10056 *Biochemistry* **2009,** *48,* 10056–10065
DOI: 10.1021/bi901212r

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Interaction between Cardiotonic Steroids and Na,K-ATPase. Effects of pH and Ouabain-Induced Changes in Enzyme Conformation[†]

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Received July 17, 2009; Revised Manuscript Received September 23, 2009

ABSTRACT: The Na,K-ATPase belongs to the P-type ATPase family of primary active cation pumps. It maintains the transmembrane gradients of Na^+ and K^+ across the cell membrane essential for cell homeostasis. The Na,K-ATPase is specifically inhibited by cardiotonic steroids like ouabain, which bind to the extracellular side of the enzyme and is of significant therapeutic value in the treatment of congestive heart failure. In order to further characterize the binding of cardiotonic steroids to shark Na,K-ATPase, we compared the strength and rate of inhibition at varying pH of two cardiac glycosides with either an unsaturated (ouabain) or saturated (dihydroouabain) lactone ring and three aglycons with either a 5-membered (ouabagenin and digitoxigenin) or a 6-membered (bufalin) lactone. Inhibition by ouabain and dihydroouabain, and especially the aglycon ouabagenin, was found to be strongly dependent on pH with an increase in IC_{50} by factors of ~6, ~20, and ~66, respectively, when pH increased from 6.5 to 8.5. The finding that ouabagenin was the most pH-sensitive inhibitor indicates that the steroid hydroxyl side chains are pivotal for this pH effect, whereas the lactone ring saturation was less important. The sugar moiety is important in compensating for the pH effect. In contrast, the IC_{50} of the two genins bufalin and digitoxigenin increased by a factor of only ~2 when pH increased from 6.5 to 8.5, indicating that the pH effect does not relay on whether the lactone is 5- or 6-membered. The rate of inhibition was retarded much more significantly by increasing pH for the glycosides than for the aglycons. Finally, we demonstrate a change in enzyme subconformations following binding of cardiotonic steroids to Na,K-ATPase phosphoenzymes using fluoride analogues of phosphoenzyme intermediates. The results are discussed with reference to the recent high-resolution crystal structures of shark Na,K-ATPase in the unbound and ouabain-bound conformation.

Classically, cardiotonic steroids like ouabain are known to be specific inhibitors of the Na,K-ATPase (*1*). This is the basis for the therapeutic action of cardiotonic steroids like digoxin and digitoxin in treatment of congestive heart failure. Inhibition of myocardiac cell Na,K-ATPase leads to elevated intracellular Na^+ concentration, which suppresses NCX, the Na^+-Ca^{2+} exchanger, thus increasing intracellular Ca^{2+} and producing an increase in the contractility of the heart and cardiac output (the inotropic effect).

The Na,K-ATPase is indispensable in maintaining cellular homeostasis in mammalians. This integral protein is an ion pump fueled by ATP and responsible for actively maintaining the electrochemical gradients for Na^+ and K^+ across the animal cell membrane, which is essential for many physiological processes like secondary active co- and countertransport and volume regulation, and forms the basis for generating the resting membrane potential.

Recently the Na,K-ATPase has been implicated in cell signaling in which endogenous ouabain activates Src interaction with Na,K-ATPase leading to ERK 1/2 phosphorylation (*2*). This effect can apparently also be induced through a lowering of the ATP level caused by Na,K-ATPase activation, which activates AMPK and thereby ERK 1/2 (*3*).

Structurally cardiotonic steroids are composed of three major components: a steroid core, a 5-membered or 6-membered lactone ring (cardenolides or bufadienolides), and a sugar moiety, which in the case of ouabain is rhamnose. Certain cardiotonic steroids (genins) lack the sugar moiety.

In spite of the huge number of investigations carried out on the effects of cardiotonic steroids on Na,K-ATPase function the details of ouabain binding to this ion pump remain elusive. It is known that ouabain binds from the extracellular side and mainly to an E_2P conformation of Na,K-ATPase (*4*). Association and especially dissociation of ouabain are very slow processes in the minute range (*5*, *6*). Traditionally, this has been explained from an induced fit, multistep binding mechanism where the lactone ring binds first, the steroid core then opens a binding cavity, and the glycoside finally stabilizes the binding (*7*).

Recently, the high-resolution crystal structures of the Na,K-ATPase from the shark *Squalus acanthias* have been determined in the $E_2P \cdot K_2$ form both without and with bound ouabain (*8*, *9*). Although widely used as a model for epithelial transport, the functional interaction of the shark Na,K-ATPase preparation with cardiotonic steroids has not previously been characterized in detail.

Several previous investigations have indicated that the most dramatic change in binding affinity of cardiotonic steroids occurs as a result of modifications of the lactone moiety (*10*). Thus, the bufadienolide bufalin with a 6-membered lactone ring has a reduced binding affinity compared to the equivalent cardenolide digitoxigenin. Moreover, saturation of the lactone ring in ouabain to form dihydroouabain (DHO),[1] which abolishes the

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[1]Abbreviations: DG, digitoxigenin; OG, ouabagenin; DHO, dihydroouabain.

 Published on Web 09/24/2009

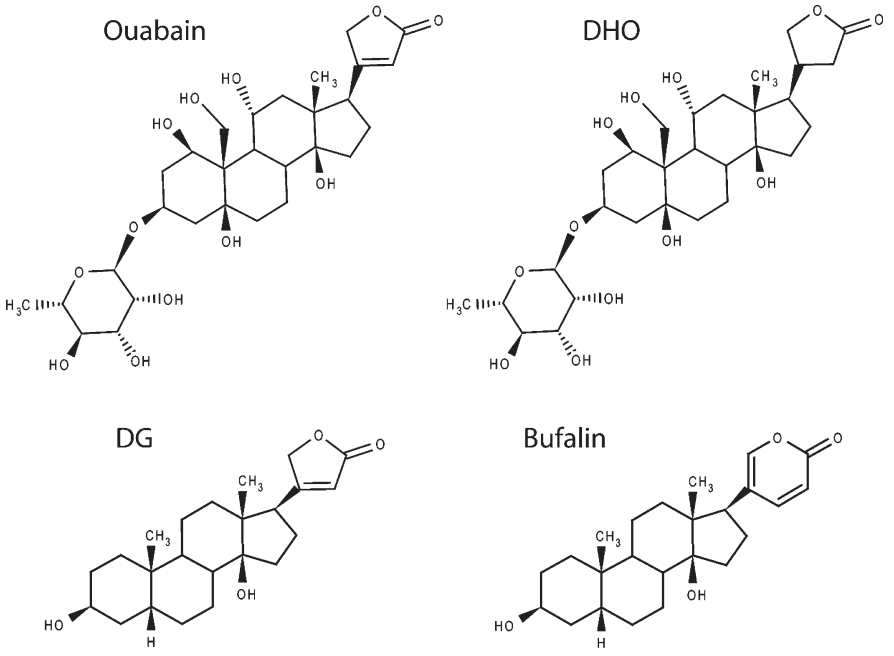

FIGURE 1: 2-D structure of cardiotonic steroids used. On top, the two cardiac glycosides ouabain, with an unsaturated lactone ring, and dihydroouabain (DHO), with a saturated lactone, are shown. OG (not shown) is the aglycon of ouabain. Below, the two aglycons digitoxigenin (DG) with a 5-membered lactone ring and bufalin with a 6-membered lactone are shown.

