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Role of homologous ASP334 and GLU319 in human non-gastric H,K- and Na,K-ATPases in cardiac glycoside binding

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Abstract

Cardiac steroids inhibit Na,K-ATPase and the related non-gastric H,K-ATPase, while they do not interact with gastric H,K-ATPase. Introducing an arginine, the residue present in the gastric H,K-ATPase, in the second extracellular loop at the corresponding position 334 in the human non-gastric H,K-ATPase (D334R mutation) rendered it completely resistant to 2 mM ouabain. The corresponding mutation (E319R) in α1 Na,K-ATPase produced a ~2-fold increase of the ouabain IC_{50} in the ouabain-resistant rat α1 Na,K-ATPase and a large decrease of the ouabain affinity of human α1 Na,K-ATPase, on the other hand this mutation had no effect on the affinity for the aglycone ouabagenin. These results provide a strong support for the orientation of ouabain in its biding site with its sugar moiety interacting directly with the second extracellular loop.
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According to the classification proposed by Axelsen and Palmgren [1], the group IIc of P-type ATPases, contains three types of ion motive ATPases composed of an α- and a β-subunit. Included in this group are the four isoforms (α1, α2, α3, and α4) of the α-subunit of Na,K-ATPase, the α-subunit of gastric proton pump or H,K-ATPase (αHKg) and the α-subunit of the third member of this group, the non-gastric H,K-ATPase (αHKng, initially named ATP1AL1 in human), colonic H,K-ATPase in rat or H,K-ATPase α2-subunit, the product of the ATP12A gene. This protein is named H,K-ATPase even if its precise ion specificity and transport stoichiometry are not yet exactly known. All these proteins share highly related sequences with more than 60% amino acid identity.

The α-subunit of the non-gastric H,K-ATPase assembles physiologically with the β1-subunit of Na,K-ATPase [2] although functional expression can be obtained with other β isoforms [3]. In this work, we have used the β-subunit of the rabbit gastric H,K-ATPase with which we obtain good functional expression of the non-gastric H,K-ATPase in *Xenopus* oocytes.

All Na,K-ATPase α isoforms and αHKng can be inhibited by ouabain (and a group of related compounds known as cardiac steroids), although with a wide range of sensitivity ranging from nanomolar for highly sensitive isoforms (Na,K-ATPase α2, α3, and α1 in most species), to micromolar for moderately sensitive (Na,K-ATPase α4) and submilimolar for poorly sensitive isoforms (Na,K-ATPase α1 of rodents, Na,K-ATPase α1 of the toad *Bufo marinus*, and the non-gastric H,K-ATPases). In contrast, the gastric H,K-ATPase is insensitive to ouabain. Resistance to

Abbreviations: αHKng, α-subunit of the non-gastric H,K-ATPase; αHKg, α-subunit of the gastric H,K-ATPase; M4 M5, 4th, 5th transmembrane segments.

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ouabain in rodents and *B. marinus* α1 isoform is known to be related to the presence of charged residues in the first extracellular loop [4], while the low sensitivity of human and other nongastric H,K-ATPase or the total resistance of gastric H,K-ATPase must be due to other primary structure differences as these proteins do not have charged residues at homologous locations.

Cardiac steroids are used for more than 200 years to treat congestive heart failure and are known for 50 years to be specific inhibitors of Na,K-ATPase. Understanding their binding site and mechanisms is important first because of their wide clinical use but also because of the presence of endogenous related compound(s) that may act as hormonal agents to control the activity of Na,K-ATPase and be implicated in the pathogenesis of conditions such as arterial hypertension or heart failure [5–7]. Another point of interest is the potential role of cardiac steroids as signaling molecules and the signal transducer role of Na,K-ATPase [8], and the potential antitumor activity of analogues of cardiac steroids [9,10]. High affinity binding of ouabain to Na,K-ATPase has been studied in details and major progress have been made recently by determining several residues directly involved in the structure of the ouabain binding site by the study of mutants in which residues belonging to the gastric and non-gastric H,K-ATPases were exchanged with those of a sensitive form of Na,K-ATPase [11,12]. Besides, the well-known role of charged residues in the first extracellular loop [13], a small number of residues in the second (transmembrane segments M3–M4) and third (M5–M6) hairpin were shown to be essential for high affinity binding. A structural model of ouabain in its binding site was proposed in which several of these residues make H-bonds with ouabain [11,12]. Several aspects of this model remain hypothetical and some residues that are functionally important are not predicted to make direct contact with the ouabain molecule. Thus, more experimental data are needed to obtain a complete and safe model of the ouabain binding site.

