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Binding of ouabain and marinobufagenin leads to different structural changes in Na,K-ATPase and depends on the enzyme conformation

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ABSTRACT

Ion pump, Na,K-ATPase specifically binds cardiotonic steroids (CTS), which leads to inhibition of the enzyme activity and activation of signaling network in the cell. We have studied interaction of Na,K-ATPase with CTS of two different types – marinobufagenin and ouabain. We have shown that both CTS inhibit activity of Na,K-ATPase with the same K_i values, but binding of ouabain is sensitive to the conformation of Na,K-ATPase while binding of marinobufagenin is not. Furthermore, binding of ouabain and marinobufagenin results in different structural changes in Na,K-ATPase. Our data allow to explain the diversity of effects on the receptor function of Na,K-ATPase caused by different types of CTS.

1. Introduction

Na,K-ATPase (NKA), an ubiquitous ion pump, provides active transport of Na^+ and K^+ across plasma membrane in all types of animal cells. During catalytic cycle the enzyme oscillates between the two main conformations: E1, with high affinity to Na^+ and ATP, and E2, which has high affinity to K^+ [1]. Resulting electrochemical gradients of Na^+ and K^+ are used by other ions and nutrient transporters for secondary active uptake of amino acids and glucose as well as for maintenance of cellular pH and Ca^{2+} homeostasis, cell volume and transmembrane potential.

NKA is specifically inhibited by cardiotonic steroids (CTS), and the enzyme is the only known target for CTS. CTS that at first were found in plants (cardenolides, among them the best known is ouabain) and then in the skin of toads (bufadienolides) were later discovered in the blood of vertebrates [2–5]. Endogenous ouabain was the first CTS to be purified from human plasma [6]. It was shown that ouabain binds to the NKA catalytic α subunit in the E2 phosphorylated (E2P) conformation [7,8]. Later, another CTS marinobufagenin (MBG) (3,5-dihydroxy-14,15-epoxy bufodienolide) was detected in human plasma and urine [5,9,10]. Both CTS act as potent vasoconstrictors [11] and increase of the MBG level was observed in acute myocardial ischemia [5] and renal ischemia [12]. CTS appear to be involved in the development of different diseases including hypertension [13].

It was demonstrated that the NKA is a receptor selectively responding to changes in the endogenous CTS level [14]. For example, binding of ouabain to NKA leads to the activation of Src-kinase and triggers signal cascades [14]. Presence of different CTS in the organism presume their different effect on the NKA receptor function. Indeed, different CTS cause diverse quantitative as well as qualitative biological responses [15]. It was shown that ouabain and MBG at the same concentration inhibit ion transport by NKA in renal epithelial cells by 50%. However they induce cell death with different values of IC_{50} (about 0.05 and 5 μM, respectively) [16]. Thus reduction of cell viability by CTS does not correlate with the inhibitory effect of ouabain and MBG on the NKA activity and

Abbreviations: NKA, Na,K-ATPase; CTS, cardiotonic steroids; MBG, marinobufagenin; FITC, fluorescein 5-isothiocyanate; 5-IAF, 5-iodoacetamidofluorescein

Author contributions: A.A.M. and O.D.L. conceived and supervised the study; I.Y.P. and V.A.M. designed experiments; E.A.K., I.Y.P. and V.A.M. performed experiments; A.A.A. performed molecular modeling studies; E.A.K., I.Y.P. and VAM analyzed data; I.Y.P., V.A.M., S.N.O., O.D.L. wrote the manuscript.

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can be due to the activation of a number of signaling cascades triggered by the formation of CTS:NKA complexes. We hypothesized that this can be a result of different structural changes in NKA when it binds ouabain and MBG. In this study we have determined the characteristics of ouabain and MBG binding with NKA and assessed conformational changes in the enzyme when it forms complexes with these CTS. We have shown that inhibition constants of NKA by MBG and ouabain are close to each other and both CTS bind to the same site in NKA. However ouabain is located deeper inside the NKA molecule compared to MBG, and binding of ouabain is sensitive to the conformation of NKA while MBG binding is not. When forming complexes with NKA, ouabain and MBG cause different structural changes in the enzyme molecule. Our data allow to explain the diversity of effects on the receptor function of Na,K-ATPase caused by different types of CTS.

