 °C.

2.3.2. H^+/K^+-ATPase purification

To purify the H^+/K^+-ATPase, apical membranes from guinea-pig distal colon (1 mg protein) were pre-incubated for 10 min at room temperature, with 0.5% BSA, 3 mM ATP, 0.2 mM PMSF in 25 mM imidazole, pH 7.4 (2 ml final volume). Treatment was initiated by the addition under agitation of 15 μl 10% SDS (1.5 mg). The suspension was incubated for 30 min at room temperature and the treatment was stopped by adding 8 ml of 0.1% BSA, pH 7.4 at 4 °C. Treated membranes were mounted on the top of a discontinuous sucrose gradient (5 ml 40%, 10 ml 28% and 5 ml 15% sucrose in 1 mM EDTA, 0.2 mM PMSF, 25 mM imidazole, pH 7.4) and centrifuged at 100,000*g* for 3 h at 4 °C in a Beckman L80 XP ultracentrifuge, with a rotor Beckman 60 Ti. Fractions (1 ml) were obtained from bottom to top, diluted with 25 ml of Solution D and centrifuged at 100,000*g* for 3 h at 4 °C. Purified H^+/K^+-ATPase was obtained in the fractions 3 and 4 (see Fig. 1C).

Purified Na^+/K^+-ATPase was prepared from small intestinal and colonic mucosa following the method described by Jorgensen [24].

2.3.3. Preservation of H^+/K^+-ATPase enzymatic activity

Apical membranes or the purified H^+/K^+-ATPase were frozen at −70 °C in Solution D containing 10% DMSO or 25% glycerol. Cryopreservatives in the resuspension medium were required to maintain H^+/K^+-ATPase activity (see Fig. 2).

2.3.4. ATPase assay

ATPase activities were measured as described by del Castillo et al. [11] and liberated inorganic phosphate (Pi) was determined as Cariani et al. [25].

Mg^{2+}-ATPase was defined as the activity obtained in the presence of Mg^{2+}. The Mg^{2+}-dependent, K^+-activated ATPase was defined as the difference in activity between that measured in the presence of Mg^{2+} plus K^+ minus that obtained in the presence of Mg^{2+} alone. The Na^+/K^+-activated ATPase activity was defined as the difference in activity between that measured in the presence of Mg^{2+} plus K^+ and Na^+ and that determined in the presence of Mg^{2+} plus K^+. ATPase activities were determined in both the presence and absence of 1 mM ouabain.

2.3.5. SDS/PAGE

Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Bollag and Edelstein [26]. Samples (10–20 μg) were resuspended in 25 μl of 60 mM Tris–HCl, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue. Samples were heated at 65 °C for 2 min, centrifuged for 1 min at 14,000 rpm in a Beckman micro-centrifuge and loaded on a 10% separating gel. Samples were run at 100 mV. Gel was stained with Coomassie blue R-250.

2.3.6. [^{32}P]-γ-ATP phosphorylation procedure

Purified H^+/K^+-ATPase (10 μg) was pre-incubated for 10 min at 10 °C in 200 μl of 5 mM $MgCl_2$, and 50 mM Tris–HCl, pH 7.0. The reaction was initiated by adding 1 μCi of [^{32}P]-ATP (100 μM ATP final concentration). Phosphorylation reaction was stopped after 10 s by adding 1 ml of 10% TCA, 0.1 mM NaH_2PO_4 and 60 μM ATP at 4 °C, then filtered through a Millipore filter (0.8 μm) and washed three times with 5 ml of 2% TCA and 20 mM H_3PO_4. Filters were transferred to a counting vial and mixed with 3 ml Aquasol®. Radioactivity was measured in a liquid scintillation counter. Unspecific binding to filters and membranes for each set of experiments was determined by incubating 10 μg of heat-denatured enzyme protein with [^{32}P]-γ-ATP. This value was less than 10% of the basal ^{32}P incorporation. Phosphorylation was expressed in nmol Pi incorporated/mg protein, estimated with a specific activity for [^{32}P]-ɣ-ATP of 3000 Ci/mmol.

The stability of Mg^{2+}-induced phosphorylation at different pH was examined as follows: once proteins were phosphorylated, two volumes of a 0.1 M buffer at different pH were added. Mes/KOH buffer was used for pH 4–5.5, Tris–HCl for pH 7.4, and carbonate/bicarbonate buffer for pH 8–9. The mixture was incubated at 4 °C for 15 min and then 910 μl ice-cold 10% trichloroacetic acid/0.1 M H_3PO_4 were added. Samples were filtered and washed as

[1] *Abbreviations used:* DTT, dithiothreitol; PMSF, phenyl-methyl-sulfonyl fluoride; Pi, inorganic phosphate; SDS–PAGE, sodium-dodecyl-sulfate polyacrylamide gel electrophoresis; df, degrees of freedom.

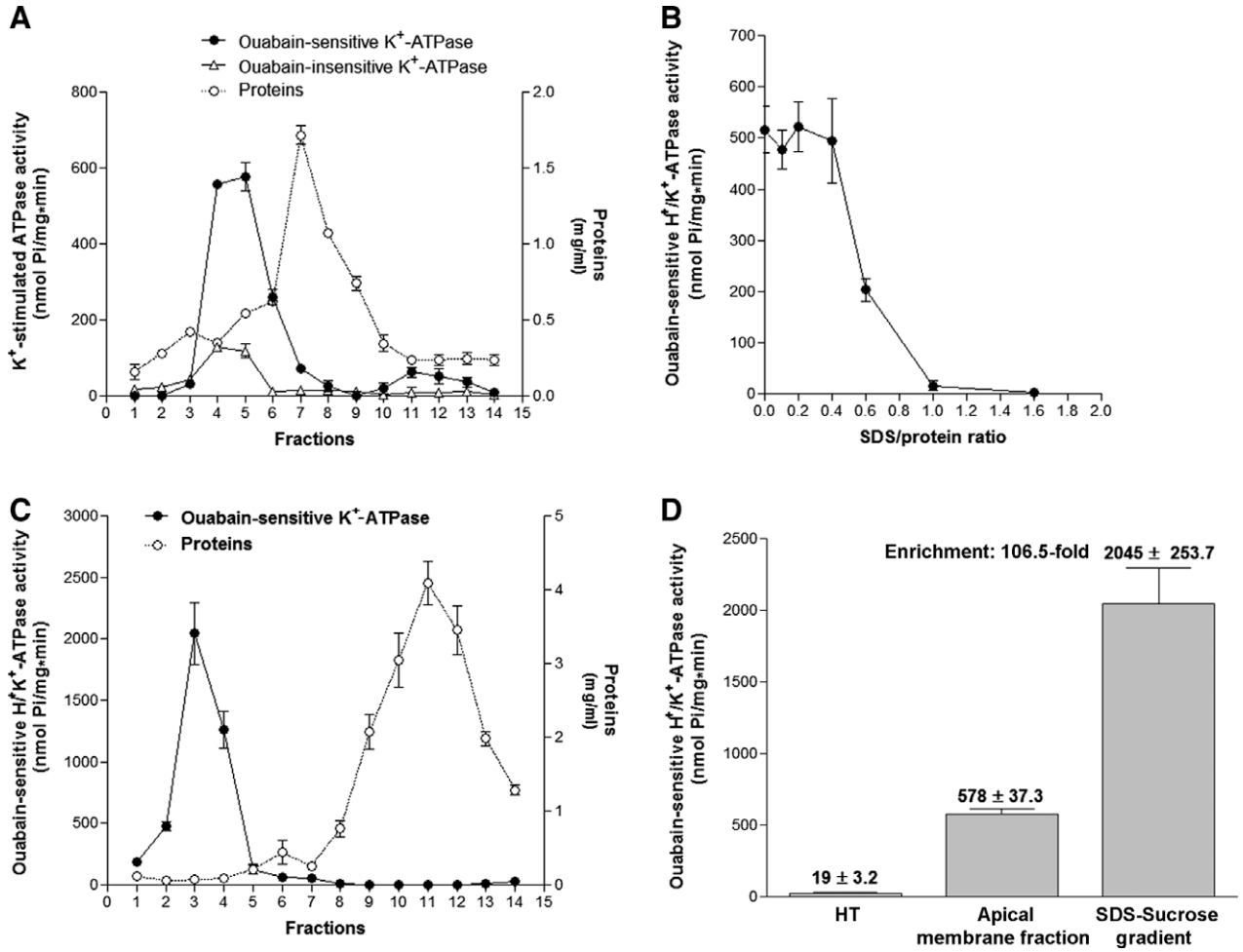

Fig. 1. Colonic ouabain-sensitive H^+/K^+-ATPase purification. (A) Distribution of ouabain-sensitive, ouabain-insensitive K^+-ATPases and proteins in a 15% Percoll® gradient. Fractions (1 ml) were collected from bottom to top and the ATPase activities and proteins were determined in each fraction. Results are mean ± SEM of 4 different preparations. (B) Effect of SDS treatment on the ouabain-sensitive K^+-ATPase of colonic apical plasma membranes. Treatment of the apical membranes was performed in a final volume of 2 ml, in the presence of 2 mM ATP and 1% bovine serum albumin (BSA) at different SDS/protein ratios. Results are mean ± SEM of 5 different preparations. (C) Distribution of ouabain-sensitive K^+-ATPase and proteins of SDS-treated apical membranes in a discontinuous sucrose gradient. Membranes were treated with SDS to a detergent/protein ratio of 1.5, mounted in a discontinuous sucrose gradient (15/28/40%), centrifuged at 100,000*g* for 1 h and 1 ml fraction (bottom to top) were obtained. Results are means ± SEM of 3 different preparations. (D) Ouabain-sensitive K^+-ATPase activity in different fractions. HT, initial homogenate; Apical membranes, apical membrane fraction from colonic epithelial cells; SDS/Sucrose gradient, purified ouabain-sensitive K^+-ATPase in final fraction obtained after SDS treatment and sucrose gradient. Enrichment refers the relationship between the H^+/K^+-ATPase activity present in the purified membrane fraction resulting of sucrose gradient and that obtained in the initial homogenate (HT).

