Biochimica et Biophysica Acta 1808 (2011) 2413–2420

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Oleic and linoleic acids are active principles in *Nigella sativa* and stabilize an E_2P conformation of the Na,K-ATPase. Fatty acids differentially regulate cardiac glycoside interaction with the pump

Yasser A. Mahmmoud [a,*], S. Brøgger Christensen [b]

[a] Department of Physiology and Biophysics, University of Aarhus, Denmark
[b] Department of Medicinal Chemistry, University of Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 23 May 2011
Received in revised form 27 June 2011
Accepted 29 June 2011
Available online 13 July 2011

Keywords:
Na,K-ATPase
Nigella Sativa
Oleic acid
Linoleic acid
Ouabain affinity
Anthroyl ouabain

ABSTRACT

Nigella sativa seed oil was found to contain a modulator of Na,K-ATPase. Separation analyses combined with ^{1}H NMR and GCMS identified the inhibitory fraction as a mixture of oleic and linoleic acids. These two fatty acids are specifically concentrated in several medicinal plant oils, and have particularly been implicated in decreasing high blood pressure. The ouabain binding site on Na,K-ATPase has also been implicated in blood pressure regulation. Thus, we aimed to determine how these two molecules modify pig kidney Na,K-ATPase. Oleic and linoleic acids did not modify reactions involving the E_1 (Na^+) conformations of the Na,K-ATPase. In contrast, K^+ dependent reactions were strongly modified after treatment. Oleic and linoleic acids were found to stabilize a pump conformation that binds ouabain with high affinity, i.e., an ion free E_2P form. Time-resolved binding assays using anthroylouabain, a fluorescent ouabain analog, revealed that the increased ouabain affinity is unique to oleic and linoleic acids, as compared with γ-linolenic acid, which decreased pump-mediated ATP hydrolysis but did not equally increase ouabain interaction with the pump. Thus, the dynamic changes in plasma levels of oleic and linoleic acids are important in the modulation of the sensitivity of the sodium pump to cardiac glycosides. Given the possible involvement of the cardiac glycoside binding site on Na,K-ATPase in the regulation of hypertension, we suggest oleic acid to be a specific chaperon that modulates interaction of cardiac glycosides with the sodium pump.

1. Introduction

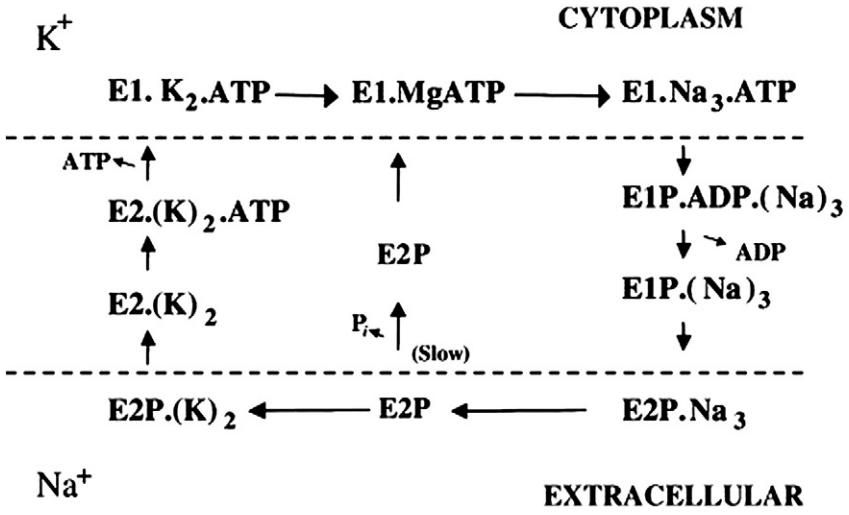
The Na,K-ATPase (sodium pump) is a member of the P_2-type ATPase family, which includes gastric H,K-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase [1]. The Na,K-ATPase is a heterodimeric membrane protein that pumps 3 Na^+ ions out- and 2 K^+ ions into the cell, at the expenditure of the energy derived from hydrolysis of one ATP molecule (Scheme 1). Consequently Na,K-ATPase establishes an electrochemical gradient for these ions across the plasma membrane, which is indispensible for many cell functions. The enzyme consists of a catalytic α-subunit that undergoes ions- and ATP-dependent conformational transitions coupling ATP hydrolysis to ion transport, and a glycosylated β-subunit that is important for function, folding, and plasma membrane delivery of the overall enzyme complex [2]. The sodium pump also contains a highly conserved site of inhibition by cardiac glycosides, which apparently represents the molecular basis for the treatment of congestive heart failure and supraventricular arrhythmias by these drugs for more than 200 years. The gastric H, K-ATPase is found in gastric parietal cells where it exchanges cytoplasmic H^+ for luminal K^+, and is responsible for a million fold H^+ gradient across the epithelial cell membrane [3].