2. Materials and methods

2.1. Na,K-ATPase purification and activity measurements

Na,K-ATPase (α1β1 isozyme [17]) was purified from duck salt glands (for details see [18–20]) to the purity grade of 95% of total protein as confirmed by polyacrylamide gel electrophoresis in the presence of SDS (Supplementary data, Fig. S1). The enzyme preparations also contain a 13 kDa protein that is similar to proteins of the FXYD family [21]. Protein concentration was determined by the method of Lowry with bovine serum albumin as a standard. Hydrolytic activity of the duck NKA reached ~1800–2000 μmol P_i (mg protein × h)$^{-1}$ at 37 °C.

Hydrolytic activity of the duck NKA was estimated as ATP cleavage in the medium containing (mM): 130 NaCl, 20 KCl, 3 $MgCl_2$, 3 ATP, and 30 imidazole, pH 7.4 at 37 °C as described elsewhere [22]. NKA concentration in the sample was 0.4–0.6 μg/ml. Dependence of the NKA hydrolytic activity on CTS concentration was fitted by the single logistic sigmoid function: $V_i = V_{max}/(1 + ([X]/K_i)^n)$ [23], where V is NKA hydrolytic activity, $[X]$ is concentration of CTS, K_i is inhibition constant and n is the Hill coefficient. Fitting was performed using Origin 7.0 software (Microcal Software, Inc.).

2.2. Fluorescence measurements

Na,K-ATPase was labeled with fluorescein 5-isothiocyanate (FITC) or with 5-iodoacetamidofluorescein (5-IAF) as described earlier [24,25]. Stoichiometric ratio was equal to 0.5 mol FITC/1 mol NKA and 0.5 mol 5-IAF/1 mol NKA. Fluorescence measurements were carried out with 0.1 μM Na,K-ATPase, at 25 °C in 10 mM imidazole, 1 mM EDTA, 3 mM P_i/Tris, 3 mM $MgCl_2$ pH 7.5, using Varian Cary Eclipse fluorescence spectrophotometer. Excitation was at 495 nm, emission at 520 nm. The bandpass for the excitation light and fluorescence were 5 nm, integration time was 0.1 s. Dependence of NKA fluorescence level on the CTS concentration was fitted by the single logistic sigmoid function [23]: $F = F_{max}/(1 + [X]/I_{50})$, where F is fluorescence level, $[X]$ is concentration of CTS, I_{50} is the concentration of CTS causing 50% changes in the NKA fluorescence. Fitting was performed using Origin 7.0 software.

2.3. Isothermal titration calorimetry (ITC)

The thermodynamic parameters of steroid binding to the duck Na,K-ATPase were measured using MicroCal iTC200 instrument, as described elsewhere [22,26]. Experiments were carried out at 25 °C in three different solutions: (buffer 1) 10 mM imidazole, 1 mM EDTA, 0.1 mM DTT, 3 mM $MgCl_2$, 3 mM Tris/Pi, pH 7.5; (buffer 2) 10 mM imidazole, 1 mM EDTA, 0.1 mM DTT, 130 mM NaCl, 20 mM KCl, pH 7.5; (buffer 3) 10 mM imidazole, 1 mM EDTA, 0.1 mM DTT, 3 mM NaCl, pH 7.5. Aliquots (2.6 μl) of ligands were injected into a 0.2-ml cell containing protein solution to achieve a complete binding isotherm. Protein concentration in the cell ranged from 5 to 20 μM, and ligand concentration in the syringe ranged from 50 to 200 μM. The resulting titration curves were fitted using the MicroCal Origin software, assuming one set of binding sites (for details see Supplementary data, Appendix). Affinity constants (K_a) and enthalpy variations (ΔH) were determined and the Gibbs energy (ΔG) and entropy variations (ΔS) were calculated from the equation: $\Delta G = -RT\ln K_a = \Delta H - T\Delta S$.

Direct binding experiments were complemented by the displacement titration calorimetry method determining an apparent binding constant (K_{app}) corresponding to the displacement of one ligand by another. In this case, NKA was initially saturated with a ligand L_1 (MBG) introduced into the calorimeter cell and then titrated with a ligand L_2 (ouabain). An apparent binding constant, corresponding to the displacement of L_1 by L_2, was thus determined. Then, knowing the binding constant K_1 of L_1, we determined the value of the binding constant K_2 of L_2 for NKA from the equation $K_2 = K_{app}(1 + K_1[L_1])$ [27].