indicated above. Filters were dissolved in 3 ml of Aquasol® and radioactivity was counted in a liquid scintillation counter. The effect of hydroxylamine on the phosphoprotein was evaluated by incubating the phosphorylated enzyme with 250 mM hydroxylammonium sulphate in 0.1 M Mes/KOH (pH 5.4). Then, samples were treated as indicated above for the pH effect.

2.3.7. Tandem-mass spectrometry

Spectrometric analysis was performed, as a service, at "The W.M. Keck Biomedical Mass Spectrometry Laboratory and The University of Virginia Biomedical Research Facility". Trypsin-digested peptides were extracted from the polyacrylamide in 50% acetonitrile/5% formic acid and used in MS analysis. The LC–MS system consisted of a Finnigan LTQ-FT mass spectrometer system with a Protana nanospray ion source interfaced to a self-packed 8 cm × 75 μm id Phenomenex Jupiter 10 μm C18 reversed-phase capillary column. Samples (0.5–5 μl) of the extract were injected and the peptides eluted from the column by an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.25 μl/min. The nanospray ion source was operated at 2.8 kV. The digest was analyzed to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. Scaffold (version Scaffold-2_00_00_final, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identification. Peptide identifications were accepted if they could be established at greater than 95% probability as specific by the Peptide Prophet algorithm [27]. Protein identification was accepted if they could be established at greater than 99.9% probability and contained at least 4 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [28].

2.3.8. RNA isolation and RT-PCRs

Total RNA was isolated using Trizol Reagent and RNA samples were treated with "amplification grade" DNase I. First-strand cDNA was synthesized using ThermoScript RT-PCR System. Substrate for PCR (25 μl final volume) included: 2 μl of RT-reaction, 1X PCR Buffer, 2.32 mM $MgCl_2$, 200 μM of each dNTP, 200 nM of each primer and 1 unit of Platinum Taq DNA Polymerase. For all RT-PCRs, cycling parameters were 94 °C for 2 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 68 °C for 2 min; and a final step of 68 °C for 10 min.

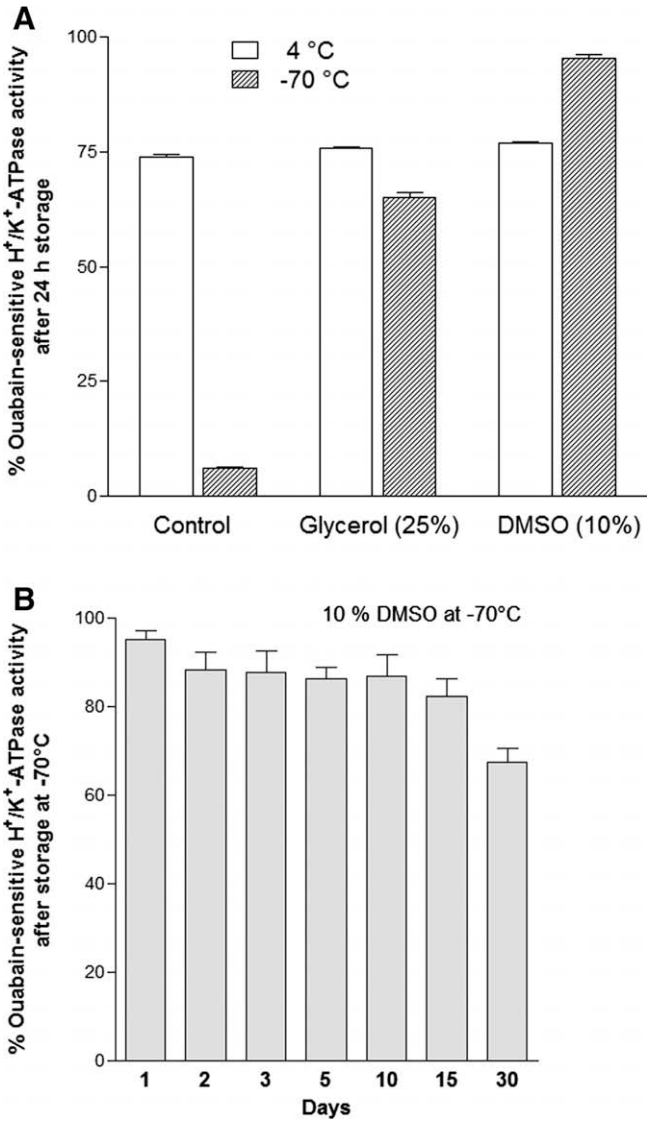

Fig. 2. Effect of storage conditions on ouabain-sensitive K^+-ATPase. (A) Purified ouabain-sensitive K^+-ATPase were stored at 4 °C and −70 °C and the effect of 10% DMSO or 25% glycerol on the enzymatic activity was evaluated at 24 h. (B) Purified ouabain-sensitive K^+-ATPase was frozen at −70 °C in the presence of 10% DMSO and enzymatic activity evaluated after 1–30 days. Results are mean ± SEM of 3 different experiments.

Primer pairs for guinea pig HKα1 and HKα2 mRNAs, rat HKα2a and HKα2b splice-variants, and β-actin were designed from GenBank cDNA sequences (see Fig. 10A). PCR products were analyzed by agarose gel electrophoresis.

Real Time RT-PCR (qRT-PCR) for each HKα2 splice variants was carried out as triplicate in a Chromo4 Real Time PCR thermal cycler (MJ Research). Reaction (20 µl final volume) included 1 µl of RT-reaction as template, 500 nM of the respective primer pair (above described) and 1X Master Mix SYBR (Fynnzymes). Cycling parameters for qRT-PCRs were: 94 °C for 15 min; followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 58 °C for 30 s, elongation at 72 °C for 30 s and reading at 80 °C for 1 s. Finally, reaction was incubated at 72 °C for 10 min and melting curve of each product was fit between 72 and 95 °C. Purified and sequenced PCR products corresponding to large (HKα2a of 229 bp) and short (HKα2b of 384 bp) splice variants of HKα2 were used as standards. Thus, 10-fold standard serial dilutions were made from 6.86×10^{12} (HKα2a) and 4.62×10^{12} (HKα2b) copies of PCR product down to 7 or 5 starting molecules, respectively.

2.3.9. Protein determination

Sample protein content was determined by a modified Coomassie blue method [29] with bovine serum albumin as standard.

2.3.10. Statistical analysis

Results are presented as means ± SEM. Difference between means was evaluated by analysis of variance and considered significant at $p < 0.05$. Adjustment of functions to experimental values was made by nonlinear regression (Marquandt-Levenberg algorithm) using a commercial program (Origin 5.0, Microcal software, Inc.). R^2 (correlation coefficient) and degrees of freedom (df) are given for each fitting procedure.

3. Results

3.1. Isolation of apical membrane from distal colon mucosa

Table 1 presents the distribution of the H^+/K^+-ATPase (apical membrane markers) and the Na^+/K^+-ATPase (basolateral membrane marker) in different fractions obtained during the apical membrane preparation. The first low-speed centrifugation resulted in 55.6% recovery and 6.7-fold enrichment of the ouabain-sensitive H^+/K^+-ATPase in the pellet (P1). In contrast, the Na^+/K^+-ATPase has a 6.7% recovery and its specific activity decreases respect to the homogenate, indicating that the H^+/K^+- and Na^+/K^+-ATPases are not located at the same sub-cellular fraction. During the second low-speed centrifugation step, most of the ouabain-sensitive H^+/K^+-ATPase is recovered in P2 (33.4% recovery and 17.6-fold enrichment) without basolateral membrane contamination, indicated by the low level of Na^+/K^+-ATPase activity in this fraction. The distribution of the ouabain-insensitive K^+-ATPase resembled better the distribution of the Na^+/K^+-ATPase, suggesting that could be associated with the colonocyte basolateral plasma membrane. However, it was still present in P2 fraction.

