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Isolation and cloning of the K^+-independent, ouabain-insensitive Na^+-ATPase

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ABSTRACT

Primary Na^+ transport has been essentially attributed to Na^+/K^+ pump. However, there are functional and biochemical evidences that suggest the existence of a K^+-independent, ouabain-insensitive Na^+ pump, associated to a Na^+-ATPase with similar characteristics, located at basolateral plasma membrane of epithelial cells. Herein, membrane protein complex associated with this Na^+-ATPase was identified. Basolateral membranes from guinea-pig enterocytes were solubilized with polyoxyethylene-9-lauryl ether and Na^+-ATPase was purified by concanavalin A affinity and ion exchange chromatographies. Purified enzyme preserves its native biochemical characteristics: Mg^{2+} dependence, specific Na^+ stimulation, K^+ independence, ouabain insensitivity and inhibition by furosemide (IC_{50}: 0.5 mM) and vanadate (IC_{50}: 9.1 μM). IgY antibodies against purified Na^+-ATPase did not recognize Na^+/K^+-ATPase and vice versa. Analysis of purified Na^+-ATPase by SDS-PAGE and 2D-electrophoresis showed that is constituted by two subunits: 90 (α) and 50 (β) kDa. Tandem mass spectrometry of α-subunit identified three peptides, also present in most Na^+/K^+-ATPase isoforms, which were used to design primers for cloning both ATPases by PCR from guinea-pig intestinal epithelial cells. A cDNA fragment of 1148 bp (*atna*) was cloned, in addition to Na^+/K^+-ATPase α1-isoform cDNA (1283 bp). In MDCK cells, which constitutively express Na^+-ATPase, silencing of *atna* mRNA specifically suppressed Na^+-ATPase α-subunit and ouabain-insensitive Na^+-ATPase activity, demonstrating that *atna* transcript is linked to this enzyme. Guinea-pig *atna* mRNA sequence (2787 bp) was completed using RLM-RACE. It encodes a protein of 811 amino acids (88.9 kDa) with the nine structural motifs of P-type ATPases. It has 64% identity and 72% homology with guinea-pig Na^+/K^+-ATPase α1-isoform. These structural and biochemical evidences identify the K^+-independent, ouabain-insensitive Na^+-ATPase as a unique P-type ATPase.

1. Introduction

In the small intestine and renal proximal tubule, transepithelial Na^+ transport depends on (i) Na^+ entry across the luminal membrane of the epithelial cells, following its electrochemical gradient, and (ii) Na^+ extrusion through the basolateral plasma membrane by active mechanisms [1]. The primary active Na^+ transport has been mainly attributed to the Na^+/K^+ pump. However, in these epithelia, active Na^+ transport is not exclusively mediated by the Na^+/K^+ exchange pump. In rabbit ileum, Nellans and Schultz [2] were unable to detect any relation between the ouabain-sensitive Na^+/K^+ exchange mechanism and the active transcellular Na^+ transport. Additionally, they reported that the maintenance of cell volume did not appear to be dependent upon the ouabain-sensitive Na^+/K^+ pump. These results suggest the existence of K^+-independent active Na^+-extrusion in the small intestine. In this sense, two different mechanisms for active Na^+ transport across basolateral plasma membrane have been described in small intestinal and proximal tubular cells [3–7]. These Na^+ pumps have been associated with two different ATPase activities, also present in these tissues [8–11]. The Na^+/K^+ pump and its Na^+/K^+-ATPase activity require K^+, are inhibited by ouabain and vanadate, but are insensitive to furosemide. In contrast, The Na^+ pump and its associated Na^+-ATPase do not require K^+, are insensitive to ouabain, but are inhibited by furosemide and vanadate [8,11]. This K^+-independent, ouabain-insensitive mechanism had been denominated the second sodium pump and has been implicated in isosmotic cell volume regulation [5,6]. Furthermore, phospho-enzyme intermediaries have been associated to the Na^+- and the Na^+/K^+-ATPases. Both enzymes can be phosphorylated from ATP or Pi in the presence of Mg^{2+} [12–15]. Recently, ouabain-insensitive Na^+-ATPase activity was found increased in kidney proximal tubule of spontaneously hypertensive rats (SHR), suggesting that this enzyme could be involved in the development of arterial hypertension [16].