2.4. Modeling

Three-dimensional model of the NKA catalytic α subunit in complex with MBG was created on the basis of the previously published 3.4 Å structure of the porcine α subunit in complex with ouabain (PDB code 4HYT) [28]. Sequence alignment of the duck (Q7ZYV1 in the UniProtKB database) and pig (P05024) NKA α subunits by UNIPROT (www.uniprot.org) showed that the amino acid residues involved in binding CTS are identical (Supplementary data, Fig. S2). MBG was substituted for ouabain in the binding site of the existing structure, with subsequent local energy minimization of the complex structure (root mean square gradient: 0.05, force field: MMFF94X, calculate forcefield partial charges: activated) after addition of hydrogens using the MOE version 2013.08 modeling software (Chemical Computing Group Inc.).

2.5. Chemicals and solutions

5-IAF and FITC were obtained from Molecular Probes, ouabain and other reagents were purchased from Sigma, MBG was kindly gifted by professor Bagrov (National Institute on Aging, National Institutes of Health, Baltimore, MD). Ouabain was diluted in the distilled water, stock solution of MBG was made using dimethyl sulfoxide. The highest concentrations of dimethyl sulfoxide used in the experiments (0.5%) did not affect neither enzyme activity, nor the fluorescence of labeled enzyme.

3. Results

3.1. Inhibition of hydrolytic activity of Na,K-ATPase by CTS

Using purified NKA from duck salt gland we have found that ouabain and MBG inhibited the hydrolytic activity of Na,K-ATPase with similar inhibition constants (K_i) equal to 1.30 ± 0.08 and 0.57 ± 0.06 μM, respectively (Fig. 1A). The Hill coefficient value (n) is close to 1 for both CTS, indicating the absence of cooperativity for the ouabain and MBG binding to NKA.

3.2. Changes in the labeled Na,K-ATPase fluorescence upon CTS binding

To examine conformational changes in the NKA alpha subunit of α1β1 isozyme two fluorescent labels FITC and 5-IAF have been used, which bind Lys501 and predominantly Cys457 of the alpha subunit, respectively [29,30]. Both these residues are located in