To better understand the mechanism of ouabain binding, in particular to the human non-gastric H,K-ATPase, we have selected a number of residues, located on the extracytoplasmic surface or in the transmembrane segments, which are divergent between Na,K-ATPase and non-gastric or gastric H,K-ATPase (see Table 1), and studied ouabain inhibition of ion transport functions in wild type and mutant αHKng and α1 Na,K-ATPase proteins in which residues were exchanged.

Table 1
Alignment of human α1 Na,K-ATPase, α non-gastric H,K-ATPase and α gastric H,K-ATPase sequences in the regions concerned by the mutations

wild type sequences		mutants	
	== M4 ==		
α1NaK	SLIL**E**$_{314}$YTWL**E**$_{319}$AVIF	α1NaK	**E**$_{314}$**K** **E**$_{319}$**R**
αHKng	AVSL**K**$_{329}$YQVL**D**$_{334}$SIIF	αHKng	**K**$_{329}$**E** **K**$_{329}$**G** **D**$_{334}$**R**
αHKg	AMCI**G**$_{325}$YTFL**R**$_{330}$AMVF		
	====== M5 ======		
α1NaK	LTSNI**P**$_{785}$EI**T**$_{788}$PFLI	α1NaK	**P**$_{785}$**A**
αHKng	LTKNI**A**$_{800}$EL**C**$_{803}$PFLI	αHKng	**A**$_{800}$**P**
αHKg	LTKNI**P**$_{796}$EL**T**$_{799}$PYLI		
	===== M8 ======		
α1NaK	AFFVSI**V**$_{927}$VVQWAD	α1NaK	**V**$_{927}$**E**
αHKng	AFFVGI**L**$_{942}$VQQIAD	αHKng	**L**$_{942}$**E**
αHKg	VFFISI**E**$_{938}$VCQIAD		

On the right are shown the mutants generated and studied in this work.

Materials and methods

Mutant constructions. Five mutants of human αHKng were created in which a residue was replaced by the amino acid present at the homologous position in the sequence of rat α1 (K329E, L942E) or α2 or α3 (K329G), in both Na,K- or gastric H,K-ATPase (A800P) or in the gastric H,K-ATPase (D334R). Similarly, four mutants of rat Na,K-ATPase α1 were created in which a residue was replaced by the amino acid present at the homologous position in the sequence of human αHKng (E314K, P785A, and V927E) or in gastric H,K-ATPase (E319R). Finally, a mutant of the human α1 Na,K-ATPase (E319R) was created in the same way (Table 1).

Mutagenesis was performed using Quickchange II XL Site-directed Mutagenesis Kit (Stratagene), following the manufacturer's instructions. All mutations were confirmed by sequencing. cRNA of α and β-subunits was prepared by *in vitro* transcription.

Expression in Xenopus oocytes. *Xenopus laevis* were anaesthetized by immersion in MS 222 (2 g/l; Sandoz, Basel, Switzerland) and ovarian tissue was removed. Frogs were killed by decapitation under MS 222 anesthesia. Stage V/VI oocytes were collected from the ovarian fragments treated with collagenase as described earlier [14]. cRNA of mutant and wild-type α-subunit (8–10 ng) and β-subunit (1 ng) was injected into the vegetal pole of the oocytes. The rat β1-subunit was expressed with the rat α1-subunit and rabbit βHK was expressed with the human αHKng. Two to three days later the oocytes were loaded with Na^+ by exposure to a K^+-free solution containing 0.2 μM ouabain in order to inhibit the oocyte endogenous Na,K-ATPase. This concentration of ouabain inhibits more than 95% of the endogenous oocyte Na,K-ATPase while it has practically no effect on resistant isoforms such as rat α1 Na,K-ATPase or non gastric H,K-ATPase [3,15,16].

Electrophysiological measurements. The functional expression of wild type and mutant Na,K-ATPase was first studied by the 2-electrode voltage clamp technique starting in the following control K^+-free solution: (in mM) Na^+ 87.4, Ca^{2+} 0.4, Mg^{2+} 0.8, *N*-methyl-D-glucamine (NMDG) 5, Cl^- 86.0, HCO_3^- 2.4, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes) 10.0. The current induced by addition of 10 mM K^+ (I_K+) was recorded as well as the current inhibited by 2 mM ouabain added to the solution containing 10 mM K^+ solution.