planarity of the ring system, decreases the binding affinity by a factor of ~20 (*10*) (cf. Figure 1). Apparently, proper positioning of the lactone moiety in the Na,K-ATPase binding pocket is delicate, and strong van der Waals contacts around the lactone ring may be important for the binding affinity. In the present investigation we have examined how the binding of cardiotonic steroids with different lactone ring structures is affected by pH.

The low-affinity ouabain-bound crystal structure (*9*) demonstrates that ouabain is wedged deeply into the transmembrane domain of Na,K-ATPase interacting mainly with the M1−M2 and M4−M6 helices. The lactone ring is placed near the K^+-binding sites and the sugar exposed to the solvent. To reach the binding site, ouabain causes rearrangements of M1−M4 transmembrane helices to create a cavity much like the one observed in the crystal structure of SERCA1a in the E_2P ground state stabilized by BeF_3^- (*11*, *12*) as also indicated by several model studies (*13*−*16*). In the present investigation we probe the structural changes following ouabain binding to Na,K-ATPase by detecting changes in the trypsin digestion patterns following ouabain binding.

EXPERIMENTAL PROCEDURES

Materials. The cardiotonic steroids ouabain, dihydroouabain (DHO), ouabagenin (OG), digitoxigenin (DG), and bufalin were obtained from Sigma-Aldrich (St. Louis, MO).

Preparation of Shark Na,K-ATPase. Crude membrane fractions (microsomes) from the rectal gland of the shark *S. acanthias* were prepared by homogenization followed by washing and isolation by centrifugation in 30 mM histidine, 1 mM EDTA, and 0.25 M sucrose, pH 6.8.

The purified microsomes were activated by a mild DOC treatment (~0.15% DOC) to extract extrinsic proteins and to open sealed vesicles. After washing and resuspension the purified membrane preparation is obtained by differential centrifugation essential as previously described (*17*). The preparation is suspended in histidine/EDTA buffer with 25% glycerol and kept at −20 °C. The preparation has a specific hydrolytic activity of ~30 units/mg at 37 °C and contains the α_1-, β_1-subunits together with the FXYD10 regulatory subunit (*18*). Protein concentrations, ranging from 3 to 5 mg/mL, were determined using Peterson's modification (*19*) of the Lowry method (*20*), using bovine serum albumin as a standard.

Inhibition Assay of Na,K-ATPase Activity. The enzyme was incubated for 2 h at 23 °C with the cardiotonic steroid concentration at the desired pH in 4 mM Mg, 1 mM P_i, and 30 mM imidazole to induce the E_2P conformation. After this preincubation the enzyme was diluted 10 times in test solution containing 130 mM NaCl, 20 mM KCl, 3 mM ATP, 4 mM $MgCl_2$, 30 mM imidazole (pH 7.5), and 0.066% albumin, and the hydrolytic activity was measured at 23 °C by the method of Baginski et al. (*21*). Alternatively, the test solutions contained the same concentration of inhibitor as in the preincubations to avoid possible dissociation of inhibitor during testing.

Trypsin Digestion. Ouabain-dependent trypsin cleavage of shark Na,K-ATPase was performed as previously described (*22*). Briefly, membranes (100 μg of protein) were suspended in a buffer containing 20 mM Tris-HCl, pH 7, 30 mM NaCl, and 5 mM $MgCl_2$. The membranes were preincubated for 5 min at 24 °C before the addition of 20 μg of trypsin. Proteolysis was allowed to proceed for 45 min at 24 °C and quenched with SDS sample buffer containing 0.5% TCA to prevent reactivation of trypsin by the detergent.

Gel Electrophoresis, Immunoblotting, and Sequencing. Protein fragments were separated using SDS−polyacrylamide gel electrophoresis (SDS−PAGE, 3% stacking gel, 9% intermediate gel, and 16% resolving gel). The gels were transferred onto PVFD membranes using a semidry blotter. Following 2 h transfer, the membranes were washed twice with phosphate-buffered saline and incubated with primary antibody (α1002−1016, kindly provided by J. V. Møller) overnight at room temperature. The membranes were washed as before and incubated with secondary goat anti-rabbit antibody for 2 h at room temperature. Proteins were visualized by treatment with ECL reagent (Amersham) according to the manufacturer's procedure. The bands

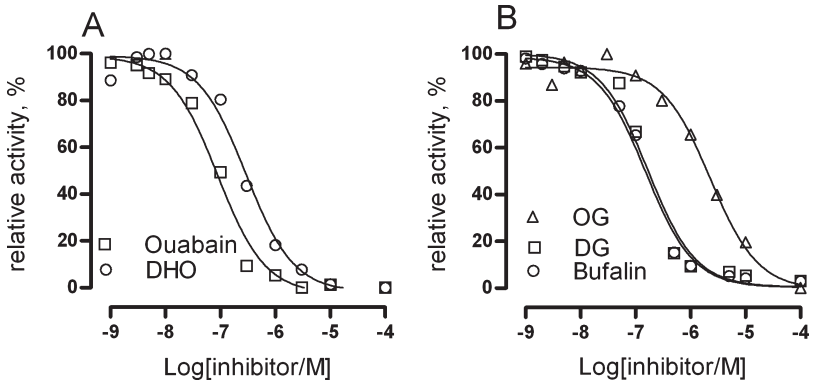

FIGURE 2: Inhibition of Na,K-ATPase activity by cardiotonic steroids at pH 7.5. Panel A shows inhibition by ouabain and dihydroouabain (DHO). Panel B shows inhibition by bufalin, digitoxigenin (DG), and ouabagenin (OG). IC_{50} values calculated from fitting to a sigmoid dose−response equation are given in Table 1.

corresponding to the α-subunit fragments were excised from the membranes and submitted to Edman degradation, using an ABI 494 sequencer. The analysis was performed at Alphalyse A/S, Odense, Denmark. Six cycles were enough for the unambiguous determination of the sequence of the α-subunit fragments.

