VETERINARSKI ARHIV 76 (6), 489-495, 2006

Low nanomolar concentrations of ouabain may induce higher activity of the Na^+/K^+-ATPase in human erythrocytes

Roberto Antolović*

Biology Department, PLIVA Research Institute Ltd., Zagreb, Croatia

ANTOLOVIĆ, R.: Low nanomolar concentrations of ouabain may induce higher activity of the Na^+/K^+-ATPase in human erythrocytes. Vet. arhiv 76, 489-495, 2006.

ABSTRACT

The inhibitor g-Strophantin, also known as ouabain, is a specific inhibitor of the Na^+/K^+-ATPase. In this work ouabain has been used for the inhibition of the Na^+/K^+-ATPase in human erythrocytes and isolated enzyme from pig kidney. Enzymatic activity of the Na^+/K^+-ATPase has been measured in a broad concentration range of ouabain in two different *in vitro* investigations. As a potent inhibitor of the sodium pump the cardiotonic steroid ouabain inhibits its enzymatic activity on both isolated human red blood cells and on the purified enzyme from pig kidney. Na^+/K^+-ATPase activity in erythrocyte can be determined by measuring the ouabain-sensitive uptake of ^{86}Rb (as a congener for potassium). This work provides evidence that very low concentrations of ouabain in the nM range can stimulate increase of Na^+/K^+-ATPase activity in human erythrocytes, in contrast to inhibition of the purified enzyme from pig kidney, where such an effect was not observed.

Key words: Na^+/K^+-ATPase, ouabain, erythrocyte, signal transduction, cardiotonic steroid

Introduction

Na^+/K^+-ATPase is a highly conserved, ubiquitous membrane protein (EC 3.6.1.37) and is a member of the P-type ATPase superfamily. The enzyme is composed of three sub-units: the α-subunit (≈113 kDa) binds ATP, sodium and potassium ions, contains the phosphorylation site and has an equal number of membrane spanning regions (ANTOLOVIĆ et al., 1991). The smaller beta sub-unit (≈35 kDa glycoprotein) is necessary for activity of the complex and its correct insertion in the plasma membrane of the animal cell. The gamma sub-unit (≈10 kDa) is the smallest sub-unit and its function in the Na^+/K^+-ATPase and is, until now, not well defined. Several isoforms of both alpha and beta sub-units have

* Contact address:
Dr. Roberto Antolović, Biology Department, PLIVA Research Institute Ltd., Prilaz baruna Filipovića 29, 10000 Zagreb, Croatia, E-mail: Roberto.Antolovic@pliva.hr

ISSN 0372-5480
Printed in Croatia

been identified (KAPLAN, 2002). The Na^+/K^+-ATPase, or sodium pump, carries out the coupled extrusion and uptake of Na^+ and K^+ ions across the plasma membranes of cells of higher eukaryotes. Na^+/K^+-ATPase is specifically inhibited by cardiotonic steroids (SCHATZMANN, 1953) which bind to a specific conformation of Na^+/K^+-ATPase formed in the catalytic cycle during the hydrolysis of ATP (HANSEN, 1984; GLYNN et al., 1985). The Na^+/K^+-ATPase is the only known pharmacological receptor for cardiotonic steroids such as digitalis. Observations, including the tight conservation of the cardiac glycoside binding site over many phila, suggest that endogenous inhibitors of the Na^+/K^+-ATPase might exist in the mammalian body. Accumulated evidence published in the last decade suggests that several compounds of cardienolide or bufadienoide structure are present in humans and animals and have an influence on the physiological role of the Na^+/K^+-ATPase in the cell. For more than 200 years, digitalis, a cardiotonic steroid and its congeners have been used to treat congestive heart failure. Modern understanding of digitalis therapy arose 50 years ago, when in 1953 Schatzmann discovered that the cardiotonic steroids are specific inhibitors of the sodium pump and that the digitalis receptor is the Na^+/K^+-ATPase of the plasma membranes. The discovery of the Na^+/Ca^{2+} exchanger in the late 1960s in mammalian cardiac muscle led to the view that inhibition of the sodium pump by cardiotonic steroids leads to an increase in the concentration of intracellular Ca^{2+} as secondary event, which in turn results in a positive inotropic effect on cardiac muscle. α_1 isoform is ubiquitously distributed in plasma membranes of cardiomyocytes, but the α_2/α_3 isoforms reside in plasma membrane areas close to the endoplasmic reticulum. Such "plasmerosomes" also contain the Na^+/Ca^{2+} exchanger protein. Inhibition of the α_2 and α_3 isoforms of Na^+/K^+-ATPase in such a restricted area leads to an exchange in cytosolic Na^+ and, indirectly, Ca^{2+} concentration. In turn, this modulates the Ca^{2+} content of the sarcoplasmic reticulum and Ca^{2+} signaling and leads finally to the positive inotropic effect of cardiac glycosides and altered gene and expression of the proteins (SCHEINER-BOBIS and SCHONER, 2001). Additionally, it became evident that in contrast to previous assumptions, the sodium pump itself may act as a hormone receptor, transducing the signal or causing an interaction with the cardiotonic steroid. Ouabain-induced cross-talk in the cell involves highly complex signaling cascades which lead to the activation of transcription factors as NF-κB and AP-1 (AIZMAN et al., 2001). Changes in ouabain concentration in the blood stream will affect the contractility, growth rate and differentiation of a great number of the cells in the tissue in a specific way.