To further purify the apical membrane preparation, P2 fraction was run in a continuous Percoll® gradient. Results are showed in Fig. 1A. The ouabain-sensitive K^+-ATPase was obtained in fractions 4–7. These fractions were pooled, centrifuge at 100.000*g* for one hour and resuspended in a DTT containing solution (Solution C). This pool was considered as the apical membrane fraction.

Table 2 presents the specific activity and recovery of ouabain-sensitive and ouabain-insensitive K^+-ATPases in the apical membrane fraction, obtained by the modified procedure. Specific activity of the ouabain-sensitive H^+/K^+-ATPase was 544.3 nmol Pi lib/mg min, representing 28.3–fold enrichment and 24.5% total activity recovery, with only 0.82% protein recovery. In contrast, ouabain-insensitive K^+-ATPase had a specific activity of 70.4 nmol Pi lib/mg min, representing 3.7-fold enrichment, with 2.9% recovery. In the apical membrane fraction, different enzymatic markers (succinate dehydrogenase for mitochondria, lactate dehydrogenase for cytoplasm, glucose-6-phosphatase for endoplasmic reticulum) had less than 0.1% recovery and 1-fold enrichment, indicating fraction purity (data not shown).

3.2. Purification of the Na^+-independent, ouabain-sensitive H^+/K^+-ATPase

The apical membrane fraction was used as starting material for the purification of the ouabain-sensitive H^+/K^+-ATPase. In this sense, apical membrane fraction was treated with SDS at different SDS/membrane protein ratios (Fig. 1B). The increase of SDS/protein ratio decreases the ouabain-sensitive H^+/K^+-ATPase activity. Total inhibition was obtained at 1 SDS/protein ratio and higher, however this effect was reversible. The centrifugation of apical membranes, treated with SDS (up to 1.5 SDS/protein ratio) in the presence of bovine

Table 1
Distribution of the H^+/K^+-ATPases (sensitive and insensitive to ouabain) and Na^+/K^+-ATPase in different fractions of the apical membrane preparation.

		Fractions				
		HT	S1	P1	S2	P2
Proteins	% Recovery	100	92.8 ± 3.10	6.4 ± 0.25	3.6 ± 0.45	1.9 ± 0.06
Ouabain-sensitive H^+/K^+-ATPase	Specific activity[a]	19.2 ± 3.23	7.3 ± 0.95	127.8 ± 9.76	64.5 ± 5.65	338.7 ± 6.42
	Total activity[b]	1775.5 ± 342.56	623.7 ± 96.26	987.0 ± 56.3	207.1 ± 6.43	592.7 ± 10.51
	% Recovery	100	36.4 ± 3.37	55.6 ± 9.61	11.7 ± 0.87	33.4 ± 2.18
Ouabain-insensitive H^+/K^+-ATPase	Specific activity[a]	18.8 ± 1.25	17.3 ± 1.10	33.4 ± 1.22	44.5 ± 2.68	33.3 ± 0.95
	Total activity[b]	1717.5 ± 75.21	1472.3 ± 102.79	219.7 ± 9.12	142.3 ± 8.02	58.8 ± 2.17
	% Recovery	100	85.4 ± 2.56	12.8 ± 0.92	8.3 ± 0.70	3.4 ± 0.19
Na^+/K^+-ATPase	Specific activity[a]	85.8 ± 3.89	84.3 ± 3.47	79.3 ± 2.89	156.4 ± 7.25	2.3 ± 0.18
	Total activity[b]	7889.8 ± 43.90	7175.7 ± 219.91	529.1 ± 32.71	499.9 ± 19.17	4.0 ± 0.91
	% Recovery	100	91.3 ± 3.55	6.7 ± 0.21	6.4 ± 0.36	0.1 ± 0.01

HT: Initial homogenate; S1 and P1: supernatant and pellet of the first centrifugation step; S2 and P2: supernatant and pellet of the second centrifugation step. Results are means ± SEM of 10 different experiments.
[a] Specific activities are expressed in nmol Pi lib/mg min.
[b] Total activities in nmol Pi lib/min.

Table 2
H^+/K^+-ATPases (sensitive and insensitive to ouabain) in the apical membrane fraction.

K^+-ATPases	Specific activity (nmol Pi lib/mg min)	% Recovery
Ouabain-sensitive	544.3 ± 37.25	24.5 ± 1.74
Ouabain-insensitive	70.4 ± 7.92	2.9 ± 0.26
Protein	–	0.82 ± 0.035

Values are means ± SEM of five different experiments.

serum albumin, on a discontinuous sucrose gradient reactivated the ATPase activity, producing a purified ouabain-sensitive H^+/K^+-ATPase preparation. Fig. 1C shows the distribution of the ouabain-sensitive H^+/K^+-ATPase and proteins in the discontinuous sucrose gradient. The ouabain-sensitive H^+/K^+-ATPase was obtained in fraction 2–4 (1% protein recovery), while most of the other apical membrane proteins were obtained in fractions 8–14. The purified ouabain-sensitive H^+/K^+-ATPase had a specific activity of 2,045 nmol Pi lib/mg min, representing 106.5-fold enrichment respect to the specific activity determined in the initial homogenate (Fig. 1D). The ouabain-insensitive K^+-ATPase activity, identified in the apical membrane fraction, remained in a pellet obtained after the sucrose gradient centrifugation (data not shown).

3.3. Preservation of H^+/K^+-ATPase activity

Ouabain-sensitive H^+/K^+-ATPase activity was substantially decreased when the enzyme was stored at −70 °C. To preserve it, glycerol and DMSO were evaluated as cryo-preservatives. Fig. 2A presents the effect of storage at 4 °C and −70 °C in the presence of 25% glycerol or 10% DMSO on ouabain-sensitive H^+/K^+-ATPase activity. Storage at 4 °C for 24 h decreased the activity in about 25%, while freezing it at −70 °C decreased the specific activity to less than 10%. This effect can be prevented by adding 25% glycerol and even better 10% DMSO to the resuspension medium. The ATPase activity can be preserved in DMSO over 80% of the initial value up to 15 days (Fig. 2B).

3.4. Characterization of the purified ouabain-sensitive H^+/K^+-ATPase

The effect of different KCl concentrations on ouabain-sensitive ATPase activity of the purified enzyme is shown in Fig. 3A. At pH 7.4, the raise of K^+ concentration produced an increase in the ATPase activity, obtaining maximal activity at about 0.5 mM with a km for K^+ of 24.5 μM. In the absence of K^+ in the medium, the enzyme showed an ouabain-sensitive ATPase activity (about 500 nmol Pi lib/mg min) a 27% of the maximal activity obtained in the presence of 5 mM K^+ (Fig. 3A). The km for K^+ increased when incubation medium pH was decreased to 7.0 (Fig. 3B). Furthermore, K^+ did not have any significant effect on the ouabain-sensitive, Mg^{2+}-dependent ATPase activity when measured at pH 6.6 (Fig. 3C).

These results suggest that H^+ could have a K^+-like effect on the H^+/K^+-ATPase. If this is the case, the ouabain-sensitive ATPase activity, in the absence of K^+, must be enhanced at higher H^+ concentrations, changing the optimal pH to lower values. To test this possibility, the ouabain-sensitive ATPase activity was measured in K^+-free and K^+-containing media at different pHs (Fig. 3D). The optimal pH for the ouabain-sensitive ATPase activity in the presence of K^+ was 7.4, while the optimum pH for this activity, in the absence of K^+, was 6.6. These data suggest that K^+ could be replaced by H^+ in the enzyme reaction cycle, however pH-induced conformational changes cannot be discarded.

The ouabain-sensitive ATPase activity was stimulated by K^+, Rb^+, NH_4^+ and Cs^+ but not by Na^+, Li^+ or *N*-methyl-glucamine (Fig. 4A). Activation by K^+ was independent of the accompanying anion. Stimulation was the same with different potassium salts (Fig. 4B).

Fig. 5A shows the effect of different nucleotide on the ouabain-sensitive K^+-ATPase. The hydrolytic activity is mainly supported by ATP. The ouabain-sensitive hydrolytic activity is low in the presence of CTP, GTP, UTP or ITP as compared to that obtained in the presence of ATP. These characteristics define this enzyme as an H^+/K^+-stimulated ATPase.

The substrate for the P- type ATPases is the Mg^{2+}-ATP. Thus, we evaluated the effect of different Mg^{2+} concentration at 5 mM ATP, different ATP concentrations at 5 mM $MgCl_2$ and different Mg^{2+}-ATP concentration at a 1:1 fixed ratio. Results are shown in Fig. 5B–D. The ATPase activity was Mg^{2+}-dependent. The raise in Mg^{2+} concentration increased ouabain-sensitive K^+-ATPase activity, reaching a maximum at 5 mM with a km of 3.4 mM (Fig. 5B). In the same way, the increase of ATP concentration enhanced the ouabain-sensitive, K^+-stimulated ATPase activity, obtaining maximum activity at 5 mM ATP and a km of 1.9 mM (Fig. 5C). ATP concentrations over 5 mM induced H^+/K^+-ATPase activity inhibition, suggesting the existence of ATP-regulatory sites. When Mg^{2+} and ATP were increased together, at 1:1 fixed ratio, a km of 0.5 mM was obtained for the complex (Fig. 5D).