For the measurements of ouabain inhibition constant (K_I) in oocytes expressing rat α1/β1 Na,K-ATPase or human αHKng/rabbit βHK , the current was recorded in the 10 mM K^+ solution and then during exposure to increasing concentrations of ouabain (50, 200, 600 μM and 2.0 mM).

For measurements of ouabain K_I in human α1β1 Na,K-ATPase which has a much higher affinity for ouabain (close to that of the *Xenopus* oocyte Na,K-ATPase) it was not possible to inhibit the endogenous component by exposure to low doses of ouabain. And also, because of the slow association rate constant of ouabain, it was not possible to use acute exposure to increasing concentration of ouabain. We therefore started with Na^+ loaded oocyte injected either with the human β1 alone or wild type or mutant α1 + β1 human Na,K-ATPase. We then exposed groups of oocytes to various concentrations of ouabain during 1 h to reach equilibrium binding and then measured rapidly (within 1 min) the current induced by 10 mM K^+ and then the current inhibited by a high concentration (2 mM) of ouabain. This concentration is expected to inhibit very

close to 100 % of the sensitive human α1β1 Na,K-ATPase. Ouabain K_I was obtained by analyzing the relationship between the remaining ouabain-sensitive current and the concentration of ouabain during the 1 h exposure. To obtain the affinity of the exogenous component the mean current measured in the oocytes expressing the β-subunit alone (endogenous component) was subtracted at each corresponding ouabain concentration.

^{86}Rb uptake measurements. ^{86}Rb uptake was performed as previously described [17]. In brief, oocytes were injected either with 8 ng ATP1AL1 cRNA or with 8 ng rat Na,K-ATPase α_1-subunit cRNA in combination with 1 ng of the β-subunit of the rabbit gastric H,K-ATPase (βHK) or 1 ng rat β1 cRNA, respectively. After loading of the oocytes with Na^+(see above) and recovery in a solution containing 90 mM NaCl, 2 mM $CaCl_2$, 5 mM $BaCl_2$, 10 mM Mops, pH 7.4, and 0.2 μM ouabain to inhibit the endogenous oocyte Na,K-ATPase, oocytes were transferred to a solution containing 5 mM KCl, 90 mM NaCl, 1 mM $CaCl_2$, 5 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, and containing 10 μM bumetanide. After addition of 5 μCi/ml ^{86}Rb (Amersham), oocytes were incubated for 12 min at room temperature with or without ouabain, before washing in a solution containing 90 mM NaCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM Hepes, pH 7.4. Individual oocytes were dissolved in 0.5% SDS and counted.

Results

Inhibition by ouabain of human αHKng mutants

^{86}Rb uptake was measured in wild-type rat α1β1 Na,K-ATPase and in wild type and mutant human αHKng coexpressed with the β-subunit of the rabbit gastric H,K-ATPase (βHK), in the absence and in the presence of ouabain. In all cases, the endogenous Na,K-ATPase was inhibited by previous exposure to 0.2 μM ouabain. As shown in Fig. 1, wild-type ATPases and all mutants were well expressed with a robust ^{86}Rb uptake after subtraction of the uptake observed in the oocytes expressing only the β-subunit. Two αHKng mutants (K329E and D334R) showed a slightly reduced transport function to about 60% of the wild-type αHKng. Ouabain, at a 2 mM concentration, inhibited more than 80% of the ^{86}Rb uptake in wild-type rat α1β1 Na,K-ATPase and αHKng/βHK, a value compatible with published values of ouabain inhibition constant (K_I) for these enzymes. All the mutants were inhibited to a similar extend by 2 mM ouabain, except D334R for which there was no significant difference between the conditions with and without ouabain. This result indicate a very high level of resistance to ouabain inhibition, similar to that observed in the gastric H,K-ATPase. Because of the difficulty to solubilize ouabain at concentration much higher than 2 mM it would not be possible to obtain direct K_I measurement with this mutant.