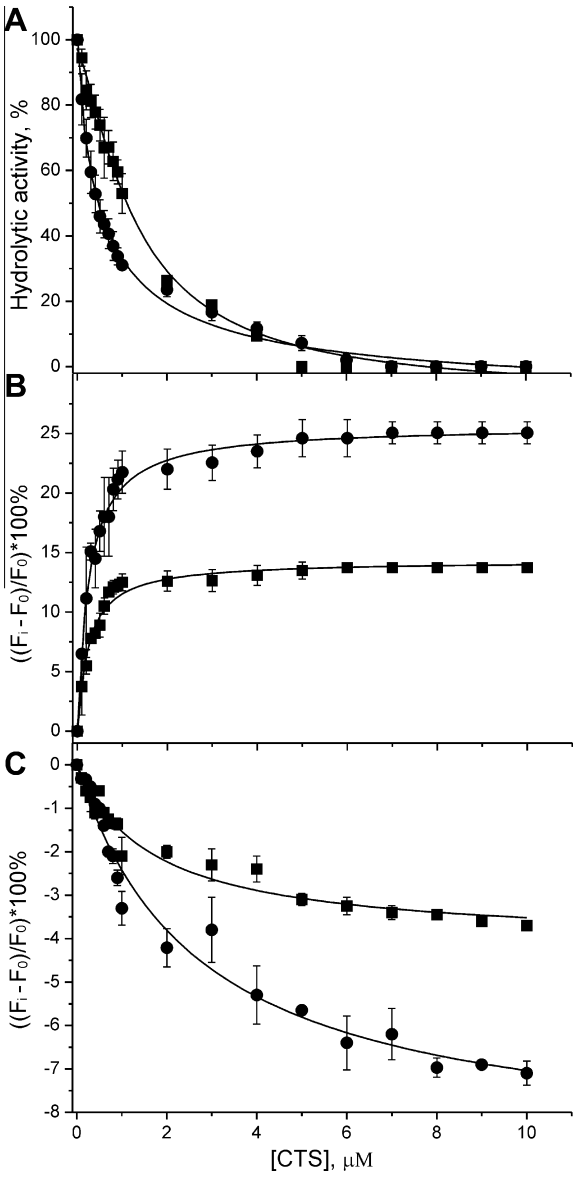

Fig. 1. Effect of ouabain and marinobufagenin binding on the hydrolytic activity and fluorescence intensity of Na,K-ATPase. (A) Inhibition of Na,K-ATPase hydrolytic activity by ouabain (squares) and marinobufagenin (circles) at 37 °C. (B and C) Changes in fluorescence of 0.1 μM FITC-Na, K-ATPase (B) and 0.1 μM 5-IAF-Na,K-ATPase (C) upon binding of ouabain and marinobufagenin at 25 °C. Results of the three independent experiments ± S.D. are presented.

the nucleotide binding domain and it was demonstrated that any change in fluorescence of these labels reflected conformational changes of NKA alpha subunit [29,30]. Addition of ouabain or MBG to the FITC-labeled NKA in the presence of Mg^{2+} and P_i (E2P conformation in which NKA has a maximal affinity to ouabain [31]) leads to increase of the fluorescence level in the concentration-dependent manner described by hyperbolic curves (Fig. 1B). The maximum change in fluorescence intensity $(F_i - F_0)/F_0 * 100\%$, where F_0 is initial fluorescence intensity, was 14% for ouabain and 25% for MBG (Fig. 1B).

Adding CTS to the NKA labeled with 5-IAF resulted in the decrease of fluorescence intensity (Fig. 1C). As in the case of FITC-labeled NKA, the change in fluorescence level was more pronounced when 5-IAF-NKA interacted with MBG. The maximum fluorescence change constituted 3.7% for ouabain and 7.1% for MBG. The difference between I_{50} values for binding of CTS with the 5-IAF and FITC labeled NKA can be explained by the fact that the labels are localized in different microenvironments. Variations in the labeled NKA fluorescence on binding MBG and ouabain point to different structural changes resulting from binding these CTS.

3.3. Thermodynamics of ouabain and MBG binding to Na,K-ATPase

To ascertain differences in binding of ouabain and MBG to NKA we used ITC, making it possible to determine thermodynamic parameters of the interaction of biomolecules directly, without labels and modification of the protein. Using ITC we estimated association (K_a) and dissociation (K_d) equilibrium constants, changes of enthalpy (ΔH) and entropy (ΔS) for the binding of ouabain and MBG to NKA in three states: E2P (buffer 1), E1/E2 (buffer 2) and E1 (buffer 3) (Table 1). A typical set of ITC data for the CTS binding to NKA in E2P state at 25 °C is shown in Fig. 2, including the "raw" calorimetric data for the ligand-into-protein titration (upper parts) and the binding curves (lower parts). The latter were fitted to a model with one CTS binding site per NKA molecule.

In the E2P state, we observed a 17-fold higher affinity for the binding of NKA to ouabain compared to MBG (K_d values equal to 0.1 and 1.7 μM, respectively) (Table 1). The obtained binding constant for ouabain is close to the value determined previously [31]. As is clear from the thermodynamic parameters (Table 1), the binding of ouabain to NKA is an enthalpy-driven process. In contrast, the MBG binding to NKA has a reduced enthalpic contribution and a larger entropic component. ITC experiments were complemented by "displacement titration", where MBG was replaced in the NKA-MBG binary complex by ouabain. The value of K_d (0.08 μM) for ouabain binding to NKA estimated from a competitive ligand binding model was virtually the same as those obtained by the direct titration (Table 1). When titrating the NKA-ouabain binary complex by MBG we did not detect any binding, which can be explained by the fact that in the E2P conformation the binding constant of MBG and NKA is by order of magnitude lower than the binding constant of ouabain (Table 1), and MBG cannot "displace" it under given conditions (Table 1). Thus we can conclude that both CTS bind to the same site of the protein.