Finally, we evaluated the effect of different inhibitors on the Na^+-independent, H^+/K^+-stimulated ATPase. Fig. 6A presents the Dose–Response curve for ouabain on the ATPase activity of the purified K^+-ATPase. Ouabain inhibits the K^+-stimulated ATPase with an IC_{50} of 1.7 μM.

The Dose–Response curve for vanadate on the ouabain-sensitive K^+-ATPase of the purified K^+-ATPase is shown in Fig. 6B. K^+-ATPase activity was inhibited by vanadate (IC_{50}: 1.6 μM). Others ATPase

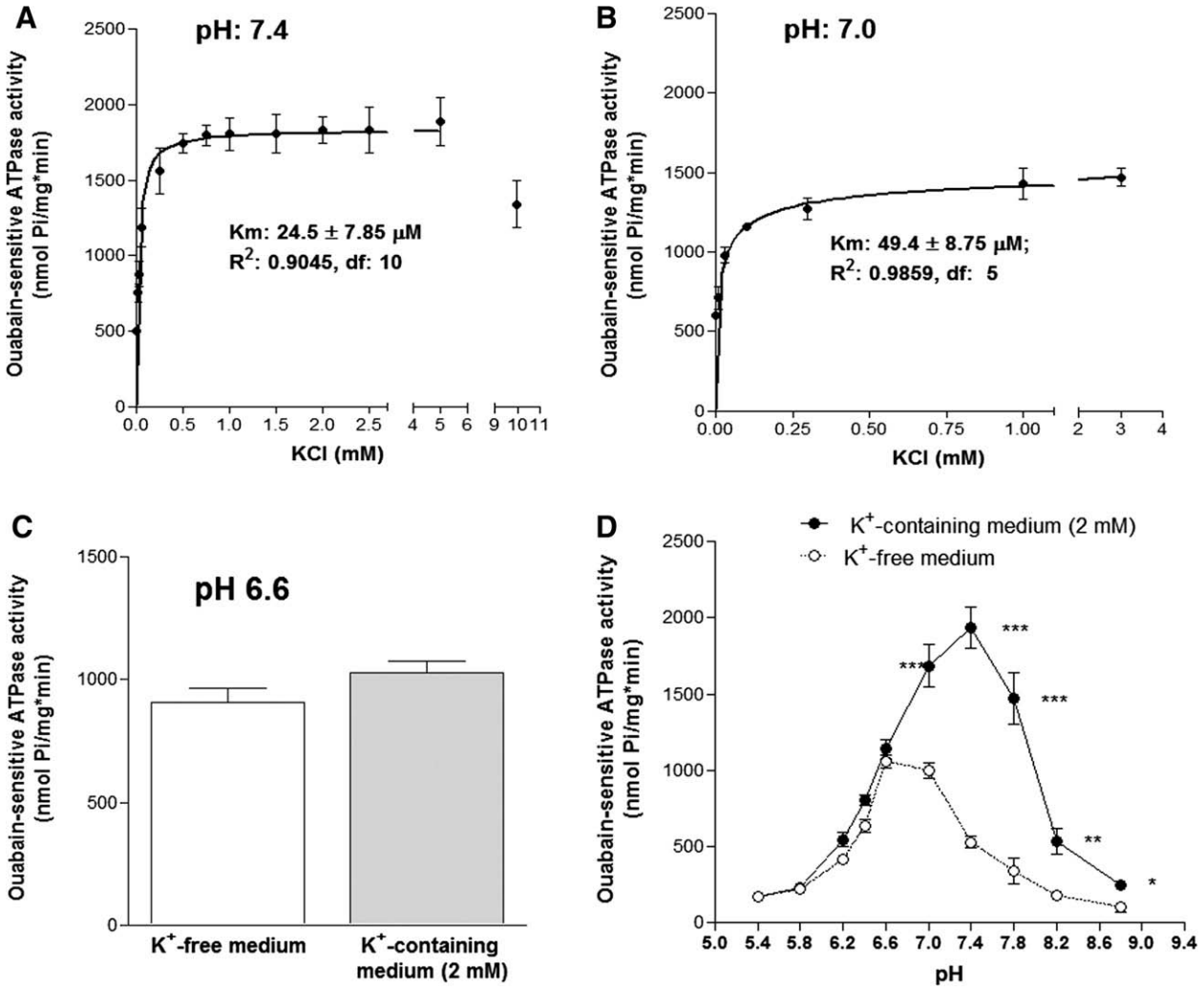

Fig. 3. Effect of different K^+ concentrations on the ouabain-sensitive K^+-ATPase activity evaluated at pH 7.4 (A) and pH 7.0 (B). Values are means ± SEM of 5 different experiments. Lines are the non-regression of experimental points adjusted to a hyperbolic function. R^2 is the regression coefficient and df are the degree of freedom for the non-lineal regression. (C) K^+ effect on the ouabain-sensitive, Mg^{2+}-dependent ATPase activity at pH 6.6. (D) Effect of different pH on the ouabain-sensitive K^+-ATPase activity, measured in K^+-free (○) and K^+-containing media (●). MES-Tris was used to adjust pH media. Values are means ± SEM of five different preparations. Differences between K^+-free and K^+-containing media at distinct pH's were analyzed by two-way ANOVA. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; without mark: not significant.

inhibitors as SCH-28080 (100 μM), a gastric H^+/K^+-ATPase inhibitor, and bafilomycin-A (100 μM), an inhibitor of V-type ATPases, had no effect on the ouabain-sensitive H^+/K^+-ATPase (Fig. 6C).

3.5. SDS–PAGE of purified ouabain-sensitive H^+/K^+-ATPase

Purified H^+/K^+-ATPase was run in 10% SDS–PAGE, under dissociating conditions. Fig. 7 shows that purified enzyme has two main bands of 100 and 50 kDa, which could correspond to α and β subunits, respectively. In the SDS–PAGE, 50-kDa subunit (β) was more intensively stained than 100-kDa subunit (α). However, it has long been known that Coomassie blue vary widely its ability to bind proteins due, at least in part, to its affinity for proteins rich in basic amino acids, which may overstate relative protein quantities in the gel. A similar pattern has been demonstrated for the Na^+/K^+-ATPase [30]. However, the purified ouabain-sensitive H^+/K^+-ATPase fraction has not Na^+/K^+-ATPase activity, suggesting that the H^+/K^+-ATPase is not related or contaminated with this enzyme.

3.6. Phosphorylation of the H^+/K^+-ATPase

P-type ATPases are able to generate phosphorylated intermediates during their catalytic cycle. Since, ouabain-sensitive H^+/K^+-ATPase is Mg^{2+}-dependent and sensitive to vanadate, characteristics of the P-type ATPases, we decided to evaluate the possibility of phosphorylated intermediate production during its catalytic cycle. In the presence of Mg^{2+} (5 mM), phosphorylation from [^{32}P]-γ-ATP was a linear function of the incubation time up to 10 s at 4 °C. Maximal phosphorylation was obtained at 30 s. Thus, phosphorylated form of the enzyme was evaluated under different conditions at 4 °C for 10 s. As present in Fig. 8A, there was an insignificant protein phosphorylation in Mg^{2+}-free medium. Addition of Mg^{2+} (5 mM) highly increased phosphorylation. K^+ (2 mM), but not Na^+, inhibited the phosphorylation, process blocked by ouabain (1 mM). In addition, phosphorylation was also inhibited by vanadate (100 μM). These results indicate that the purified H^+/K^+-ATPase forms phosphorylated intermediate from [^{32}P]-γ-ATP, which is Mg^{2+}-dependent, sensitive to K^+, stabilized by ouabain and inhibited by vanadate.