Traditional medicine has been a wealthy source of chemotherapeutics [4]. Several modulators of P-type pumps have also been found in medicinal plants [5–7]. The seeds of *Nigella sativa* L. have traditionally been used to treat several health problems. To date, about 6 active principles have been isolated from the seed oil [8]. We have been interested in identifying modulators of P-type ATPases in medicinal plants (e.g., [9]). In this study, we have initially observed that an extract of *N. sativa* seeds contains a factor that inhibits ATP hydrolysis by Na, K-ATPase and H,K-ATPase. The active modulator was found to consist of oleic and linoleic acids as the major components. Significantly, the predominant levels of these two fatty acids have been shown to build up in the plasma of animals challenged with high salt intake [10]. Additionally, oleic acids have unambiguously been shown to have a blood pressure lowering effect [11]. Furthermore, the highly conserved cardiac glycoside site on the Na,K-ATPase has been implicated in the regulation of blood pressure [12]. The above considerations motivated

Abbreviations: EDTA, Ethylenediaminetetraacetic acid; EP, phosphoenzyme; DMSO, Dimethyl sulfoxide; *pNPP*, *para*-nitrophenyl phosphate; TCA, trichloroacetic acid

* Corresponding author at: Department of Physiology and Biophysics, Ole Worms Alle 6, Bld. 1180, DK-8000 Aarhus C, Denmark. Tel.: +45 89422927; fax: +45 86129599.
E-mail address: yam@biophys.au.dk (Y.A. Mahmmoud).

0005-2736/$ – see front matter.
doi:10.1016/j.bbamem.2011.06.025



Scheme 1. Minimum reaction cycle of the Na,K-ATPase, according to the Post-Albers model [2].

us to perform detailed analysis to precisely define the mechanism whereby oleic and linoleic acids modulate the properties of pig kidney Na,K-ATPase. We provide evidence that an increase in the level of oleic and linoleic acids in the membrane locks the sodium pump in a potassium-free E_2P conformation, the form that binds cardiac glycosides with high affinity.

2. Methods

2.1. Preparation and hydrolytic activity of Na,K-ATPase

Plasma membranes from pig kidney red outer medulla were isolated according to a modified Jørgensen's method described earlier [13]. In brief, minced tissue pieces were homogenized in 30 mM imidazole buffer pH 7.4, containing 250 mM sucrose and 1 mM EDTA. The homogenate was subjected to several differential centrifugation steps to obtain a microsomal plasma membrane fraction. Microsomes were treated with a mild concentration of sodium dodecyl sulfate to open sealed vesicles and dissociate several peripheral proteins from the membrane. The final preparation had a specific activity of 1.4–2.0 $mmol.h^{-1}.mg^{-1}$ protein at 37 °C. Protein concentration was determined using a BioRad detergent compatible kit (Cat# 500–0113), according to the manufacturer's instructions. ATPase activity was measured by incubating the enzyme with buffer and substrates (see separate figure legends) at 37 °C followed by measuring phosphate liberated from ATP, according to the method of Baginsky [14]. Control activities were measured in identical conditions but in the presence of 1 mM ouabain. ATPase measurements made in the presence of low ATP concentrations were performed in the presence of tracer amount of ^{32}P-ATP. In the later case, ATP hydrolysis was determined by measuring released ^{32}P, isolated after conversion to phosphomolybdate, essentially as described earlier [15]. *Para*-nitrophenyl phosphatase (*p*NPPase) activity was measured in a reaction mixture containing 30 mM histidine pH 7.0, 5 mM MgCl, 10 mM *para*-nitrophenyl phosphate (*p*NPP), 5 μg protein, and the K^+ concentrations mentioned in the legend to Fig. 10. Ouabain inhibitable *para*-nitrophenyl release was determined using a colorimetric method, by measuring the absorbance at 410 nm. *Para*-nitrophenyl has a molar extinction coefficient of $1.8 \cdot 10^4\ M^{-1}\ cm^{-1}$.

