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Identification, purification and partial characterization of a 70 kDa inhibitor protein of Na^+/K^+-ATPase from cytosol of pulmonary artery smooth muscle

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ABSTRACT

Aims: We sought to identify, purify and partially characterize a protein inhibitor of Na^+/K^+-ATPase in cytosol of pulmonary artery smooth muscle.
Main methods: (i) By spectrophotometric assay, we identified an inhibitor of Na^+/K^+-ATPase in cytosolic fraction of pulmonary artery smooth muscle; (ii) the inhibitor was purified by a combination of ammonium sulfate precipitation, diethylaminoethyl (DEAE) cellulose chromatography, hydroxyapatite chromatography and gel filtration chromatography; (iii) additionally, we have also purified Na^+/K^+-ATPase $\alpha_2\beta_1$ and $\alpha_1\beta_1$ isozymes for determining some characteristics of the inhibitor.
Key findings: We identified a novel endogenous protein inhibitor of Na^+/K^+-ATPase having an apparent mol mass of ~70 kDa in the cytosolic fraction of the smooth muscle. The IC_{50} value of the inhibitor towards the enzyme was determined to be in the nanomolar range. Important characteristics of the inhibitor are as follows: (i) it showed different affinities toward the $\alpha_2\beta_1$ and $\alpha_1\beta_1$ isozymes of the Na^+/K^+-ATPase; (ii) it interacted reversibly to the E_1 site of the enzyme; (iii) the inhibitor blocked the phosphorylated intermediate formation; and (iv) it competitively inhibited the enzyme with respect to ATP. CD studies indicated that the inhibitor causes an alteration of the conformation of the enzyme. The inhibition study also suggested that the DHPC solubilized Na^+/K^+-ATPase exists as $(\alpha\beta)_2$ diprotomer.
Significance: The inhibitor binds to the Na^+/K^+-ATPase at a site different from the ouabain binding site. The novelty of the inhibitor is that it acts in an isoform specific manner on the enzyme, where α_2 is more sensitive than α_1.

Introduction

The enzyme Na^+/K^+-ATPase exists in the plasma membrane of animal cells and maintains a high intracellular K^+ to Na^+ ratio by coupling movement of these ions to the hydrolysis of ATP. The enzyme is composed of two subunits, a larger α subunit having a molecular mass of ~112 kDa, and the smaller glycosylated β subunit, the molecular mass of which ranges between 40 and 60 kDa depending on the extent of glycosylation. A third subunit, termed γ subunit has also been identified recently (Blanco and Mercer 1998).

Like many other essential proteins in the cell, the Na^+/K^+-ATPase has been shown to be expressed as several isozymes in different tissues and the expression appears to occur in developmental-specific manner. The α subunit has four isoforms identified to date: α_1, α_2, α_3 and α_4; while there are three isoforms of the β subunit: β_1, β_2 and β_3. The α_1 isoform is expressed ubiquitously but the α_2 isoform is present largely in the skeletal muscle, heart and vascular smooth muscle. The α_3 isoform is found almost exclusively in neurons and ovaries. The α_4 isoform is expressed in sperm (Lopina 2001). We have recently demonstrated that the Na^+/K^+-ATPase exist as $\alpha_1\beta_1$ and $\alpha_2\beta_1$ isozymes in pulmonary artery smooth muscle (Ghosh et al. 2009a).

Cardiac glycosides, for example, ouabain has been shown to inhibit Na^+/K^+-ATPase activity by binding to the extracellular face of the enzyme. Apart from cardiac glycosides, some modulators are also known to modify the Na^+/K^+-ATPase activity. Some of these compounds, for example, endobain (Rodriguez et al. 1998) elicit its effect in the similar fashion to that of ouabain by acting on the E_2 conformation of the enzyme; while some chromium complexes, for instance, $Cr(H_2O)_4$-Ado*PP*[CH_2]P elicit their inhibitory effect on the enzyme upon binding to the E_1 site of the enzyme (Hamer and Schoner 1993). Besides these endogenous inhibitors, reports are also available about the existence of some activators of the enzyme, for example, PEC-60 (Kairane et al. 1994).