3.7. Chemical nature of the phosphoprotein bond of the H^+/K^+-ATPase phosphorylated intermediate

As shown in Fig. 8B, phosphoprotein was stable at acidic pH while unstable at alkaline pH. Additionally, hydroxylamine reduced the phosphorylation by more than 80% (Fig. 8C). These

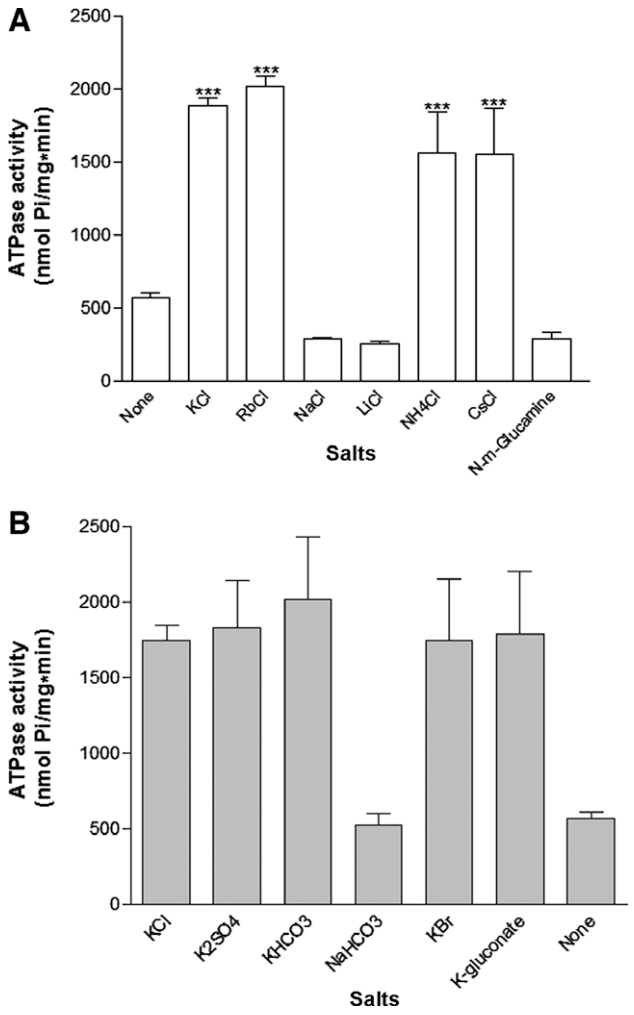

Fig. 4. Effect of different monovalent cations (A) and anions (B) on the Mg^{2+}-dependent ATPase activity of the purified ouabain-sensitive K^+-ATPase. Different salts were added at 2 mM final concentration. Values are means ± SEM of five different experiments.

two independent experimental evidences suggest that the phosphoprotein bond of the H^+/K^+-ATPase phosphorylated intermediate is an acyl-phosphate.

3.8. Tandem-mass spectrometry of the H^+/K^+-ATPase

MS/MS-spectrometric analysis of the 100-kDa subunit of the purified H^+/K^+-ATPase identified 25 peptides (shadow in Fig. 9A) that represent a 37.5% of reported guinea pig HKα2 amino acid sequence (GI:452168). Additionally, MS/MS-analysis of the 50-kDa subunit of the purified H^+/K^+-ATPase identified 4 peptides (shadow in Fig. 9B) that represent a 14.71% of reported guinea pig Na^+/K^+-ATPase β1-subunit amino acid sequence (GI:7024443). MS/MS-spectrometry also showed that the preparation was contaminated with bovine serum albumin, used to preserve the enzyme during SDS-treatment.

3.9. Expression of H^+/K^+-ATPase isoforms and splice variants by RT-PCR

In mammals, H^+/K^+-ATPase activity has been attributed to two isoforms of H^+/K^+-ATPase α-subunit, the gastric (HKα1) and the non-gastric (HKα2) isoforms, encoded by two different genes. In the colon, the ouabain-sensitive H^+/K^+-ATPase seems to be associated to HKα2 gene. The expression of these genes was evaluated in guinea pig and rat. Fig. 10B shows the RT-PCR products for HKα1 (lanes 1–3) and HKα2 (lanes 4–6) mRNAs in stomach (lanes 1 and 4), distal colon (lanes 2 and 5) and kidney (lanes 3 and 6) from guinea pig. The specific RT-PCR product for HKα1 (471 bp) was only amplified in gastric tissue. In contrast, HKα2 RT-PCR product (541 bp) was only generated from distal colon and kidney. Identical results were obtained with rat tissues (data not shown).

Two splice-variants of the non-gastric isoform have been described in rat kidney [16]. Expression of these variants was evaluated in rat because HKα2b splice-variant has not been reported in guinea pig. Fig. 10C shows the electrophoretical analysis of RT-PCR products for HKα2a (lanes 1–3) and HKα2b (lanes 4–6) splice variants in rat small intestine (lanes 1 and 4), distal colon (lanes 2 and 5) and kidney (lanes 3 and 6). HKα2a (229 bp) and HKα2b (384 bp) splice variants were only amplified in distal colon and kidney, suggesting co-expression in these tissues.

Fig. 10D presents qRT-PCR for HKα2a (black bars) and HKα2b (grey bars) splice variants in rat distal colon (left columns) and kidney (right columns). Both variants were higher expressed in distal colon than kidney. HKα2b (short variant) was the predominant splice-variant in distal colon (HKα2b/HKα2a ratio of 11.4), while the expression of both variants in the kidney was similar (HKα2b/HKα2a ratio of 0.9). β-actin expression was identical in all samples (white bars).

4. Discussion

In the apical plasma membranes of colonocytes, there are at least two different Na^+-independent K^+-ATPase activities, an ouabain-sensitive and other ouabain-insensitive, which seems associated with the transepithelial K^+ absorption in the distal colon [1,2]. At the moment, it is not clear if the native H^+/K^+-ATPase is an α/β-heterodimer, as suggested by co-expression experiments, and if any Na^+/K^+-ATPase β-subunit constitutes part of the native enzyme. These questions could be answered purifying the ouabain-sensitive H^+/K^+-ATPase.

Both, ouabain-sensitive and ouabain-insensitive K^+-ATPase activities have been identified in the apical membrane of colonocytes [11]. In this sense, this membrane was used as starting material to purify the ouabain-sensitive K^+-ATPase. An apical membrane preparation was modified to obtain more initial protein. The main modification to Meyer zu Düttingdorf et al. procedure [23] was the substitution of multiple centrifugation and filtration steps by a Percoll® gradient, producing an apical membrane fraction with a specific activity for the ouabain-sensitive K^+-ATPase of 544.3 nmol Pi/mg min, with 28.3-fold enrichment and 24.5% recovery of the enzymatic activity, in contrast to 4% recovery obtained by Meyer zu Düttingdorf et al.

Apical membrane preparations are usually frozen and thawed before the determination of the enzymatic activities; however the ouabain-sensitive H^+/K^+-ATPase is deactivated by freezing/thawing procedures (Fig. 2). The use of DMSO (10%) or glycerol (25%) in the resuspension medium preserves the enzymatic activity, for at least 15 days (Fig. 2). At the moment, the maximal specific activities reported for the ouabain-sensitive K^+-ATPase is 70 nmol Pi/mg min [11], in contrast to the 544.3 nmol Pi/mg min accounted here. This observation could be explained by deactivation of the enzyme under freezing/thawing conditions. The molecular mechanism for the protective effect of DMSO is unknown but it has been proposed that the methyl groups of the compound interact with membrane phospholipids, stabilizing the membrane structure under freezing/thawing conditions [31,32].

The ouabain-sensitive and ouabain-insensitive K^+-ATPase activities present in apical membrane preparations can be separated. During the preparative procedure, the relative distribution of these two K^+-activated ATPases activities was different. As shown in Ta-