In the E1/E2 state the affinity of NKA to ouabain decreased by an order of magnitude in comparison to E2P state and binding changed from the enthalphy to entropy-favorable process (Table 1). In the E1 state we could not detect binding of ouabain to NKA. At the same time the affinity of MBG to NKA was practically the same in all three states (Table 1), but thermodynamic profile of the binding process changed. Shift of NKA from the E2 to E1 state leads to the reduction of entropy component and increase of enthalpy contribution to binding.

3.4. Molecular modeling of the Na,K-ATPase structural changes upon MBG binding

In the initial structure of the NKA:ouabain complex (PDB code 4HYT) ouabain forms five hydrogen bonds with the residues Gln111, Glu117, Asn122, Glu312, Thr797 and six hydrophobic contacts with the residues Leu125, Ile315, Phe316, Phe783, Phe786, Leu793, located in the transmembrane helices M1, M2, M5 and M6 in the enzyme alpha subunit (Fig. 3B) [7]. In the 4HYT structure ouabain was replaced with MBG (Fig. 3C) with subsequent energy minimization for the complex (Fig. 3D). In the energy-optimized model, the steroid core of MBG is positioned by ~5 Å closer to the binding cavity entrance compared to ouabain (Fig. 3E and F). In the complex with NKA, MBG forms one hydrogen bond with Glu312 and five hydrophobic contacts with the same amino acids as ouabain, Ile315, Phe316, Phe783, Phe786, and Leu793. However interactions occur with different atoms of CTS (Fig. 3D). The NKA:ouabain complex energy minimization did not lead to shifting of the ouabain position in the binding site.

Table 1
Thermodynamic parameters of the duck Na,K-ATPase (NKA) binding to ouabain and marinobufagenin determined by isothermal titration calorimetry.

Sample	NKA state[a]	Ligand	K_a[b] (M^{-1})	K_d[c] (μM)	ΔH[d] (kcal/mol)	$T\Delta S$[e] (kcal/mol)	ΔG[f] (kcal/mol)
NKA	E2P	Ouabain	9.7×10^6	0.1	−11.5	−2.0	−9.5
NKA	E2P	MBG	6.0×10^5	1.7	−1.3	6.6	−7.9
NKA-MBG[g]	E2P	Ouabain	11.9×10^6	0.08			−9.7
NKA-ouabain[g]	E2P	MBG	ndis[h]		ndis		
NKA	E1/E2	Ouabain	1.3×10^6	0.77	−0.6	7.7	−8.3
NKA	E1/E2	MBG	6.5×10^5	1.5	−2.9	5.0	−7.9
NKA	E1	Ouabain	nd[i]		nd		
NKA	E1	MBG	2.8×10^5	3.6	−5.0	2.4	−7.4

[a] The measurements of CTS binding to NKA in different states were performed in the three different solutions. E2P state: 10 mM imidazole, 0.1 mM DTT, 1 mM EDTA, 3 mM $MgCl_2$, 3 mM Tris/P_i, pH 7.5; E1/E2 state: 10 mM imidazole, 1 mM EDTA, 0.1 mM DTT, 130 mM NaCl, 20 mM KCl, pH 7.5; E1 state: 10 mM imidazole, 1 mM EDTA, 0.1 mM DTT, 3 mM NaCl, pH 7.5. All measurements were performed three to four times at 25 °C.
[b] K_a – affinity constant; standard deviation did not exceed ±20%.
[c] K_d – dissociation constant; calculated as $1/K_a$.
[d] ΔH – enthalpy variation; standard deviation did not exceed ±10%.
[e] $T\Delta S$– entropy variation; calculated from the equation $\Delta G = \Delta H - T\Delta S$.
[f] ΔG – Gibbs energy; calculated from the equation $\Delta G = RT\ln K_a$.
[g] The model of competitive ligand binding was used (see Section 2).
[h] ndis – the displacement was not observed.
[i] nd – not detectable under the given experimental conditions. It means that the K_a value $<1 \times 10^5$ M^{-1}.