Materials and methods

^{86}RbCl (Amersham Biosciences) with specific radioactivity of 1 mCi/mL Rb, and Silicon oil AR 200 and 20 (Wacker Chemie, Munich, Germany) were used. All other chemicals were of the highest available purity.

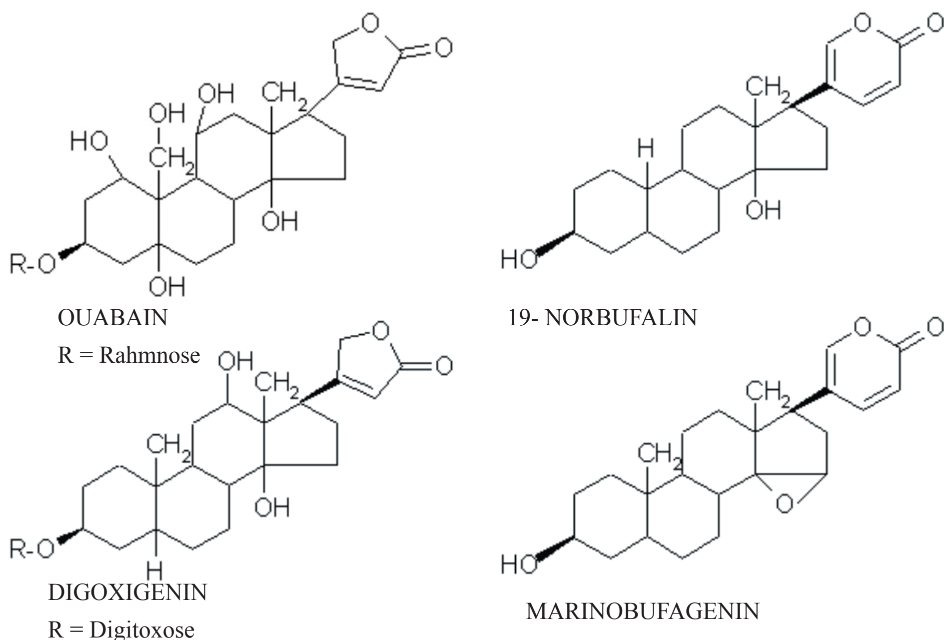

Fig. 1. Structures of the known inhibitors of the sodium pump. Ouabain and digoxigenin both with the unsaturated five-membered lactone ring at the C17 position belong to the cardienolide class and 19-norbufagenin, and manrinobufagenin with unsaturated six-membered lactone ring on the C17 position belong to the bufadienolids class.