Digital Imaging. Analyses of SDS gels and immunoblots were performed using ImageQuant TL software (Amersham Biosciences).

Enzyme Reacted with MgP_i and Fluoride Analogues of Phosphate. To induce high-affinity binding of cardiotonic steroids to Na,K-ATPase, it was stabilized in the E_2P conformation by incubation in MgP_i as described above. In order to stabilize the enzyme in either the E_2P product state or the E_2P ground state, the enzyme was reacted with fluorides essentially as described by Danko et al. (*23*). The MgF_4^{2-} product state was induced by incubating the enzyme in the presence of 25 mM Na^+ in 5 mM $MgCl_2$, 5 mM NaF, and 30 mM imidazole at the desired pH (*23*). The BeF_3^- ground state was produced in a similar way by replacing NaF with 5 μM $BeSO_4$.

Phosphorylation and Dephosphorylation. Na,K-ATPase was phosphorylated at 0 °C from [γ-^{32}P]ATP (25 μM) in a medium containing 1 mM $MgCl_2$, 30 mM NaCl, and 30 mM imidazole, pH 6.5 or 8.5. Spontaneous and ADP-supported dephosphorylation of Na,K-ATPase at 0 °C after phosphorylation was measured by chasing with cold ATP (1 mM) in the absence (spontaneous dephosphorylation) or presence of 1.5 mM ADP followed by addition of an acid-stopping solution in the presence of 30 mM NaCl, 10 mM $MgCl_2$, and 30 mM imidazole (pH 6.5 or 8.5) as previously described (*24*).

Statistics and Curve Fitting. Results are expressed as mean ± SEM. Inhibition by cardiotonic steroid was evaluated by fitting to a sigmoid dose−response equation:

$$y = y_0 + \frac{y_{max} - y_0}{1+10^{\log IC_{50} - x}}$$

where y_0 and y_{max} are baseline and maximum activity. IC_{50} is the inhibitor concentration that gives 50% inhibition.

Comparison between best-fit values was performed using an F test, and $p < 0.05$ was considered significant.

Structural figures were prepared by PyMol (http://www.pymol.com).

RESULTS

Inhibitory Potencies of Cardiotonic Steroids. In order to determine the affinity for inhibition by equilibrium binding of cardiotonic steroids, Na,K-ATPase was preincubated with different concentrations of inhibitor (10^{-9} M to 10^{-4} M) in the presence of MgP_i and imidazole (pH 7.5) to ensure stabilization of the high-affinity E_2P conformation for cardiotonic steroid binding.

Following this, the Na,K-ATPase activity was determined at optimal turnover conditions, i.e., at 130 mM Na^+, 20 mM K^+, 4 mM Mg^{2+}, 3 mM ATP, and 30 mM imidazole (pH 7.5). Preincubation and subsequent activity determination were performed at 23 °C. Under these conditions full inhibition at 10^{-4} M ouabain was achieved by preincubation for less than 3 min (cf. Figure 4) whereas >30 min was needed at 10^{-9} M ouabain. However, at high pH values and low concentrations of inhibitor significantly longer equilibration periods were needed (cf. Table 2). Thus, to determine inhibition constants for cardiotonic steroids, a preincubation of 120 min in MgP_i was employed. Yoda and Yoda (*25*) have previously demonstrated that dilution of enzyme with bound aglycons may cause instability of the complex and dissociation of the aglycon. However, this was not the case with the present method, since testing of activity without dilution of inhibitor, i.e., with the same varying aglycon concentration in the test solution as during preincubation, gave identical results as without added inhibitor in the test solution.

In Figure 2A the inhibitory potency of the two cardenolides ouabain and dihydroouabain, where the 5-membered lactone rings are either unsaturated or saturated, is compared at pH 7.5. In Figure 2B inhibition of the Na,K-ATPase activity by the cardenolide genins ouabagenin and digitoxigenin, with 5-membered lactones, is compared to the bufadienolide genin bufalin, where the lactone is 6-membered (cf. Figure 1) also at pH 7.5. The inhibitor concentration for half-maximal inhibition, IC_{50}, is about 80 nM for ouabain and 290 nM for dihydroouabain. For the two genins bufalin and digitoxigenin IC_{50} was about 130 and 100 nM, i.e., a little lower apparent affinity compared to ouabain, whereas IC_{50} for ouabagenin was significantly larger, amounting to about 2.3 μM (Table 1).

The long incubation period for cardiotonic steroids was necessary especially for ouabain and DHO. Thus using a 3 min equilibration time the IC_{50} increased for ouabain and DHO to 400 and 540 nM, respectively, at pH 7.5, i.e., by a factor of ~5 and ~2, respectively, emphasizing the slow rate of association for these two hydrophilic cardiotonic steroids. In contrast, the short equilibration time did not affect the inhibitor constants for the hydrophobic bufalin and DG.

Steady-state inhibition of cardiotonic steroids determined by adding the inhibitor directly to the test solution containing enzyme, Na^+, K^+, and Mg^{2+} and starting the reaction after 5 min by addition of ATP was significantly lower (by a factor of almost 20) than measured after preincubation in Mg^{2+} and P_i, reflecting, in part, the unfavorable steady-state distribution of conformations for ouabain binding in the absence of ATP and the presence of K^+ that opposes ouabain binding as also previously demonstrated (*6*, *10*). Furthermore, during such turnover conditions the inhibitor potency was independent of pH in contrast to the effects of pH on binding of cardiotonic steroids to enzyme stabilized in the E_2P state by MgP_i, as described below.

In Figure 3 the pH dependence of high-affinity inhibition by the different cardiotonic steroids is determined by preincubation at low (pH 6.5) and high (pH 8.5) pH values followed by measurement of catalytic activity at pH 7.5. It is evident that high pH significantly decreases the inhibitory effect of ouabain (IC_{50} increases from 70 nM to about 400 nM, i.e., by a factor of ~6). It was even more significant for DHO; here, IC_{50} increased by a factor of ~20 from 160 nM to ~3 μM. The difference in IC_{50} between ouabain and DHO increases significantly as pH increases (from a factor of ~2 to a factor of ~8, Table 1). For the two genins bufalin and digitoxigenin the increase in IC_{50} by pH is much less dramatic, from 123 to 284 nM for bufalin (a factor of ~2) and from 138 to 332 nM for DG (a factor of ~2.5).