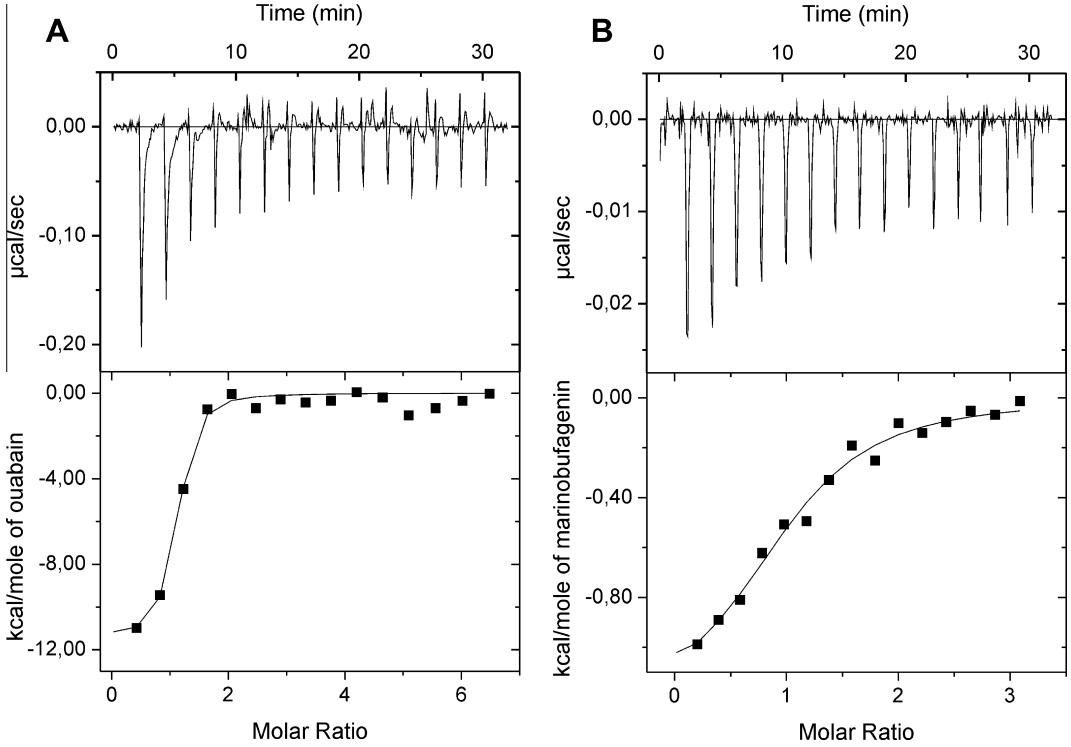

Fig. 2. ITC data for ouabain (A) and marinobufagenin (B) binding to Na,K-ATPase (12 μM) in the E2P state. Titration curves (upper panel) and binding isotherms (lower panel) are shown for the Na,K-ATPase interaction with ouabain and marinobufagenin at 25 °C.

4. Discussion

Biological effects of CTS can be divided into the two major groups – positive ionotropic effect which is associated with the inhibition of NKA, and activation of specific signal transduction pathways mediated by NKA. Circulating concentrations of endogenous CTS (from a few nanomolar to picomolar) [2,13,32] are most likely too low to induce inhibitory effect on the NKA ion transport. The huge diversity in cardenolide and bufadienolide effects can be attributed to different ability for activating specific transduction pathways [33]. The latter might be a result of the fact that diverse CTS can induce the NKA conformations where the enzyme has different capacity to bind intracellular ligands such as Src-kinase, BAX, Bcl-2, calveolin1, ankyrin, MLP and PI3-kinase [13,34–36].

We have shown that ouabain and MBG, while inhibiting the NKA activity with similar K_i, induce changes in the fluorescence of NKA conjugated with FITC and 5-IAF which have different magnitude (Fig. 1B and C). According to the work of [24,37] changes in the fluorescence of these labels reflect structural changes in NKA. The Lys501 residue conjugated with FITC, is located in the ATP-binding site of the nucleotide binding domain (Fig. 4). In the NKA molecule 5-IAF bonded with Cys457 [30] is located in