The Mg^{2+} dependence, vanadate sensitivity and phosphorylation of the enzyme during its catalytic cycle suggest that the K^+-independent Na^+-ATPase could be a P-type ATPase [17,18]. It is a membrane protein family that actively transports cations or amino-phospholipids, coupling this process with ATP hydrolysis [17–19]. This family includes the Na^+/K^+-ATPase, H^+/K^+-ATPases, Ca^{2+}-ATPases, Cu^{2+}-ATPases and flippases [19].

Despite the extensive biochemical, functional and pharmacological evidences indicating the existence of the K^+-independent, ouabain-

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insensitive Na^+-ATPase in different tissues [20], it had not so far been possible to identify any protein or gene related with this ATPase activity. In this study, the membrane protein complex associated with the K^+-independent Na^+-ATPase was identified, isolated and characterized. In addition, the cDNA that codes for the catalytic subunit of this ATPase was cloned, sequenced and silenced. These results define this enzyme as a new P-type ATPase.

2. Materials and methods

Present work was approved by IVIC Bioethical Committee and carried out in accordance with EC Directive 86/609/EEC for animal experiments.

2.1. Basolateral plasma membrane preparation

Basolateral plasma membranes of guinea-pig small intestinal cells were obtained as described by del Castillo and Robinson [21]. Membranes were resuspended in 250 mM sucrose, 0.1 mM PMSF and 50 mM Tris–HCl, pH 7.2, to a protein concentration greater than 2 mg/ml and stored at −70 °C until their use.

2.2. ATPase activity determination

The ATPase activities were determined as described [8,22] and expressed in nmol of phosphate liberated per mg of protein per minute, after the subtraction of a blank obtained on adding membrane suspension only after the reaction was stopped. ATPase activity determined in the presence of Mg^{2+} alone is referred as Mg^{2+}-ATPase. The difference in activity between the Mg^{2+}-ATPase and activity in the presence of both magnesium and sodium is denoted as the Na^+-ATPase. Both Mg^{2+}- and Na^+-ATPases are insensitive to 1 mM ouabain. The difference in activity obtained in the presence of Mg^{2+}, Na^+ and 1 mM ouabain or Mg^{2+}, Na^+, K^+ and ouabain, and the activity determined in the presence of Mg^{2+}, Na^+ and K^+ are considered to be due to the Na^+/K^+- ATPase.

2.3. Solubilization of membrane-bound ATPases

Basolateral plasma membranes (1 mg/ml) were solubilized in a buffer containing 8 mM Hepes, 13 mM imidazole, 150 mM KCl, 2 mM ATP, 30% glycerol (pH 7.2) and different amounts of either octylglucoside, octaethylene-glycol-monododecyl ether ($C_{12}E_8$) or polyoxyethylene-9-lauryl ether ($C_{12}E_9$). After incubation at 4 °C for 30 min, insoluble material was separated by centrifugation at 105,000 x *g* for 90 min at 4 °C in a 50Ti rotor using a Beckman L2-65B centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). Supernatant was removed and used as a source of solubilized Na^+-ATPase. Phenyl-methylsulfonyl fluoride and a protease-inhibitor cocktail (leupeptin, pepstatin and aprotinin) were added to the suspension during the solubilization step.

2.4. Na^+/K^+-ATPase purification

Na^+/K^+-ATPase from guinea-pig renal medulla was purified, using sodium-dodecyl-sulfate (SDS) as detergent, in a discontinuous sucrose gradient as described [23]. Purified enzyme was resuspended in 10% sucrose, 25 mM imidazole and 1 mM EDTA, at pH 7.5 and stored at −70 °C.