In order to test whether the different pH sensitivity was due to the lactone moiety or to the sugar, we compared the inhibitory potency of ouabain with ouabagenin, which has identical steroid and lactone but lacks the rhamnose. As seen from Figure 3E, IC_{50} for ouabagenin is shifted significantly more by pH than ouabain. Thus for OG IC_{50} increases from ~490 nM at pH 6.5 to 33 μM at pH 8.5, i.e., by a factor of more than 66, compared to a factor of only ~6 for ouabain. This large pH effect must therefore be assigned to the lack of the sugar moiety, but only in the presence of the three OH groups on the β face of the steroid at C1, C5, and C19 (cf. Figure 1) since bufalin and DG, with no OH side chains at these positions, are much less pH sensitive.

Table 1: Inhibitor Constant (IC_{50}) for Various Cardiotonic Steroids at Different pH Values

	IC_{50} (μM)				
pH	ouabain	DHO	bufalin	DG	OG
6.5	0.070 ± 0.008	0.158 ± 0.010	0.123 ± 0.001	0.138 ± 0.001	0.493 ± 0.004
7.5	0.081 ± 0.005	0.292 ± 0.024	0.134 ± 0.001	0.104 ± 0.001	2.28 ± 0.20
8.5	0.403 ± 0.003	3.06 ± 0.03	0.284 ± 0.001	0.332 ± 0.042	32.7 ± 3.0

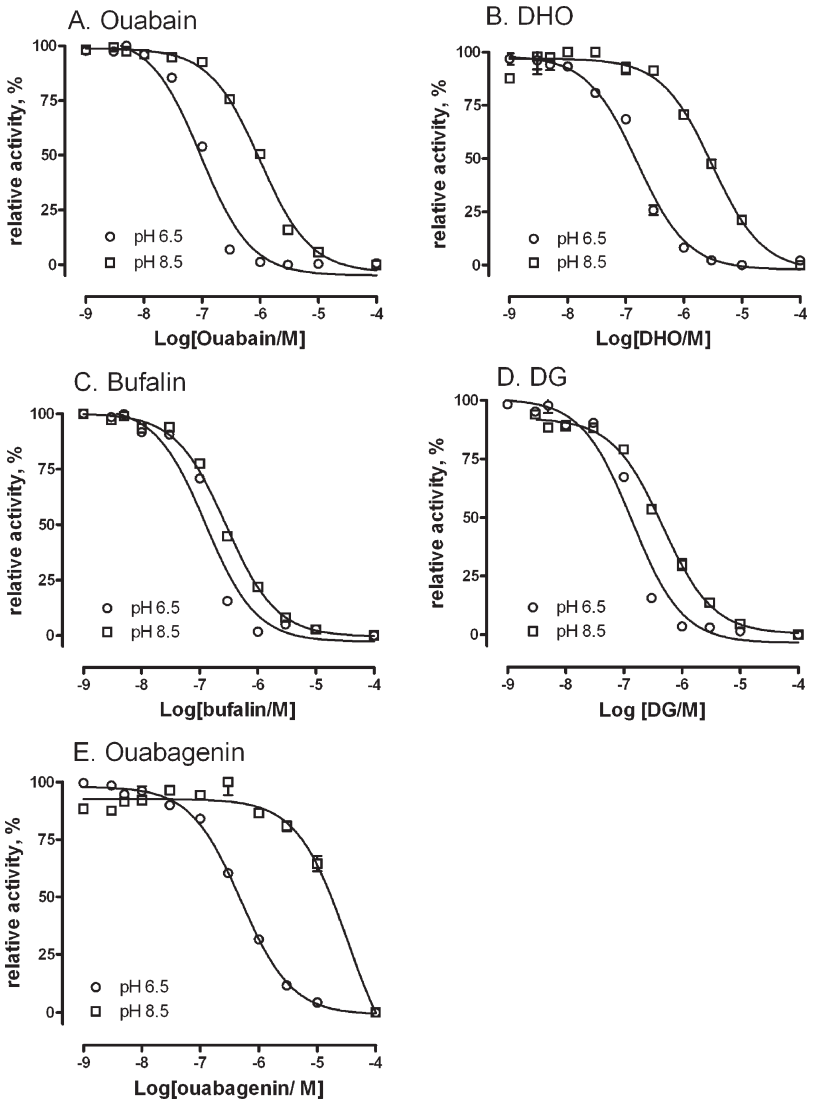

FIGURE 3: pH dependence of inhibition by cardiotonic steroids at pH 6.5 and 8.5. IC_{50} values are given in Table 1.

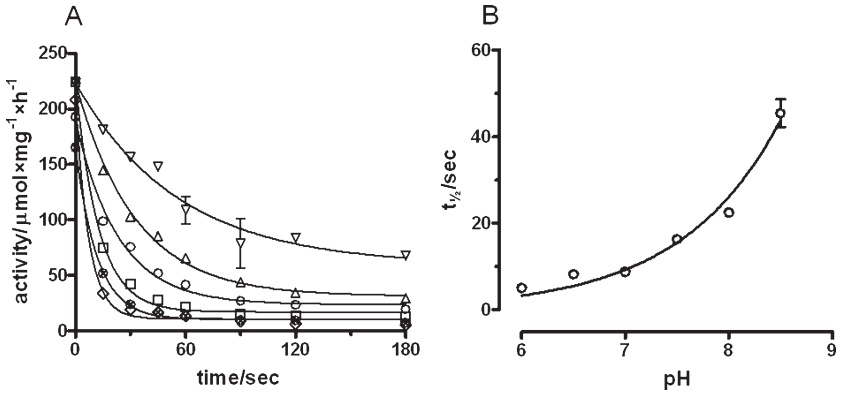

FIGURE 4: Time−course of ouabain inhibition (10^{-4} M) at pH values between 6.0 and 8.5. In panel A progressive inhibition during a 3 min period is shown. In panel B the half-life ($t_{1/2}$) of inhibition is plotted against pH.