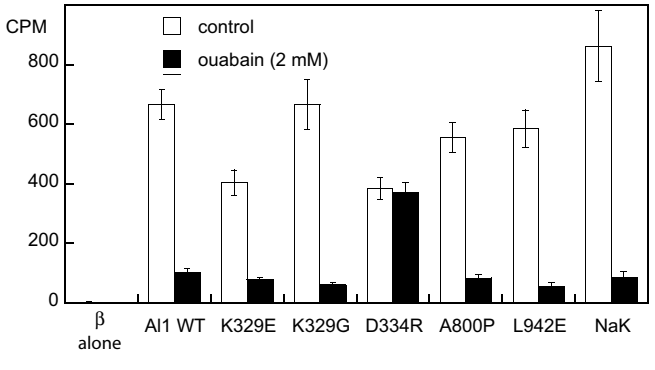

Fig. 1. Transport function of wild type and mutant αHKng expressed in *Xenopus* oocytes. ^{86}Rb uptake was measured as described in the text after inhibition of the endogenous oocyte Na,K-ATPase by overnight exposure to 0.2 μM ouabain. White and black columns represent the uptake observed in the absence and in the presence of 2 mM ouabain, respectively. The number of measurements is between 18 and 22 in each group.

Inhibition by ouabain of Na,K-ATPase mutants

The current activated by addition of 10 mM K^+ to a previously K^+-free solution (I_K) and the current inhibited by 2 mM ouabain (I_{ouab}) added in the presence of 10 mM K^+ were measured in the wild type and mutant Na,K-ATPase. All the mutants had a good electrogenic functional expression but two of them (E319R and V927E) had a significantly reduced expression to about 50% and 70% of wild type, respectively. In all cases the current inhibited by 2 mM ouabain was similar to the current activated by K^+, indicating that no major change of ouabain affinity had occurred.

To assess more precisely the affinity for ouabain, the effect of progressively higher concentrations of ouabain (0, 0.05, 0.2, 0.6, and 2 mM) were applied in the presence of 10 mM K^+ resulting in the inhibition curves shown in Fig. 2. Ouabain K_I values were similar in the wild-type Na,K-ATPase and in the E314K and V927E mutants, 153, 173, and 157 μM, respectively, while the apparent affinity for ouabain was decreased about twofold (K_I

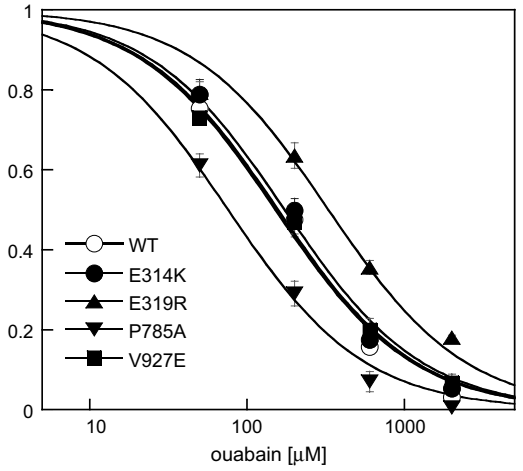

Fig. 2. Steady state ouabain concentration/inhibition curves of the K^+ activated outward current in oocytes expressing wild type and mutant rat α1/β1 Na,K-ATPase. Exposure to ouabain was performed in the presence of 10 mM K^+ extracellular concentrations. Solid lines show the best fitting normalized current (I_{norm}) curves to the equation $I_{norm} = 1 - 1/\left(1 + \frac{K_I}{[ouabain]}\right)$.

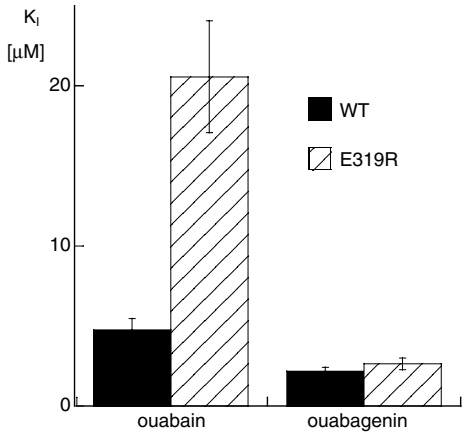

Fig. 3. Ouabain and ouabagenin inhibition constants (K_I) in wild type and E319R mutant α1/β1 human Na,K-ATPase. The results are the mean of 9–11 measurements in each group.

328 μM) for the E319R mutant, and increased about twofold (K_I 75 μM) for the P785A mutant.