There is a long standing controversy about the minimum catalytic unit of the enzyme Na^+/K^+-ATPase i.e., whether it exists as αβ protomer or higher oligomers (Repke and Schon 1973; Stein et al. 1973; Plesner 1987). Both the αβ protomeric and $(\alpha\beta)_2$ diprotomeric models gained support from various experiments (Ward and Cavieres 1993; Hamer and Schoner 1993).

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Herein, we report the identification, purification and characterization of an apparently specific Na^+/K^+-ATPase inhibitor isolated from bovine pulmonary artery smooth muscle cytosol. The inhibitor is different from ouabain with respect to its mode of action. The apparent molecular mass of the inhibitor appears to be 70 kDa. We also investigated the inhibitory potency of the inhibitor against purified $\alpha_1\beta_1$ and $\alpha_2\beta_1$ isozymes of the Na^+/K^+-ATPase in the absence or presence of ouabain, as well as its ability to block high affinity [^{3}H] ouabain binding to these isozymes. Additionally, we suggest that the purified Na^+/K^+-ATPase upon detergent solubilization of the plasma membrane could remain in dimeric $(\alpha\beta)_2$ form.

Materials and methods

Materials

Sephacryl S200 was purchased from GE Health Care Biosciences Ltd., Hong Kong. BCA protein assay kit was the product of Pierce (Rockford, IL). [γ-^{32}P] ATP (specific activity 30 C_i/mmol) and [^{3}H] ouabain (specific activity 18 C_i/mmol) were the products of New England Nuclear, Wilmington, DE. Rabbit polyclonal anti-mannosidase II was purchased from U.S. Biological (Swampscott, MA). Mouse anti-calnexin was obtained from BD Transduction Laboratories (San Jose, CA). Mouse anti-integrin and mouse anti Lamp-1 were the products of Calbiochem (La Jolla, CA). Polyclonal antibody of lactate dehydrogenase (LDH) (host goat) was obtained from Fitzerald Industries International Inc (Concord, MA). Polyclonal histone H3 antibody was the product of Abcam Ltd. (Cambridge, UK). Na^+/K^+-ATPase non-selective polyclonal α subunit (anti LEAVE), α_1 specific polyclonal antibody (anti NASE) and α_2 specific polyclonal antibody (anti HERED) were kindly provided by Prof. Tomas Pressley (Department of Physiology, Texas Tech University Health Sciences Center, Lubbock, TX). Na^+/K^+-ATPase β-subunit (β_1, β_2, and β_3) polyclonal antibodies were kindly provided by Prof. Pablo Martin Vassalo (Department of Biochemistry and Molecular Biology, University of La Lagune, La Lagune, Tenerife, Spain). All other chemicals were the products of Sigma Chemical Co. (St. Louis, MO).

Methods

Isolation of pulmonary artery smooth muscle tissue

Bovine pulmonary artery collected from slaughter house was washed several times with Hank's buffered physiological saline (HBPS, pH 7.4), kept at 4 °C, and was used for further processing within 1 h after collection. The intimal and serosal (outer) layers were removed and the tunica media i.e., the smooth muscle tissue was collected by following the procedure of Chakraborti et al. (1996), then characterized histologically as described by Mandal et al. (2003) and used for the present studies.

Isolation of plasma membrane fraction and cytosol from pulmonary artery smooth muscle tissue