Table 2: Comparison of Half-Life ($t_{1/2}$) for Inhibition of Na,K-ATPase by Various Cardiotonic Steroids

	$t_{1/2}$ (s)		
	pH 6.5	pH 7.5	pH 8.5
ouabain, 10^{-4} M	8.2 ± 0.2	16.3 ± 0.9	45.4 ± 7
DHO, 10^{-4} M	4.6 ± 0.3	9.2 ± 0.3	29.2 ± 0.8
ouabain, 10^{-5} M	23.4 ± 3	42.5 ± 6	563 ± 7
DHO, 10^{-5} M	20.3 ± 3	29.5 ± 2	492 ± 8
bufalin, 10^{-5} M	4.8 ± 0.3	6.3 ± 0.4	16.7 ± 1.8
DG, 10^{-5} M	5.8 ± 0.2	7.0 ± 0.3	27.7 ± 3.0
OG, 10^{-5} M	25.9 ± 3.7	48.7 ± 3.5	267 ± 23

Time−Course of Cardiotonic Steroid Inhibition. Figure 4A shows ouabain (10^{-4} M) inhibition of hydrolytic activity of Na,K-ATPase preincubated in MgP_i for various periods of time. As indicated, the inhibition increases exponentially with time of preincubation. The half-life ($t_{1/2}$) for inhibition increases progressively with pH during preincubation (Figure 4B). Thus the half-life for ouabain inhibition at 10^{-4} M increases from about 5 s at pH 6 to about 45 s at pH 8.5. At a 10 times lower concentration (10^{-5} M) the half-life for ouabain increases from 23 to 563 s. Indeed, the inhibition by cardiotonic steroids is a slow process, as also previously shown (*5*, *6*). Similar data for DHO, OG, bufalin, and DG are listed in Table 2. As indicated, the rate of ouabain and DHO inhibition is approximately equal. At 10^{-4} M the inhibition of bufalin and DG is too fast to be measured accurately by this method. However, at 10^{-5} M bufalin and DG inhibition is faster than inhibition by ouabain and DHO by a factor of approximately 4 at pH 6.5 and a factor 30 at pH 8.5. Inhibition of OG is the slowest of the aglycons tested. At pH 6.5 it is as slow as ouabain, whereas at pH 8.5 it is approximately twice as fast. Thus the lack of a sugar moiety speeds up the rate of inhibition, whereas OH side groups in the steroid moiety significantly slow it down.

The E_1P/E_2P Conformational Equilibrium. Since binding of cardiotonic steroids takes place mainly to the E_2P conformation, we investigated whether the pH effect on inhibitory potency was a result of a change in the poise of the E_1P/E_2P equilibrium. We have previously demonstrated that the E_1P/E_2P conformational equilibrium of shark Na,K-ATPase in the presence of 30 mM Na^+ and pH 7.4 is strongly poised toward the E_2P conformation since dephosphorylation was virtually independent of ADP, indicating the virtual absence of ADP-sensitive E_1P (*21*). In order to detect whether this conformational equilibrium is affected by pH, ADP sensitivity of dephosphorylation was compared at pH 6.5, 7.5, and 8.5. In Figure 5 the results of such experiments are shown, and as seen, the effect of ADP in accelerating dephosphorylation increases significantly at high pH, indicating a shift in the E_1P/E_2P conformational equilibrium toward E_1P. Thus, at pH 6.5 and 7.5 ADP decreased the half-life of dephosphorylation by a factor of 2.3 (from 2.6 ± 0.17 and 2.9 ± 0.17 s^{-1} to 1.1 ± 0.06 and 1.3 ± 0.06 s^{-1}, respectively), whereas at pH 8.5 it increased by a factor of 4 (from 3.6 ± 0.28 s^{-1} to 0.92 ± 0.07 s^{-1}).

Ouabain-Induced Changes in the Trypsin Digestion Pattern. In the following ouabain-induced conformational changes were probed on stable E_2P analogues using fluoride analogues of phosphate: MgF_4^{2-}, AlF_4^-, and BeF_3^-. $E_2{\cdot}MgF_4^{2-}$ and $E_2{\cdot}AlF_4^-$ are the transition- and product-state analogues of phosphoenzymes, whereas $E_2{\cdot}BeF_3^-$ is the E_2P ground-state analogue (*23*). In Figure 6 the higher MW part of the digestion patterns of Na,K-ATPase stabilized in the different phosphoenzyme conformations in the presence of 25 mM Na^+ is shown before and after binding of 1 mM ouabain and compared to the digestion pattern of the Na,K-ATPase dephospho form. As seen by comparing the two first lanes in Figure 6 (controls), trypsin cleavage of enzyme incubated in 20 mM Tris is substantial during the 45 min incubation, leaving almost entirely a 19 kDa band (not shown). However, the cleavage pattern for the phospho forms (or their fluoride analogues) is less complete. Without ouabain a 80 kDa band is present in the upper part of the SDS gel, which is especially prominent in enzyme stabilized in the E_2P states by MgP_i and MgF_4^{2-} but weaker in AlF_4^- and BeF_3^-. After binding of cardiotonic steroids the 80 kDa band weakens or disappears completely. Instead, a 95 kDa band appears, most clearly observed in MgF_4^{2-} and AlF_4^-. The presence of Na^+ (or K^+) in the phosphoenzyme incubation buffers is necessary to observe these trypsin digestion patterns. Without occluded cations digestion is much more complete, leaving almost only the 19 kDa fragment. The effects on the Na,K-ATPase trypsin digestion patterns for the three cardiotonic steroids, ouabain, DG, and bufalin, are in general very similar.

If the $E_2{\cdot}MgF_4^{2-}$ state is produced in the presence of 5 mM K^+ instead of 25 mM Na^+, a state similar to the recently published 2.4 Å crystal structure (*8*), incubation with 10 mM ouabain produced a similar change in the digestion pattern (Figure 6, right panel). This demonstrates that low-affinity ouabain binding is still taking place to the K^+-bound phosphoenzyme although K^+ binding antagonizes the binding of ouabain (*9*).