Enzyme and assays. Na^+/K^+-ATPase in the range of 28-32 $U \cdot mg^{-1}$ protein was isolated from pig kidneys by a modification of Jørgensen's procedure (JØRGENSEN, 1974). One enzyme unit (U) is defined as the amount of the enzyme catalyzing the hydrolysis of 1 μmol ATP at 37 °C under the conditions of the coupled spectrophotometric assay (SCHONER et al., 1967). Impurities from other ATPases were below 0.4%, as estimated by difference from a control with ouabain. Protein concentration was determined by the method of LOWRY et al. (1951) using Lab-Trol as a standard. Lab-Trol is a mixture of proteins and enzymes used for the calibration of assays in clinical chemical analysis. All buffers used were adjusted to their respective pH value at room temperature.

^+Rb uptake in human erythrocytes. The blood was taken by venipuncture in the presence of heparin as an anticoagulant. The cells were subsequently washed three times with Krebs-Ringer buffer (KRP, 140 mM NaCl, 5 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgSO_4$,

1.4 mM $CaCl_2$, and 2.5 mM glucose, pH 7.4) and incubated with or without ouabain in the Krebs-Ringer buffer for 15 min. at 37 °C. $^{86}Rb^+$ was added to a final concentration of 2 μCi/mL, and the reaction was allowed to proceed for 15 min. at 37 °C. $^{86}Rb^+$ uptake was terminated by washing thrice with ice-cold Krebs-Ringer buffer. The cells were then lysed with lysis buffer (50 mM NaOH, 1% Triton X-100) and incubated for 5 min. at room temperature. The radioactivity of the cell lysates was determined after adding scintillation mixture solution. Protein concentration per well was determined according to the Lowry assay and averaged. For this, cells contained in three wells were taken through the procedure without the addition of a label.

Results

Na^+/K^+-ATPase activity was influenced in the presence of ouabain. The ouabain-specific inhibition of the sodium pump has been shown on the purified enzyme from pig kidney and on isolated human erythrocytes. The inhibition rate for ouabain of the Na^+/K^+-ATPase purified from pig kidney was in the μmolar range, as shown in Table 1. This is in concordance with in the literature-published ouabain concentrations which exhibit half the maximal inhibition of the Na^+/K^+-ATPase. Low concentrations of the inhibitor in nanomolar range did not influence hydrolytic activity of the sodium pump tested *in vitro* assay, as reported in material and methods.

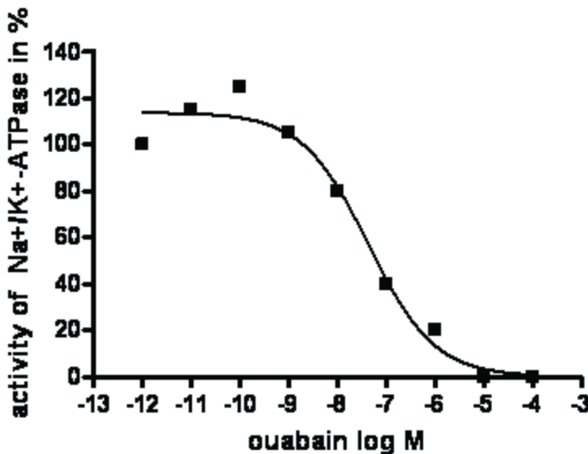

Fig. 2. Inactivation of isolated Na^+/K^+-ATPase from pig kidney by ouabain as a function of its concentration. Activity of the Na^+/K^+-ATPase measured by the Rubidium uptake in the human erythrocytes in the presence of different concentrations of ouabain. Values are normalized and compared to the Na^+/K^+-ATPase activity of the control erythrocytes incubated with ^{86}Rb in absence of ouabain.

In contrast to the enzymatic inhibition of the Na^+/K^+-ATPase of purified enzyme the Rubidium uptake into the human erythrocytes did show a difference in the Na^+/K^+-ATPase activity in the presence of the very low ouabain concentrations. Nanomolar concentrations of ouabain increased the activity of the Na^+/K^+-ATPase in human erythrocytes. Although the activity of the sodium pump in human erythrocytes increased at low ouabain concentrations the IC_{50} for ouabain value was the same as the isolated enzyme from the pig kidney, as shown in Table 1.