Inhibition of the E319R mutant of the human Na,K-ATPase α-subunit by ouabain and ouabagenin

The lower affinity of E319R for ouabain is consistent with the results recently reported by Qiu et al. [11] who showed that this residue is directly involved in the structure of the ouabain binding site. These authors propose a structural model in which E319 directly interacts with the sugar moiety of ouabain through a H-bound. This hypothesis requires that ouabain is oriented in its binding site with its sugar part between the extracellular part of M4 and that of the M1–M2 hairpin. To test for a direct link between E319 and the rhamnose part of ouabain, we compared the inhibition of wild type and E319R mutant Na,K-ATPase by the glycoside ouabain and its aglycone ouabagenin. Because of resistance to ouabain and the potentially even lower affinity of the aglycone, we could not define precisely the affinity of ouabagenin for the rat α1 Na,K-ATPase. We have therefore generated the E319R mutant of the human α1-subunit of Na,K-ATPase and performed these studies by measuring steady state inhibition after 1-h incubation at various concentrations of ouabain or ouabagenin, as described in Materials and methods.

As shown in Fig. 3, there was a large difference in the apparent affinity between wild-type human α1 Na,K-ATPase and the E319R mutant ($P < 0.001$), while there was no significant difference for ouabagenin. These results support the presence of a direct interaction between E319 and the sugar moiety of ouabain.

Discussion

Major progress have been made recently in the characterization of the high affinity cardiac steroid-binding site present at the extracellular surface of Na,K-ATPase, particularly by the use of amino acid exchange between Na,K-ATPase and the related gastric or non-gastric H,K-ATPases [11,12]. The determinants of the low affinity ouabain inhibition of the non-gastric H,K-ATPase is less well understood and was addressed in the present work.

Qiu et al. [12] have shown that the position of E319 (E312 in their numbering) position is one of the most important residues for high affinity ouabain binding of Na,K-ATPase. Here, we show that mutating this glutamic acid residue in αHKng to arginine (mutant D334R) renders the non-gastric H,K-ATPase completely resistant to ouabain. Arginine-330 is a highly conserved residue present in all known gastric H,K-ATPase sequences at the position homologous to D334 in αHKng. It seems thus likely that this sequence divergence between gastric and non-gastric H,K-ATPase is crucial to the difference in low affinity ouabain binding between these two related proteins.

In Na,K-ATPase, the E319R mutant had also a lower apparent affinity for ouabain, an observation consistent with those recently reported by Qiu et al. [11] who showed that a similar mutant had a reduced ouabain binding and proposed that it is directly involved in the structure of the ouabain binding site and interacts with ouabain through a H-bound. The difference was however only a twofold change in the case of Na,K-ATPase compared to a much larger change for the homologous mutation in αHKng.

Two main orientations of ouabain in its binding site have been proposed. The possibility to label a cysteine in M1 with the steroid derivative HDMA (in which the reactive group is located at the position of the sugar of ouabain) suggested that the ouabain was oriented with its sugar part towards the first hairpin formed by the M1 and M2 transmembrane segments [18]. A more precise position of ouabain in its binding site has been proposed by Qiu et al. [11,12] who proposed that E319, a residue located at the extracellular end of M4, directly interacts with the rhamnose part of ouabain through a H-bound. Other authors [19] have proposed a rather opposite orientation of ouabain in its binding site with the sugar moiety pointing rather to the transmembrane segments 9 and 10. Our results show that a mutation of E319 has a large effect on the affinity for the glycoside ouabain but does not influence the binding of the corresponding aglycone supporting the hypothesis put forward by Qiu et al. [11,12].

Because P785 of α1 Na,K-ATPase is located close to the cation binding site and rather distant from the putative ouabain binding site, the small effect of the P785A mutation in rat α1 may be explained by an allosteric type of effect due to a change in cation affinity or conformational equilibrium.

In summary, our results confirm the importance of a charged residue in the extracellular part of the 4th transmembrane segment of α1 Na,K-ATPase and show that this position is also crucial for the effect of ouabain on the human non-gastric H,K-ATPase. In addition, we provide

experimental data supporting a direct interaction of the sugar moiety of ouabain with the second extracellular loop allowing to define the orientation of the ouabain molecule in its binding site.