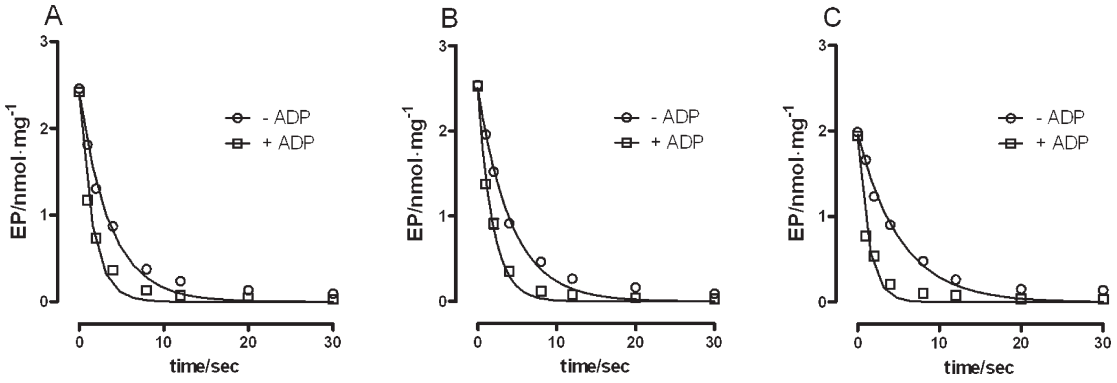

FIGURE 5: Spontaneous and ADP-supported dephosphorylation of Na,K-ATPase at pH 6.5 (panel A), 7.5 (panel B), and 8.5 (panel C). The curves are one-phase decay curves with half-lives 2.55 ± 0.17 and $1.11 \pm 0.06\ s^{-1}$ (A), 2.93 ± 0.17 and $1.3 \pm 0.06\ s^{-1}$ (B), and 3.63 ± 0.28 and $0.92 \pm 0.07\ s^{-1}$ (C).

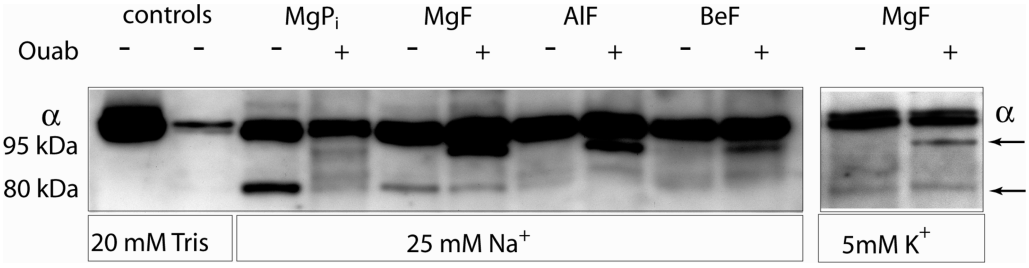

FIGURE 6: Immunoblot of trypsin-treated Na,K-ATPase. The left panel shows untreated enzyme (lane 1) and trypsin-cleaved enzyme (lane 2) incubated in 20 mM Tris followed by tryptic cleavage of enzyme stabilized in different E_2-phosphoenzyme conformations, E_2P (MgP_i), $E_2 \cdot MgF_4^{2-}$ (MgF), $E_2 \cdot AlF_4^-$ (AlF), and $E_2 \cdot BeF_3^-$ (BeF) (lanes 3−10), incubated without or with ouabain (1 mM) in the presence of 25 mM Na^+. The right panel shows the effect of ouabain (10 mM) on trypsin digestion of enzyme incubated in 5 mM K^+ and stabilized in the $E_2 \cdot MgF_4^{2-}$ state.

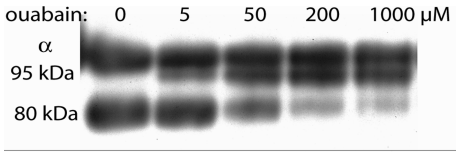

FIGURE 7: Trypsin digestion pattern of enzyme stabilized in the $E_2 \cdot MgF_4^{2-}$ conformation in the presence of 25 mM Na^+ and incubated with increasing concentration of ouabain.

Table 3: N-Terminal Sequence of the 80 and 95 kDa Tryptic Digestion Fragments

	control	+ouabain
fragment	80 kDa	95 kDa
N-terminal sequence	I^{270}ATLAS	N^{162}MVPGG

In Figure 7 the digestion pattern of Na,K-ATPase in $E_2 \cdot MgF_4^{2-}$ state was examined in the presence of increasing concentrations of ouabain. As the ouabain concentration increases the digestion pattern gradually changes from one where an ~80 kDa digestion fragment dominates into one where an ~95 kDa fragment is dominating.

In Table 3 the N-terminal sequences of the 80 kDa (−ouabain) and 95 kDa (+ouabain) fragments are given, and in Figure 8 the locations of the determined trypsin splits are compared to the shark crystal structure in $E_2P \cdot K_2$ stabilized with MgF_4^{2-} (*8*). One split (tryp1) is at Lys161 located at a small helix, M2′, connecting the M2 transmembrane helix with the A-domain. This split is the one exposed by ouabain binding and produces the larger 95 kDa fragment in the SDS gels (Figures 6 and 7). The second split at Arg269, which is protected by ouabain binding, is located on another small helix, A3, on the opposite face of the Na,K-ATPase α-subunit connecting the A-domain with the M3 transmembrane helix (Figure 8). A split here gives rise to the smaller 80 kDa fragment in the SDS gel. Thus ouabain "protects" a tryptic cleavage in A3−M3 and "exposes" the cleavage site in M2−M2′, most clearly observed in the $E_2 \cdot MgF_4^{2-}$ and $E_2 \cdot AlF_4^-$ conformations (Figure 6).

DISCUSSION

The shark Na,K-ATPase has previously been demonstrated to bind ouabain with high affinity. The binding isotherms were shown to be complex and compatible with the presence of two dissociation constants of 0.7 and 42 nM (*26*). In the present study we compare the inhibitory potency and rate of inhibition at different pH values of shark Na,K-ATPase stabilized in an E_2P conformation by various cardiotonic steroids. The effects of the sugar moiety and steroid core of cardiotonic steroids were investigated by comparing the cardiac glycoside ouabain with the aglycons OG and DG, and to investigate the effects of structural changes in the lactone moiety, the inhibitory effects of bufalin, which has a 6-membered lactone, and DHO, containing a saturated 5-membered lactone, were measured (cf. Figure 1).

The effects of pH on the binding of cardiotonic steroids to Na,K-ATPase have previously been studied in only a few cases. Yoda and Yoda (*25*) demonstrated that the association rate constant for binding of ouabain and digoxigenin to Na,K-ATPase incubated in Mg^{2+} and P_i decreases by a factor of ~3 as pH is increased from 6.5 to 8.0. In accordance with this Moczydlowski and Fortes (*27*) showed that in MgP_i the binding of anthroyl-ouabain to Na,K-ATPase is faster at pH 6.2 than at pH 7.5.