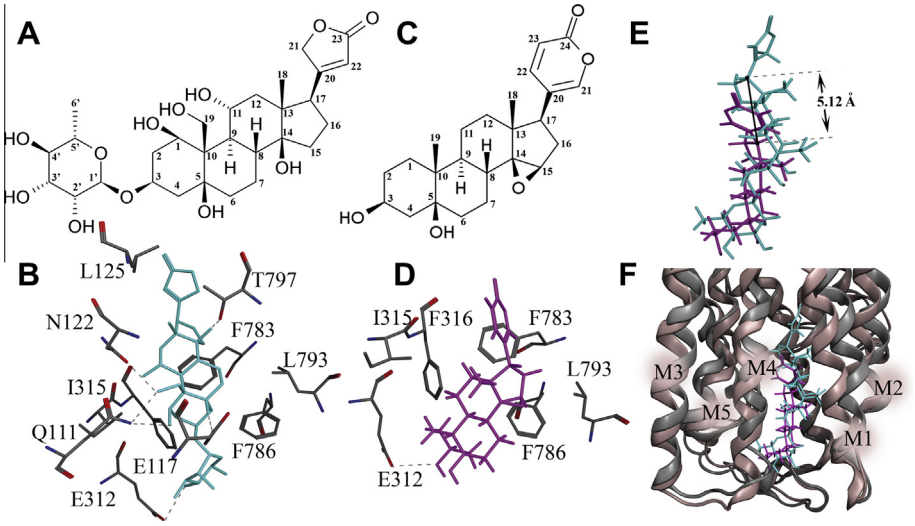

Fig. 3. Location of ouabain and marinobufagenin in the structure of Na,K-ATPase. (A) Chemical structure of ouabain and (B) its binding site in the structure of the Na,K-ATPase:ouabain complex (PDB code 4HYT). Amino acid residues of NKA involved in the complex formation are indicated. Hydrogen bonds between the cardiotonic steroids and Na,K-ATPase are shown by dotted lines. (C) Chemical structure of marinobufagenin. (D) Modeling of the marinobufagenin binding site in Na,K-ATPase based on the structure of NKA:ouabain complex (PDB code 4HYT). (E) Superposition of ouabain (cyan) and marinobufagenin (magenta) in the binding site of Na,K-ATPase. The distance between ouabain and marinobufagenin C17 atoms is 5.1 Å. (F) Superposition of the complexes Na,K-ATPase:ouabain and Na,K-ATPase:marinobufagenin. The structural elements of Na,K-ATPase in the complex with marinobufagenin are shown in magenta and in the complex with ouabain in gray. M1, M2, M3, M4 and M5 are transmembrane segments of the Na,K-ATPase alpha subunit.

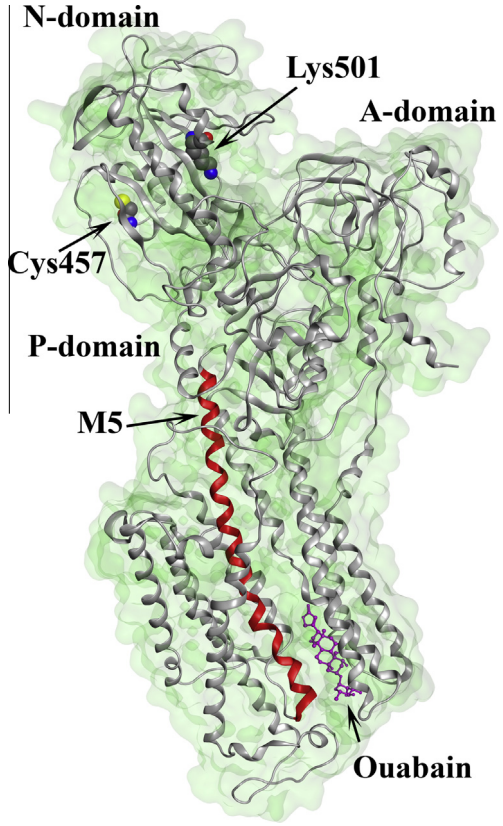

Fig. 4. Cartoon representation of the Na,K-ATPase:ouabain complex (PDB code 4HYT). Ouabain is represented by magenta sticks, αM5 is marked in red, Lys501 and Cys457 are shown as ball-and-stick representation with standard color coding for atom types (carbon, gray; oxygen, red; nitrogen, blue; sulfur, yellow; phosphorus, pink; magnesium, brown).

the nucleotide binding domain on the opposite side of the ATP-binding site. According to our data, binding of ouabain and MBG to NKA leads to the FITC fluorescence increase, and decrease in the 5-IAF fluorescence, more pronounced for MBG (Fig. 1B and C). These data suggest that binding of both CTS shifts the conformation of NKA from the E2P state, and MBG produces more significant structural changes than ouabain.