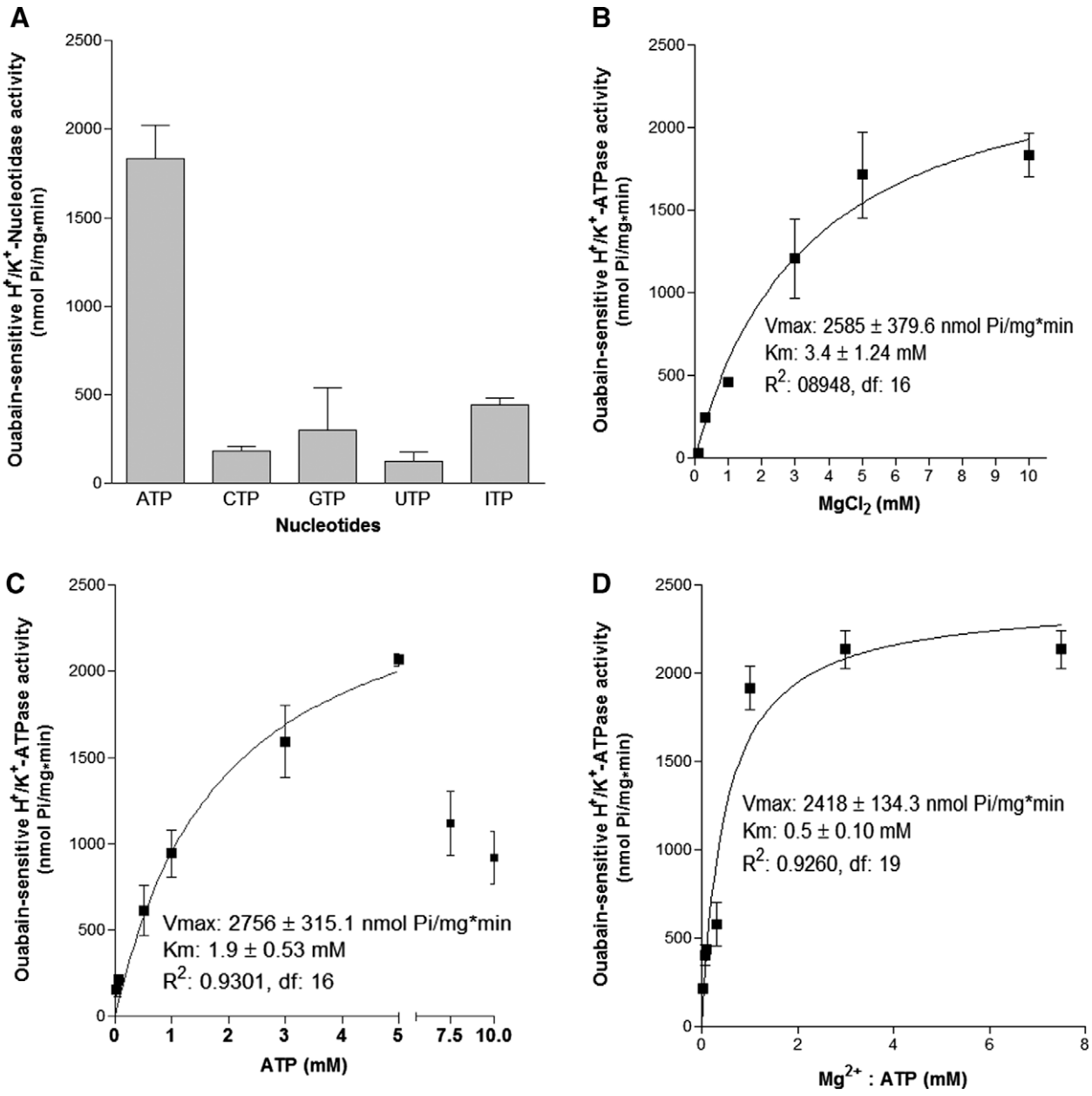

Fig. 5. (A) Effect of different nucleotides on the phosphatase activity of the ouabain-sensitive, K^+-ATPase. Nucleotides were added at 5 mM final concentration. (B) Effect of different Mg^{2+} concentrations on the ouabain-sensitive, K^+-stimulated ATPase activity at 5 mM ATP concentration. (C) Effect of different ATP concentrations on the ouabain-sensitive, K^+-stimulated ATPase activity at 5 mM Mg^{2+} concentration. (D) Effect of different Mg: ATP concentrations (added at a fixed 1:1 ratio), on the ouabain-sensitive, K^+-stimulated ATPase activity. Values are means ± SEM of 3 different preparations.

ble 1, 93.7% of the ouabain-insensitive K^+-ATPase remains in supernatants S1 and S2, while most of the ouabain-sensitive K^+-ATPase was in P1 and P2 pellets, indicating physical separation. Remaining ouabain-insensitive K^+-ATPase activity, present in apical membrane fraction, was eliminated during ouabain-sensitive K^+-ATPase purification steps, suggesting that they are different biochemical entities.

Strategy for ouabain-sensitive H^+/K^+-ATPase purification was based on the colonic apical membrane treatment with anionic detergent (SDS) and separation in a discontinuous sucrose gradient. This strategy has been used to purify the Na^+/K^+-ATPase [24] and the gastric H^+/K^+-ATPase [33]. Purified enzyme fraction has a specific activity of 2,045 nmol Pi/mg min that corresponds to 106.5-fold enrichment respect to the initial homogenate, with 15% recovery. SDS–PAGE of this fraction showed that the enzyme is constituted by α (100 kDa) and β (50 kDa) subunits. Tandem-mass spectrometric analysis revealed that the α-subunit (100 kDa) corresponds to the guinea pig non-gastric isoform of H^+/K^+-ATPase (HKα2, GI:452168) and the β-subunit (50 kDa) could correspond to the β1-subunit of the Na^+/K^+-ATPase (GI:7024443), as suggested by co-expression experiments [17–20]. Although β3-subunit of Na^+/K^+-ATPase could integrate the native H^+/K^+-ATPase, it seems to be unlikely since this protein was not identified by MS/MS-analysis of the purified ouabain-sensitive H^+/K^+-ATPase and its co-expression with HKα2 gene induced an intracellular restricted ouabain-insensitive K^+-ATPase activity [22,34]. These data indicate that the purified enzyme is constituted by HKα2 and β1-subunit of the Na^+/K^+-ATPase.

Two HKα2 splice-variants have been reported in rat kidney [16]. These variants are identical except that the HKα2a includes 108

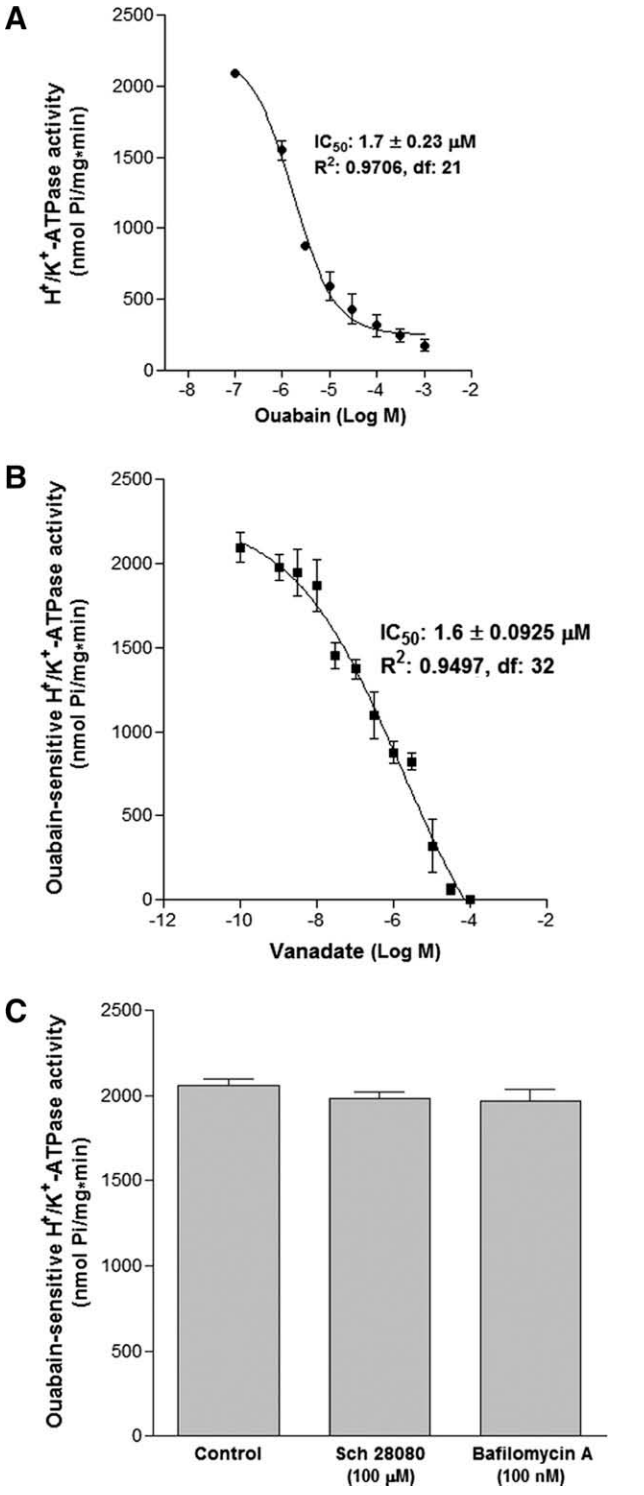

Fig. 6. Effect of different inhibitors on the ouabain-sensitive, K^+-stimulated ATPase activity. (A) Effect of ouabain. (B) Effect of vanadate. (C) Effect of SCH-28080 and bafilomycin-A.

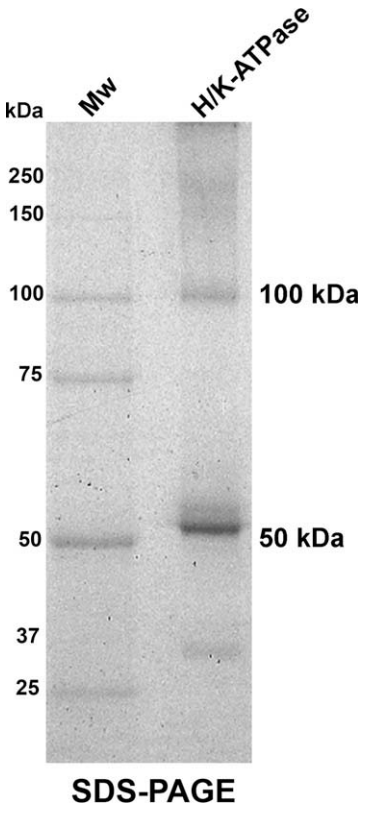

Fig. 7. SDS–PAGE of the purified ouabain-sensitive K^+-ATPase, stained with Coomassie blue.

additional amino acids at the N-terminal end. MS/MS-spectrometry of the α-subunit identified five peptides (KTLEIYSVELDGTK, ELDLDDHK, LTSEELEQK, YGTNIIR and DGPNALSPPK) present in this end, suggesting that at least HKα2a forms part of the native enzyme. On the other hand, the HKα2b has been reported as predominant in distal colon [16]. In fact, qRT-PCR quantification of HKα2 splice-variants revealed that both are expressed in distal colon, being HKα2b the predominant (Fig. 10D). Probably, both variants are present in the native enzyme. The concurrent presence of two splice variants in the same cell does not necessary involve functional redundancy. HKα2 splice-variants have different promoters, which respond to different transcriptional factors that could determine their preferential expression [16,35].