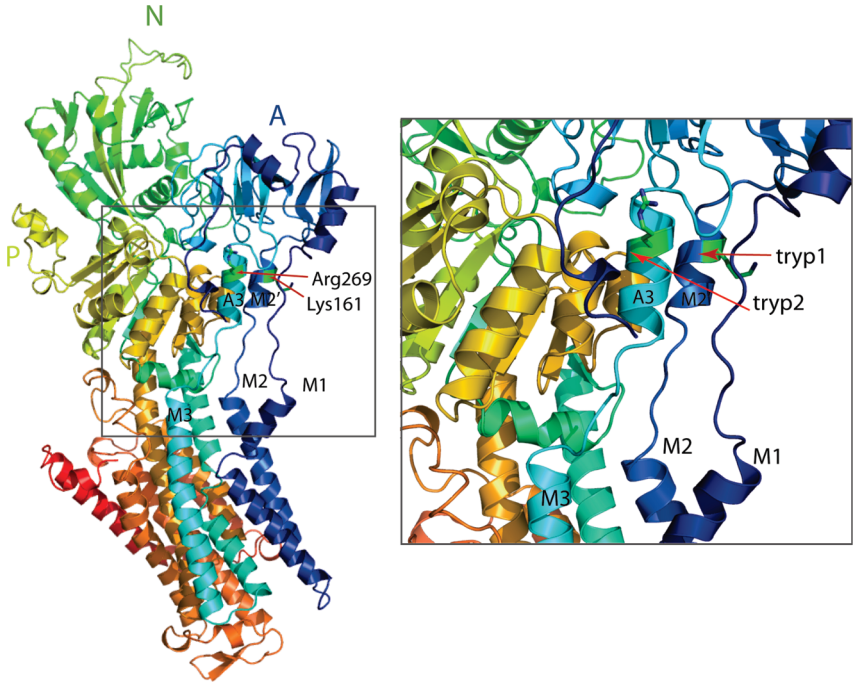

FIGURE 8: Location of the two trypsin splits in the $E_2P \cdot K_2$ crystal structure of Na,K-ATPase α-subunit: one after Lys161 (tryp1) in the short helix following M2 (M2′) and the other after Arg269 (tryp2) in the short helix before M3 (A3). PyMol was used to visualize the structure (Protein Data Bank accession number 2ZXE).

From Figure 2A,B and Table 1 it is clear that the inhibitory potency for the tested cardiotonic steroids at pH 7.5 indicated by the inhibitor constant IC_{50} is highest for ouabain (IC_{50} = 81 nM) followed by the two aglycons DG and bufalin (IC_{50} = 104 and 134 nM, respectively). The inhibitor constant for DHO was somewhat higher than for ouabain, amounting to 292 nM, whereas the lowest inhibitor potency was observed for OG with an IC_{50} of 2.28 μM. Thus the parameters determining the inhibitory potency are complex depending on the presence of sugars, the side chains of the steroid moiety, and the structure of the lactone ring.

In a recent investigation Paula et al. (*10*) compared binding affinities of a range of cardiotonic steroids to the E_2P conformation of Na,K-ATPase with the low-affinity inhibitory potency of Na,K-ATPase activity during Na,K-ATPase turnover in the presence of Na^+, K^+, and ATP. They find bufalin 7 times and DG 2 times more potent inhibitors than ouabain whereas they have 5 and 2 times lower binding affinities. The same tendency is even more pronounced if ouabain and ouabagenin were compared. Here the genin has a 311 times lower binding affinity but is only half as potent an inhibitor. Paula et al. (*10*) explain this from the fact that the binding affinity is primarily reflecting interactions between the cardiotonic steroid and the M1−M2 loop of the Na,K-ATPase α-subunit, whereas the inhibitory potency mainly relies on interactions with the M5−M6 helices. However, as pointed out by the authors themselves, inhibition during turnover conditions may be the result of interactions with several enzyme species, which makes the contributions of the consequent conformational changes ambiguous. During turnover conditions the enzyme conformations to which the cardiotonic steroid preferentially binds (i.e., the E_2P conformations) are accessible only very temporarily, favoring binding of inhibitors with a high association rate. Such ambiguities are not present by the method used here, where the inhibitor is equilibrated with the favorable enzyme conformation prior to assay of activity. Accordingly, the inhibitory potency of OG is found to be smaller than that of ouabain by a factor of 28 in the present investigation.

When pH is increased from 6.5 to 8.5, the inhibitory potency by cardiotonic steroids of shark Na,K-ATPase activity is significantly decreased; i.e., IC_{50} increases (Figure 3 and Table 1). This effect is most pronounced for ouabagenin, ouabain, and dihydroouabain and to a much lesser degree for bufalin and DG. Therefore, the pH sensitivity seems to rely mainly on the side chains of the steroid moiety where ouabain and its derivatives have several OH groups. Comparing ouabain and its aglycon ouabagenin (Figures 3 A and E), it is clear that the presence of a sugar moiety makes this pH effect much less pronounced.

The rate of inhibition is found to be significantly faster for the two hydrophobic aglycons bufalin and DG than for the water-soluble glycosides ouabain and DHO (Figure 4 and Table 2). However, the inhibition rate of OG is comparable, or even slower, than ouabain and DHO, indicating again that the OH side chains of the steroid are the main hindrance for inducing the high-affinity inhibition. Having reached the binding site the dissociation seems to depend mainly on the sugar moiety, which stabilizes binding since variations in IC_{50} have previously been demonstrated to depend primarily on variations in the dissociation rate constant (*6*).

Previous suggestions have postulated that cardiotonic steroids move through an extracellular channel cavity formed in the E_2P ground state of Na,K-ATPase in order to reach the binding site. Thus, in contrast to models that postulated a superficial binding of cardiotonic steroids to the extracellular face of Na,K-ATPase (*28*, *29*), other models hypothesize the sugar moiety as directed to the extracellular space whereas the lactone ring is wedged deeply into the membrane (*14*, *16*, *30*). The newly published crystal structure of the low-affinity ouabain-bound shark Na,K-ATPase state clearly demonstrates the latter to hold true. Indeed, as the cardiotonic steroid binding proceeds deeper into the transmembrane domain, interactions of the lactone moiety with M4−M6 helices take place and inhibition results