The smooth muscle plasma membrane and cytosol fractions were isolated by following the procedure as previously described (Chakraborti et al. 1996). Briefly, the bovine pulmonary artery smooth muscle tissue was homogenized in ice-cold homogenizing media (containing 0.25 M sucrose–100 mM Tris buffer (pH 7.4) and 100 μM PMSF). The homogenate was then centrifuged at 20,000 *g* in a high speed refrigerated centrifuge (Hitachi) for 20 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 100,000 *g* in an ultracentrifuge (Beckman) for 1 h. The pellet was then suspended in the homogenizing buffer and centrifuged again at 100,000 *g* for 1 h. The resulting supernatant was considered as the cytosol fraction. The pellet was resuspended in 10% sucrose–100 mM Tris buffer (pH 7.4), then layered onto a sucrose gradient containing 30%–40% sucrose (containing 10 mM Tris buffer, pH 7.4), and centrifuged at 110,000 *g* in an ultracentrifuge (Beckman) for 1 h at 4 °C. The fraction at the 30–40% sucrose interface was collected and centrifuged at 120,000 *g* for 1 h at 4 °C in an ultracentrifuge (Beckman). The pellet was suspended in the homogenizing buffer and used as the plasma membrane fraction.

Identification of Na^+/K^+-ATPase and its isoforms in pulmonary artery smooth muscle plasma membrane

All protein samples were solubilized with Laemmli sample buffer and then heated at 80 °C. Ten micrograms of each sample was separated in 7.5% SDS-PAGE. After electrophoresis, the proteins were transferred electrophoretically on nitrocellulose membranes by following the method previously described by Towbin et al. (1979) with some modification as described by Chakraborti et al. (2005). Membranes were incubated for 1 h in 5% non-fat milk in 50 mM Tris–saline containing 0.05% Tween 20 at pH 7.5 (TTBS). The membranes were then incubated overnight in the primary antibody in TBS at 22 °C. After that the membranes were rinsed three times in TTBS and incubated for 2 h in horseradish peroxidase conjugated appropriate secondary antibodies. The membranes were then washed three times with TTBS (20 min each), and then developed with 0.2 mM 4-chloro-1-naphthol.

Purification of 70 kDa inhibitor protein of Na^+/K^+-ATPase

Ammonium sulphate precipitation

Solid ammonium sulfate was added to the cytosolic fraction to 40% saturation. The suspension was then centrifuged at 12,000 *g* for 45 min. The pellet was collected and dissolved in a buffer containing 25 mM Tris–HCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.25 M sucrose (pH 7.5) and dialyzed against the same buffer for 24 h with several changes.

DEAE-Cellulose Chromatography

The dialyzed fraction of the ammonium sulphate precipitate was loaded onto a Bio-Rad Econo Pack column (10 ml) filled with diethylaminoethyl (DEAE) cellulose matrix, which was equilibrated with a buffer containing 25 mM Tris–HCl (pH 7.5), 0.2 mM EDTA and 0.5 mM β-mercaptoethanol. The elution was performed with a 0.03–0.25 M NaCl gradient and the $Abs_{280\ nm}$ of the eluates were monitored to detect the protein peak. The fractions containing the inhibitor were pooled and then dialyzed with the same buffer without EDTA for 24 h with 4 changes. The dialyzed material was then concentrated with Amicon ultrafiltration using YM10 membrane (Molecular weight cut off 10 kDa).

Hydroxyapatite chromatography

The concentrated sample from DEAE-Cellulose was applied to a hydroxyapatite column [Bio-Rad Econo Pack column (10 ml)], which was pre-equilibrated with 10 mM potassium phosphate buffer (pH 6.8), and protein was eluted by washing the column with the same buffer. It was then dialyzed against 10 mM Tris–HCl buffer (pH 7.5) containing 0.5 mM β-mercaptoethanol for 48 h with four changes and concentrated with Amicon ultrafiltration using YM10 membrane (Molecular weight cut off 10 kDa).

Gel filtration with Sephacryl S200

The concentrated material obtained after hydroxyapatite chromatography was further purified by gel filtration at 4 °C on a Sephacryl S200 Column (1.4×70 cm) connected with AKTA Prime plus FPLC system which was equilibrated and eluted with a buffer containing 25 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl with a flow rate of 0.4 ml/min. Proteins were monitored spectrophotometrically at 280 nm. Fractions showing Na^+/K^+-ATPase inhibitory activity were pooled, concentrated by Amicon ultrafiltration using YM10 membrane (molecular weight cut off 10 kDa), and dialyzed against the same buffer for 2 h at 4 °C.