Purified ouabain-sensitive H^+/K^+-ATPase has a km for potassium of 24.5 µM, similar to 55 µM reported in colonic mucosa homogenate [10] but different to 0.75 mM obtained for the ATPase in the apical membrane [11]. In co-expression experiments, km for K^+ varied between 0.45–1.4 mM [17,19,20]. These differences could be due to preparative and/or co-expression procedures. H^+/K^+-ATPase is Mg^{2+}-dependent and preferentially hydrolyzes ATP, km for Mg-ATP was 0.5 mM. The enzyme was activated by K^+, Cs^+, and NH_4^+ but insensitive to Na^+ and Li^+. Potassium activation was independent of the accompanying anion. The enzyme was inhibited by ouabain (IC_{50}: 1.7 µM) and vanadate (IC_{50}: 1.6 µM), but insensitive to SCH-28080 (an inhibitor of the gastric H^+/K^+-ATPase) or bafilomycin-A (an inhibitor of the vacuolar H^+ ATPase). The optimal pH of the ouabain-sensitive H^+/K^+-ATPase was 7.4. The km for K^+ increased from 24.5 µM at pH 7.4 to 49.4 µM at pH 7.0 (Fig. 3), indicating a K^+-affinity decrease associated to an increase in H^+ concentration. Furthermore, purified enzyme has an ouabain-sensitive ATPase activity, in the absence of potassium. In addition, 2 mM K^+ did not have any significant effect on the ouabain-sensitive, Mg^{2+}-dependent ATPase activity, measured at pH 6.6 (Fig. 3C). These data suggest H^+-K^+ competition.

The enzyme was able to form phosphorylated intermediate from [^{32}P]-γ-ATP during its catalytic cycle. This intermediary was Mg^{2+}-dependent, sensitive to K^+, stabilized by ouabain and inhibited by vanadate (Fig. 8). Phosphoprotein was sensitive to alkaline pH and hydroxylamine, indicating that the bond is an acyl-phosphate. These data confirm the hypothesis that the H^+/K^+-ATPase is a P-type ATPase.

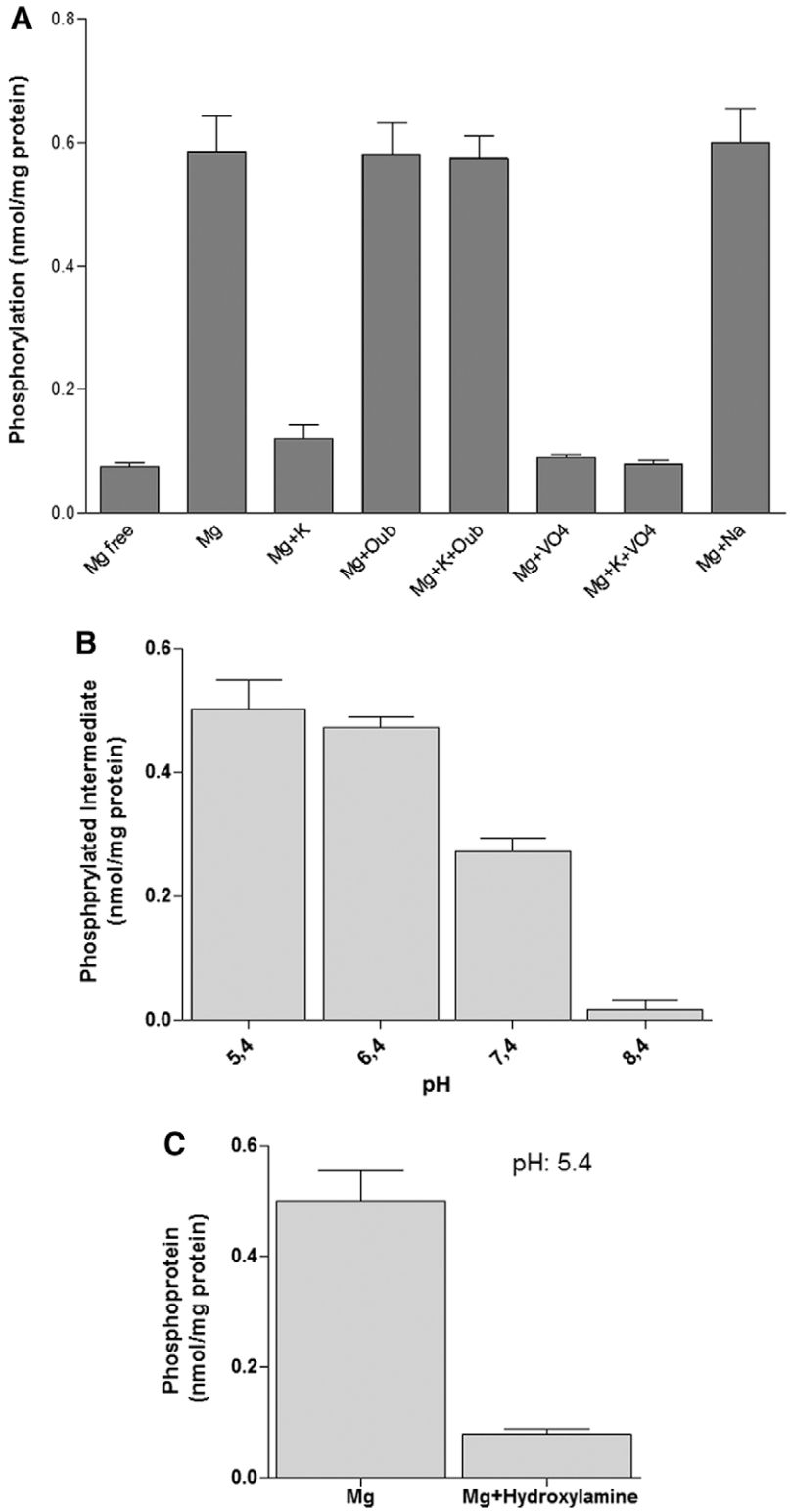

Fig. 8. (A) Phosphorylation of purified H^+/K^+-ATPase from [^{32}P]-ATP. Enzyme (10 μg) was pre-incubated with 100 mM Tris–HCl (Mg^{2+}-free); 100 mM Tris–HCl plus 5 mM $MgCl_2$ (Mg^{2+}); 100 mM Tris–HCl, 5 mM $MgCl_2$, 2 mM KCl ($Mg^{2+} + K^+$); 100 mM Tris–HCl plus 5 mM $MgCl_2$, 1 mM ouabain (Mg^{2+}+Oub); 100 mM Tris–HCl, 5 mM $MgCl_2$, 2 mM KCl, 1 mM ouabain (Mg^{2+}+K^++Oub); 100 mM Tris–HCl, 5 mM $MgCl_2$, 100 μM vanadate (Mg^{2+} + VO4); 100 mM Tris–HCl, 5 mM $MgCl_2$, 2 mM KCl, 100 μM vanadate ($Mg^{2+} + K^+$ + VO4); and 100 mM Tris–HCl, 5 mM $MgCl_2$, 2 mM NaCl ($Mg^{2+} + Na^+$) at 10 °C for 10 min. Phosphorylation was initiated by adding [^{32}P]-ATP (100 μM) and stopped after 10 s by TCA/H_3PO_4 precipitation as described in methods. Results are mean ± SEM of four different experiments. (B) pH stability of the H^+/K^+-ATPase phosphorylated intermediate. Phosphorylation reaction was initiated by adding [^{32}P]-ATP. After 10 s, the pH of incubation medium was changed to the desired values by the addition of 2 volumes of different buffers and stopped 15 min later by adding TCA/H_3PO_4 solution, as indicated in methods. (C) Effect of hydroxylamine on the phosphorylated intermediate of the Na^+-ATPase. Enzyme was phosphorylated for 10 s and incubated for 10 additional sec in the presence of 250 mM hydroxylamine at pH 5.4. Then, samples were treated as indicated for the pH effect. Results are mean ± SEM of three independent experiments.