Acknowledgments

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References

[1] K.B. Axelsen, M.G. Palmgren, Evolution of substrate specificities in the P-type ATPase superfamily, J. Mol. Evol. 46 (1998) 84–101.
[2] N.B. Pestov, T.V. Korneenko, R. Radkov, H. Zhao, M.I. Shakhparonov, N.N. Modyanov, Identification of the beta-subunit for nongastric H-K-ATPase in rat anterior prostate, Am. J. Physiol. Cell Physiol. 286 (2004) C1229–C1237.
[3] G. Crambert, J.-D. Horisberger, N.N. Modyanov, K. Geering, Human nongastric H^+-K^+-ATPase: transport properties of ATP1al1 assembled with different beta-subunits, Am. J. Physiol. Cell Physiol. 283 (2002) C305–C314.
[4] J.B. Lingrel, T.A. Kuntzweiler, Na^+,K^+-ATPase, J. Biol. Chem. 269 (1994) 19659–19662.
[5] M.G. Nicholls, A.M. Richards, L.K. Lewis, T.G. Yandle, Welcome to ouabain—a new steroid hormone, Lancet 338 (1991) 543–544.
[6] M.G. Nicholls, A.M. Richards, L.K. Lewis, T.G. Yandle, Ouabain—a new steroid hormone, Lancet 346 (1995) 1381–1382.
[7] M.P. Blaustein, Endogenous ouabain—role in the pathogenesis of hypertension, Kidney Int. 49 (1996) 1748–1753.
[8] Z.J. Xie, A. Askari, Na^+/K^+-ATPase as a signal transducer, Eur. J. Biochem. 269 (2002) 2434–2439.
[9] B. Stenkvist, Cardenolides and cancer, Anticancer Drugs 12 (2001) 635–636.
[10] H. Lin, J.L. Juang, P.S. Wang, Involvement of Cdk5/p25 in digoxin-triggered prostate cancer cell apoptosis, J. Biol. Chem. 279 (2004) 29302–29307.
[11] L.Y. Qiu, E. Krieger, G. Schaftenaar, H.G.P. Swarts, P.H.G.M. Willems, J.J.H.H. De Pont, J.B. Koenderink, Reconstruction of the complete ouabain-binding pocket of Na,K-ATPase in gastric H,K-ATPase by substitution of only seven amino acids, J. Biol. Chem. 280 (2005) 32349–32355.
[12] L.Y. Qiu, H.G.P. Swarts, E.C.M. Tonk, P.H.G.M. Willems, J.B. Koenderink, J.J.H.H. De Pont, Conversion of the low affinity ouabain-binding site of non-gastric H,K-ATPase into a high affinity binding site by substitution of only five amino acids, J. Biol. Chem. 281 (2006) 13533–13539.
[13] E.M. Price, D.A. Rice, J.B. Lingrel, Structure-function studies of Na,K-ATPase. Site-directed mutagenesis of the border residues from the H1–H2 extracellular domain of the α-subunit, J. Biol. Chem. 265 (1990) 6638–6641.
[14] P. Jaunin, J.-D. Horisberger, K. Richter, P.J. Good, B.C. Rossier, K. Geering, Processing, intracellular transport and functional expression of endogenous and exogenous α–β3 Na,K-ATPase complexes in *Xenopus* oocytes, J. Biol. Chem. 267 (1992) 577–585.
[15] M. Burnay, G. Crambert, S. Kharoubi-Hess, K. Geering, J.-D. Horisberger, Electrogenicity of Na,K- and H,K-ATPase activity and presence of a positively charged amino acid in the fifth transmembrane segment, J. Biol. Chem. 278 (2003) 19237–19244.
[16] J.-D. Horisberger, S. Kharoubi-Hess, Functional differences between α-subunit isoforms of the rat Na,K-ATPase expressed in *Xenopus* oocytes, J. Physiol. (London) 539 (2002) 669–680.
[17] A.T. Beggah, P. Jaunin, K. Geering, Role of glycosylation and disulfide bond formation in the β-subunit in the folding and functional expression of Na,K-ATPase, J. Biol. Chem. 272 (1997) 10318–10326.
[18] R. Antolovic, W. Schoner, K. Geering, C.M. Canessa, B.C. Rossier, J.-D. Horisberger, Labeling of a cysteine in the cardiotonic glycoside binding site by the steroid derivative HDMA, FEBS Lett. 368 (1995) 169–172.
[19] S.M. Keenan, R.K. Delisle, W.J. Welsh, S. Paula, W.J. Ball, Elucidation of the Na^+,K^+-ATPase digitalis binding site, J. Mol. Graph. Model. 23 (2005) 465–475.