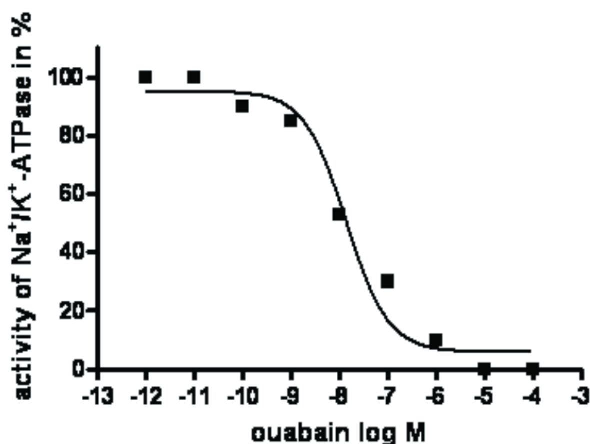

Fig. 3. Inactivation of isolated Na^+/K^+-ATPase from pig kidney by ouabain as a function of its concentration. Purified Na^+/K^+-ATPase (60 μg) was incubated with different concentrations of ouabain for 30 min. at 37 °C in the presence of 3mM ATP, 3 mM $MgCl_2$ and 150 mM NaCl.

Table 1.Various concentrations of ouabain yielding half maximal inhibition of Na^+/K^+-ATPase calculated with non linear regression in GraphPad Prism software.

Ouabain	Na^+/K^+-ATPase from pig kidney	Na^+/K^+-ATPase from human erythrocytes
IC_{50}	1.37×10^{-8} M	4.34×10^{-8} M

Discussion

In certain cell types the signalling Na^+/K^+-ATPase and its protein partners are compartmentalized in coated pits (i.e., caveolae) on the plasma membrane. Caveolae are membrane microdomains that were first identified as flask-shaped vesicular invaginations of plasma membrane enriched in cholesterol, glycosphingolipids, and sphingomyelin (LIU et al., 2002; RAZANI et al., 2002). Binding of ouabain to the signalling Na^+/K^+-ATPase activates the cytoplasmic tyrosine kinase Src, resulting in the formation of an active "binary receptor"

that phosphorylates and assembles other proteins into different signalling modules. This in turn activates multiple protein kinase cascades including mitogen-activated protein kinases and protein kinase C isozymes in a cell-specific manner. Crosstalk among the activated pathways eventually results in changes in the expression of a number of genes (SCHONER, 2002). Although ouabain stimulates hypertrophic growth in cardiac myocytes and proliferation in smooth muscle cells, it also induces apoptosis in many malignant cells. Because the Na^+/K^+-ATPase has to interact with Src, EGFR, and other proteins to transmit the ouabain signal, it is possible that the signalling enzyme may have to be preassembled with its signalling partners in caveolae. Caveolins are 21-24 kDa membrane-associated scaffolding proteins, with multiple cellular functions that typify caveolae (LIU et al., 2002). Mammals express three different caveolin genes that encode five different protein isoforms, the expression and distribution of which are tissue-specific (RAZANI et al., 2002). Caveolins directly interact with cholesterol and many signalling proteins, including receptors, Src family kinases, and adapter proteins. Recent studies have indicated that many of these interactions are mediated through digoxin and ouabain, and vertebrate-derived aglycones such as bufalin and marinobufagenin. Although they have been considered for a long time only as drugs, there is now ample evidence to indicate that some of these compounds can function as steroid hormones (SCHONER, 2002; LAREDO et al., 1997).

Increased activity of the Na^+/K^+-ATPase in human erythrocytes in the presence of nM concentrations of cardiotonic steroid ouabain may suggest that the Na^+/K^+-ATPase in erythrocytes might be a part of the caveolin-similar structures which are activated by the steroid glycosides during which effect of insertion of additional Na^+/K^+-ATPase occurs in the plasma membrane.

Conclusion

This work has produced evidence that ouabain as a potent inhibitor of the sodium pump has the same inhibition rate for the isolated Na^+/K^+-ATPase in the micromolare range and that in the red blood cells, respectively, but in the presence of the low nanomolar range ouabain increase the basal enzymatic activity of the Na^+/K^+-ATPase of isolated human erythrocytes *in vitro*.