2.5. Continuous sucrose gradient

Solubilized plasma membranes, suspended in a buffer containing 8 mM Hepes, 13 mM imidazole, 150 KCl and 0.01% $C_{12}E_9$, were loaded onto a 10–35% sucrose gradient, which was centrifuged for 16 h at 105,000 x *g* at 4 °C in a Beckman L2-65B ultracentrifuge, using a Beckman 60Ti rotor. Fractions (1 ml) were collected from bottom to top and analyzed for protein and Na^+- and Na^+/K^+-ATPase activities. Fractions with ATPase activities were concentrated using YM-50 Centricon concentrator and stored at 4 °C before use.

2.6. Gel filtration chromatography

Supernatant, containing soluble proteins, was applied to a column of Sepharose 6B (2 cm × 90 cm) from Sigma-Aldrich (Saint Louis, MO, USA) equilibrated and eluted at 4 °C with a buffer containing 8 mM Hepes, 13 mM imidazole, 150 mM KCl, 10% glycerol (pH 7.2) and 0.01% $C_{12}E_9$, at a flow rate of 15 cm/h. Fractions (2 ml) were collected using a Beckman Fraction Recovery A system and analyzed for protein and ATPases. The fractions showing ATPase activity were pooled, concentrated in a YM-50 Centricon concentrator to 1 mg/ml and stored at −70 °C.

2.7. Concanavalin A–Sepharose affinity chromatography

Fractions with ATPase activity, obtained from the Sepharose 6B column, were pooled and applied to a concanavalin A–Sepharose column from Sigma-Aldrich (1.2 × 8 cm) equilibrated with a buffer containing 8 mM Hepes, 13 mM imidazole, 150 mM KCl and 0.01% $C_{12}E_9$ (pH 7.2). The column was washed with 60 ml of the same buffer. Bounded protein was eluted with 30 ml of 8 mM Hepes, 13 mM imidazole, 150 mM KCl, 10% glycerol (pH 7.2) buffer containing 0.5 M glucose. Eluted fractions (2 ml) were assayed for protein and ATPase activities. Fractions containing Na^+-ATPase activity were pooled and concentrated using a YM-50 Centricon concentrator.

2.8. Q-Sepharose ion exchange chromatography

Fractions with Na^+-ATPase activity, obtained from the concanavalin A-sepharose 6B column, were pooled, brought up to 20 mM NaCl and loaded on a Q-sepharose Fast Flow column from Amersham Biosciences (0.5 cm × 1 cm) pre-equilibrated with 5 ml of start buffer (8 mM Hepes, 13 mM imidazole, 20 mM NaCl, 10% glycerol and 0.01% $C_{12}E_9$). Proteins were eluted with equilibration buffer, followed by a linear salt gradient (20 mM–500 mM NaCl). Fractions (1 ml) were collected at a flow rate of 1 ml/min and were assayed for protein and ATPase activities. Under these conditions, most of the Na^+-ATPase activity was eluted with 20 mM NaCl buffer.

2.9. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described [24]. 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, heated at 65 °C for 2 min, centrifuged for 1 min at 14,000 x *g* and loaded on a 10% separating gel. Samples were run at 100 mV and gel was stained with Coomassie blue R-250.

2.10. Non-denaturant PAGE

Polyacrylamide gel electrophoresis under non-denaturant conditions was carried out as described [25]. Briefly, samples (10 μg) were loaded onto a discontinuous 10% PAGE at pH 8.8 and room temperature. Samples were run at 120 mV and gel was stained with Coomassie blue R-250.

2.11. Two-dimensional electrophoresis

Purified enzyme (300–500 μg) was precipitated with acetone at −20 °C and centrifuged at 14,000 x *g* for 5 min, and the pellet was resuspended in 185 μl of re-hydration buffer (7 M urea, 2 M thiourea, 4%