Crystallographic data show that the ouabain binding site is located in the cavity formed by the transmembrane segments of the NKA alpha subunit (Figs. 3F and 4) [7]. There it forms six hydrophobic contacts and five hydrogen bonds with NKA. Our data show that MBG binds in the same cavity though it forms only a single hydrogen bond and five hydrophobic contacts with NKA (Fig. 3D). The MBG molecule is positioned by ~5 Å closer to the cavity entrance than ouabain. The deformation of the cavity as a result of conformational changes in the molecule will impair NKA binding with the relevant CTS. Apparently, this causes a decrease in ouabain affinity to NKA with its transition to the E1 state (Table 1). The fact that MBG is buried relatively less deep in the binding cavity explains lower dependence of MBG affinity to conformational changes in the NKA molecule.

Molecular modeling results show that ouabain and MBG binding result in different positioning of the NKA transmembrane segments forming the binding site (Fig. 3F), which consequently causes a range of structural changes in the cytosolic part of the molecule. These changes may be conveyed to the cytosolic part of the molecule mainly via interaction of CTS with the most extended transmembrane segment αM5 reaching the nucleotide binding domain (Fig. 4).

It is believed that NKA in the E2P state has the highest affinity to ouabain [7]. Thus other CTS binding assays are usually carried out under the same conditions [8,31]. According to our ITC data affinity of MBG shows week dependence on the NKA state (E2P or E1), while the ouabain affinity to NKA dramatically decreases in E1 state (Table 1). However the inhibition constants of NKA hydrolytic activity by MBG and ouabain are similar. Hence when the enzyme oscillates between the E2 and E1 states, its affinity for various CTS becomes essentially the same. This is confirmed by our results on the NKA binding with CTS when the enzyme is in the intermediate state (E1/E2) (Table 1). These results explain the effect of divergent values of the binding affinity and inhibitory potency, which was

identified earlier for the majority of the 37 CTS analyzed by Paula et al. [38]. We believe that these differences occur due to the sensitivity of CTS to conformational changes in NKA.

Decline of the enzyme activity under conditions of oxidative stress or low ATP levels, will facilitate ouabain binding that is sensitive to changes in the enzyme conformation. Indeed, the increase in sensitivity to ouabain of the failing human myocardium was linked, among other factors to decreased activity of the sarcolemmal NKA [39]. Our data show that the efficiency of MBG binding depends largely on its concentration rather than the enzyme conformation. This concurs with the observation that a range of pathological conditions, namely myocardial infarction, chronic kidney disease, renal ischemia and preeclampsia are accompanied by changes in the concentration of MBG [5,12,32,40,41]. It can be conjectured that MBG binding resulting in the decrease of NKA activity, increases the efficiency of ouabain binding, which can therefore serve as the MBG modulator. At present it is known that ouabain binding can reduce the toxicity of other CTS [42], possibly due to the activation of anti-apoptotic cascades [43]. Consequently different sensitivity of the CTS binding to NKA conformation may be important for their function.

As shown by our data ouabain and MBG affect the NKA conformation in a different way, which in turn may support binding of various proteins to the enzyme. At present NKA is known to bind over 10 proteins. For some of these it was shown that this binding is regulated by ouabain [35]. It is possible that CTS binding might not only trigger signaling cascades, but alter the NKA sensitivity to the regulatory proteins such as PKC, BAX, and Bcl-2 affecting the enzyme activity and regulating its presence in the membrane [34].

The effectiveness of protein binding with NKA can change depending on a range of factors. Some of the proteins (e.g. PI3-kinase, caveolin, ankyrin and proapoptotic Bcl-2 proteins) have overlapping binding sites located at or near the NKA actuator domain [34,44–46] and therefore cannot bind simultaneously. Possibly protein binding with the actuator domain occurs after the enzyme inhibition by CTS and its conformational rearrangements leading to a better access for the relevant proteins to the binding site located in the Na,K-ATPase alpha subunit. Binding of NKA with ouabain and MGB stabilizes a range of the NKA conformations possibly supporting different receptor functions of the enzyme.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.08.011.

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