References

AIZMAN, O., P. UHLÉN, M. LAI, H. BRISMAR, A. APERIA (2001): Ouabain, a steroid hormone that signals with slow calcium oscillations. Proc. Natl. Acad. Sci. USA 98, 13420-13424

ANTOLOVIC, R., H. J. BRÜLLER, S. BUNK, D. LINDER, W. SCHONER (1991): Epitope mapping by amino-acid-sequence-specific antibodies reveals that both ends of the α-subunit of Na^+/K^+-ATPase are located on the cytoplasmic side of the membrane. Eur. J. Biochem. 199, 195-202

GLYNN, I. M., J. L. HOWLAND, D. E. RICHARDS (1985): Evidence for the ordered release of rubidium ions occluded within the Na,K-ATPase of mammalian kidney. J. Physiol. 368, 453-469.

HANSEN, O. (1984): Interaction of cardiac glycosides with (Na+ + K+)-activated ATPase. A biochemical link to digitalis-induced inotropy. Pharmacol. Rev. 36, 143-163.

JØRGENSEN, P. L. (1974): Purification and characterization of ($Na^+ + K^+$)-ATPase. III. Purification from the outer medulla of mammalian kidney after selective removal of membrane components by sodium dodecylsulphate. Biochim. Biophys. Acta 356, 36-52.

KAPLAN, J. H. (2002): Biochemistry of Na, K-ATPase. Annu. Rev. Biochem. 71, 511-535.

LAREDO, J., J. R. SHAH, Z. R. LUM, B. P. HAMILTON, J. M. HAMLYN (1997):, Angiotensin II stimulates secretion of endogenous ouabain from bovine adrenocortical cells via angiotensin type 2 receptors. Hypertension 29, 401-407.

LIU, P., M. RUDICK, R. G. ANDERSON (2002): Multiple functions of caveolin-1. J. Biol. Chem. 277, 41295-41298.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, R. J. RANDALL (1951): Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 264-275.

RAZANI, B., S. E. WOODMAN, M. P. LISANTI (2002): Caveolae from cell biology to animal physiology. Pharmacol. Rev. 54, 431-467

SCHEINER-BOBIS, G., W. SCHONER (2001): A fresh facet for ouabain action. Nature medicine 7, 1288-1289.

SCHONER, W., C. VON ILBERG, R. KRAMER, W. SEUBERT (1967): On the mechanism of Na^+- and K^+-stimulated hydrolysis of adenosine triphosphate. 1. Purification and properties of a Na^+- and K^+-activated ATPase from ox brain. Eur. J. Biochem. 1, 334-343.

SCHONER, W. (2002); Endogenous cardiac glycosides, a new class of steroid hormones. Eur. J. Biochem. 269, 2440-2448.

Received: 20 December 2005
Accepted: 3 November 2006

ANTOLOVIĆ, R.: Niske molekularne koncentracije ouabaina potiču veću aktivnost Na^+/K^+-ATPaze u čovječjim eritrocitima. Vet. arhiv 76, 489-495, 2006.

SAŽETAK

g-Strophantin koji je poznat i pod imenom ouabain je specifični inhibitor Na^+/K^+-ATPase. U ovom je radu ouabain korišten u inhibiciji enzimske aktivnosti Na^+/K^+-ATPase u čovječjim eritrocitima i izoliranom enzimu iz bubrega svinje. Kao jaki inhibitor natrijeve pumpe kardiotoni steroid, ouabain inhibira njenu aktivnost u oba slučaja u izoliranim čovječjim eritrocitima i pročišćenom enzimu. Aktivnost Na^+/K^+-ATPase u eritrocitima može biti proučavana koristeći se modelom aktivnog unosa radioaktivnog rubidija (^{86}Rb) kao analoga kalija u samu stanicu. Ovaj rad prikazuje da vrlo niske nano-molarne koncentracije ouabaina mogu potaknuti povećanje enzimske aktivnosti Na^+/K^+-ATPase u čovječjim eritrocitima u usporedbi s izoliranim enzimom u kojeg taj učinak nije primijećen.

Ključne riječi: Na^+/K^+-ATPase, ouabain, eritrociti, signalna transdukcija, kardiotonički steroid