A **GI:452168|AT12A_CAVPO - Cavia porcellus potassium-transporting ATPase alpha-2 subunit**

MRR**KTLEIYSVELDGTK**DTKQLGQEEGKKCNELDLKKSSQKEELKK**ELDLDDHKLTSEELEQKYGTNIIR**GLSSTRAAELLAR**DGPN**
ALSPPKQTPEIIKFLKQMIGGFSILLWVGAILCWIAYGIQYASNQSGSLDNVYLGVVLALVVILTGIFAYYQEAK**STNIMSSFSKMI**
PQEALVTRDAEKK**VIPAEQLVVGDIVEIKGGDQIPADIRLLFSQGCKVDNSSLTGESEPQPRSAEFTHENPLETK**NIAFYSTTCLEG
TATGMVINTGDRTIIGR**IASLASGVGNEK**TPIATEIEHFVHIVAGVAVSIGILFFIIAVSLKYRVLDSIIFLIGIIVANVPEGLLAT
VTVTLSLTAKRMAKKNCLVK**NLEAVETLGSTSVICSDK**TGTLTQNRMTVAHLWFDSQIFTADTSESQSN**QAFDQSSGTWASLSK**IIA
LCNRAEFRPGQENVPIMK**RVVVGDASETALLK**FSEVILGDVMEIRKRNRK**VAEIPFNSTNK**FQLSIHETEDPGDPRFLMVMKGAPER
ILEK**CSTIMINGQEQPLDKNNANAFHTAYMELGGMGER**VLGFCHLYLPAHEFPENYSFDVDTMNFPTSNLCFVGLLSMIDPPRSTVP
DAVAKCRSAGIKVIMVTGDHPITAKAIAK**SVGIISANSETVEDIAKRCNIAVEQVNK**QDAR**AAVVTGMELK**DMTPEQLDEILANYPE
IVFARTSPQQKLIIVEGCQR**QNAVVAVTGDGVNDSPALKKADIGIAMGIAGSDAAKNAADMVLLDDNFASIVTGVEEGR**LIFDNLKK
TIAYTLTKNIAELCPFLVYIIVGLPLPIGTITILFIDLGTDIIPSIALAYEK**VESDIMNR**KPRHKKDRLVNHQLAIYSYLHIGLMQ
ALGAFLVYFTVYAQQGFWPTSLIQLRVK**WEQDYVNDLEDSYGQQWTR**YQRKYLEWTGYTAFFVGIMVQQIADLIIRKTR**RNSIFQQG**
LFRNKVIWVGITSQIIVALILSCGLGSITALNFTMLRVQYWFVAVPHAILIWVYDEVRKLFLRLYPGSWWDKNMYY

B **GI:7024443|AT1B1_CAVPO - Cavia porcellus sodium/potassium-transporting ATPase beta-1 subunit**

MARGKAKEEGSWKKFIWNSEKKEFLGRTGGSWFKILLFYVIFYGCLAGIFIGTIQVMLLTISELKPTYQDR**VAPPGLTQIPQIQK**TE
ISFRPADPK**SYEAYVLNIYR**FLEKYKDAAQKDDMIFEDCSTVPSEPKERGDFNHERGERKVCRFKLEWLGNCSGQNDDSYGYR**DGKP**
CIIIKLNRVLGFKPKPPKNDSSETVEIYSTMKYNPYVLPVQCTGKREEDKDKIGSVEYFGLGGYAGFPLQYYPYYGKLLQPKYLQPL
LAVQFTNLTTDTEVRIECK**AYGENIGYSEK**DRFQGRFDVKIEVKS

Fig. 9. Tandem-mass spectrometry of α (100 kDa)- and β (50 kDa)-subunits of the purified ouabain-sensitive H^+/K^+-ATPase. (A) Peptides (in shadow) of the purified H^+/K^+-ATPase α-subunit (100 kDa), identified by MS/MS, that correspond to the amino acid sequence of the HKα2 (GI:452168). (B) Peptides (in shadow) of the purified H^+/K^+-ATPase β-subunit (50 kDa), identified by MS/MS, that correspond to the amino acid sequence Na/K-ATPase β1-subunit (GI:7024443).

A

Primer Pairs	Gene	Specie	gi	Product size (bp)
5'-CAGAAGACCAGTCAGGGCAAACGTT-3' 5'-GGTAGAGCTGGCAGAAACCAAGAAC-3'	HKα1	*R. norvegicus*	203036	471
5'-GAGAGTTGTGGTTGGCGATGCTTCA-3' 5'-GGCTGTGATGGGATGATCTCCTGTA-3'	HKα2	*C. porcellus*	431136	541
5'-CCAACATCTTCGCCTTCACCACTCA-3' 5'-TCTGTTTTCCGGCGCATACTGTGGC-3'	HKα2a	*R. norvegicus*	203034	229
5'-CGCAGTGAGCCTATTTGAAACTTCA-3' 5'-TTTCTGTTTTCTGGAAGAGAAGCAG-3'	HKα2b	*R. norvegicus*	2735423	384
5'-GGTCATCACTATCGGCAATG-3' 5'-TAATCTCCTTCTGCATCCTG-3'	β-actin	*R. norvegicus*	42475962	215

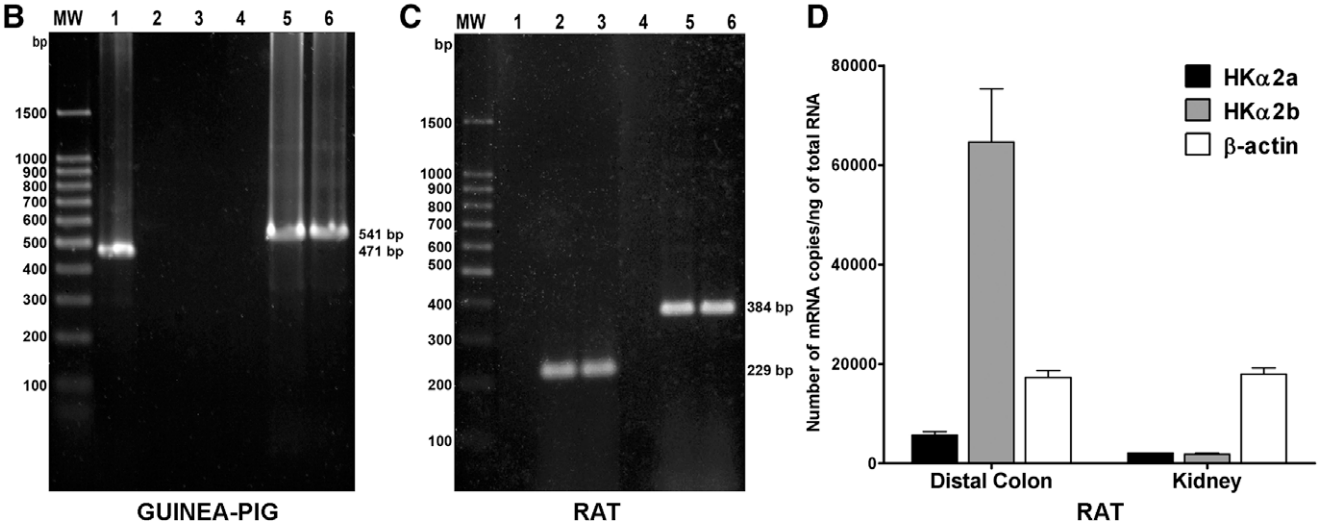

Fig. 10. Primers used to identify HKα1, HKα2, HKα2a, HKα2b and β-actin genes. (B) Expression of mRNA for the H^+/K^+-ATPase gastric (HKα1) and colonic (HKα2) isoforms by RT-PCR. The mRNA expression of gastric (lanes 1–3) and colonic (lanes 4–6) isoforms of the H^+/K^+-ATPase were determined by specific RT-PCRs in gastric epithelium (lanes 1 and 4), distal colon (lanes 2 and 5) and kidney (lanes 3 and 6) of guinea pig. (C) Expression of the two mRNA splice variants of the non-gastric isoform of the H^+/K^+-ATPase in rat tissues. RT-PCRs for HKα2a (lanes 1–3) and HKα2b (lanes 4–6) were performed in small intestine (lanes 1 and 4), distal colon (lanes 2 and 5) and kidney (lanes 3 and 6). Total RNA (1 μg), previously treated with DNase I, were used as substrate for RT-PCRs. PCR products (10 μl) were run in 2% agarose gel electrophoresis and stained with SYBR Green I. RT-PCR product size is indicated at the right. MW: 100 bp DNA Ladder (Promega). (D) Quantitative expression level of the two HKα2 mRNA splice variants by qRT-PCR. Real time PCR for HKα2a (black bars), HKα2b (gray bars) and β-actin (open bars) were carried out in rat distal colon (left) and kidney (right). Results represent the average of estimated mRNA molecules present in the samples per nanogram of total RNA, used as template, of three independent experiments.

5. Conclusions

The colonic ouabain-sensitive H^+/K^+-ATPase has been purified for the first time and its biochemical evaluation defines it as a unique P-type ATPase, constituted by α/β-heterodimer, whose α-subunit (100 kDa) is encoded by the HKα2 gene and β-subunit (50 kDa) seems to correspond to the Na^+/K^+-ATPase β1-isoform. This ATPase constitutes the biochemical unit of the colonic ouabain-sensitive H^+/K^+ pump.

Acknowledgment

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