2.2. Fractionation of the constituents of Nigella sativa L

The crude seed oil (13.7 g) was dissolved in methanol–water (9:1, 80 mL). The solution is extracted with petroleum ether (bp. 40–65 °C). The two phases were separated and concentrated in vacuo. The residue of the methanol–water phase was partitioned between water and toluene (80 mL of each) and the obtained aqueous phase subsequently extracted with ethyl acetate. All four phases were concentrated in vacuo. Only the toluene phase revealed a significant activity with respect to Na, K-ATPase and H,K-ATPase inhibition. The residue of the toluene phase (0.5 g) was fractionated by column chromatography over silica gel (50 g) using toluene added 1% of acetic acid (400 mL), toluene-ethyl acetate (19:1) added 1% of acetic acid (200 mL), toluene-ethyl acetate (9:1) added 1% of acetic acid (400 mL), toluene-ethyl acetate (4:1) added 1% of acetic acid (400 mL), toluene-ethyl acetate (3:2) added 1% of acetic acid (200 mL). Fractions with a similar TLC profile were combined. Fractions eluting between 330 and 520 mL were combined and concentrated in vacuo to give 357 mg of a residue. The residue showed a significant Na,K-ATPase and H,K-ATPase inhibitory activity. The ^{1}H NMR spectrum revealed that the residue was a mixture of oleic acid and linoleic acid (1:1). GCMS after methylation with trimethylsilyl diazomethane revealed the presence of methyl esters of oleic acid, linoleic acid and a small amount of palmitic acid. Analytical grade free fatty acids (>99%) were obtained from Sigma.

2.3. Phosphoenzyme measurements

The phosphorylation reaction was performed by diluting the enzyme at 0 °C with 10-folds of an ice-cold solution. The final phosphorylation mixture contained 20 mM histidine pH 7.0, 0.2 mM EGTA, NaCl (concentrations are mentioned in figure legends), 1 mM $MgCl_2$, and 25 μM Tris–ATP containing ~20.000 cpm ^{32}P-[ATP] per reaction. The acid stable phosphoenzyme (EP) was quenched after 10 s with ice-cold mixture containing 5% TCA, 3 mM ATP, and 2 mM Na_2HPO_4, as described previously [16]. Phosphorylation in the presence of FFA was performed by pre-incubating the enzyme with FFA for 5 min at room temperature (either in the absence or in the presence of Na^+), followed by chilling on ice for few minutes prior to phosphorylation start. The EP was collected by centrifugation and its radioactivity determined using scintillation counting. Data were expressed as EP phosphoenzyme formed in the presence of NaCl minus that formed in the presence of KCl (replacing NaCl). All phosphorylation data are presented as mean ± s.e.m. of triplicate measurements. Phosphorylation from inorganic phosphate was performed by incubating the enzyme for 15 min at room temperature in the same buffer but in the presence of 6 mM $MgCl_2$ and 40 μM Na_2HPO_4 containing ~20.000 cpm ^{32}P.

2.4. Assay for K^+ occlusion

K^+ occlusion was measured by simple gel filtration method [17] using 5 cm plastic columns loaded with Dowex 50WX8, which were