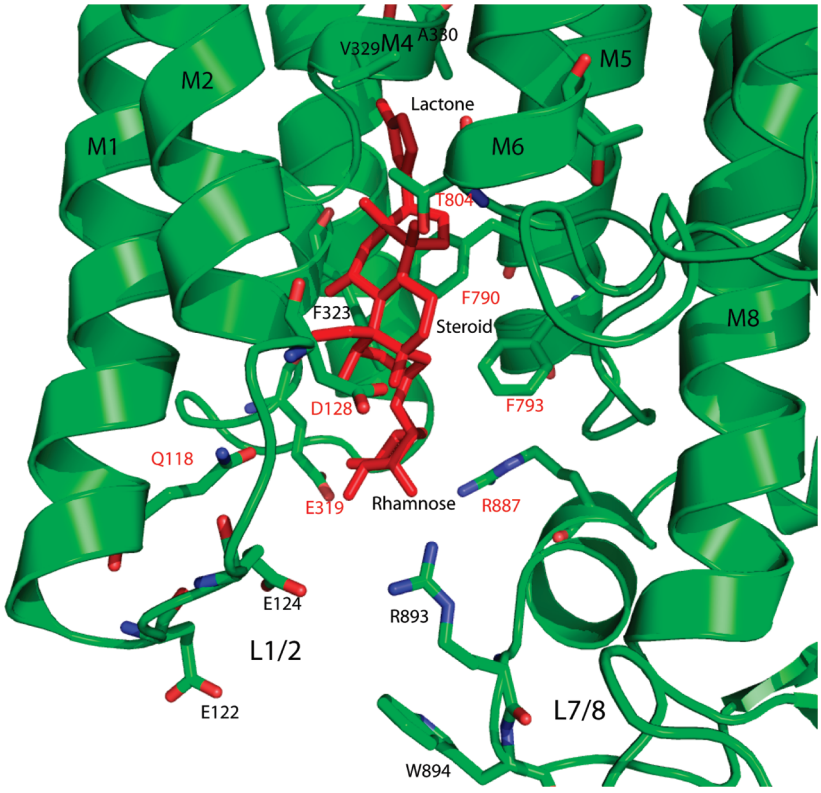

FIGURE 9: The Na,K-ATPase binding vestibule for cardiotonic steroids. Residues labeled in red are previously demonstrated by mutagenesis to be important for cardiotonic steroid binding. Ouabain is shown in sticks. Note the unwinding of M4 near the lactone of ouabain. The structure was drawn using PyMol and the Protein Data Bank accession number 3A3Y.

as a consequence of the decreased plasticity of the protein (*9*). Figure 9 shows the binding vestibule in shark Na,K-ATPase using the new 2.8 Å low-affinity ouabain-bound crystal structure. It is located where an access channel in the E_2P ground state of Ca-ATPase is also observed. Since the equilibration of bufalin and DG is much faster (in the minute range) than for ouabain and DHO (in the hour range), a hydrophobic barrier seems to exist along the access pathway to allow the inhibitors to reach the binding site. Thus, as the lactone and steroid moieties enter the hydrophobic binding site near M4−M6, the solvation of the inhibitor decreases, accounting for the change in free energy associated with binding (*30*). Yoda et al. (*31*) have previously demonstrated that the association rate constant depends primarily on the steroid moiety and not on the sugar moiety. In the present study the same tendency is found since ouabain, DHO, and OG have half-lives between 30 and 50 s whereas $t_{1/2}$ for bufalin and DG is 6−7 s (pH 7.5, Table 2). This is readily explained by comparing the ouabain-bound and unbound crystal structures (*8*, *9*). Thus several Phe residues are present in the upper part of the putative access channel (Phe323 of M4, Phe790 and Phe793 of M5; cf. Figure 9) where they form a hydrophobic cluster with I322 (M4) and L800 (M6) at the position where the steroid core of ouabain binds (*9*). To make room for ouabain binding, the M1−M4 helices are rearranged, and the extracellular part of the M4 helix unwinds. The Phe323 and Phe790 side groups move aside to make room for the steroid moiety, and Thr804 forms a hydrogen bond with the C14 hydroxyl between the C and D steroid rings.

As seen from Table 2, the rate of inhibition by cardiotonic steroids decreases significantly at increasing pH. This effect is most pronounced for ouabain and its derivatives. Thus for ouabain (10^{-5} M), $t_{1/2}$ increases from about 23 to 563 s when pH increases from 6.5 to 8.5 (Table 2). At the same time the IC_{50} value increases from about 70 to 400 nM. In comparison, the rate of inhibition of bufalin increases by a factor of ~3 for the same increase in pH, and IC_{50} only increases twice. Apparently, the sugar moiety seems to be pivotal for the difference in pH sensitivity. However, the sugar-induced pH effect on IC_{50} depends strongly on the steroid moiety. This is especially clear comparing ouabain and OG (Figure 3 and Table 1). Thus, whereas the rate of inhibition is almost identical for the two at all pH values tested, the IC_{50} values are drastically different. Therefore, the unfavorable OH groups of OG can be partially compensated for by the presence of the sugar moiety of ouabain. The low-affinity crystal structure of the ouabain-bound Na,K-ATPase explains why (*9*). The rhamnose of ouabain is likely to be coordinated by residues Glu319 and Arg887 and expected to be pH dependent. In the ouabain-bound Na,K-ATPase Glu319−Arg887 together with Asp128−Arg978 are likely to form salt bridges, and protonation of Glu/Arg residues will increase the chance for formation of the ouabain-binding vestibule. Contrary to the hydroxyl group at C14 on the β face of the steroid moiety, which forms a hydrogen bond with Thr804, the three other hydroxyl groups on its β face are unbonded and seem sterically unfavorable. In accordance with this, digitoxin with no hydroxyls on the steroid has a four times higher affinity than digoxin, with a hydroxyl at C12 (*10*). The significant pH sensitivity of OG inhibition indicates that some protein residues responsible for inducing the high-affinity state by closing the binding cavity are charged (*9*). The coordination of the sugar of the two glycosides ouabain and DHO by Asp128−Arg879 will probably also depend on pH. Yoda and Yoda have previously demonstrated that the dissociation of glycosides depends on the sugar moiety (*35*). In accordance with this, the 3′-α-OH group of

rhamnose in the high-affinity ouabain-bound Na,K-ATPase homology model (*9*) comes within hydrogen bond distance from Glu122 located in the L1/2 loop of Na,K-ATPase (Figure 9). Together, this implies that the sugar coordination of glycosides induces a conformational change to a closed high-affinity state. Indeed, replacement of the neutral amino acids Gln111 and Asn122 of sheep Na,K-ATPase (Gln118 and Asn129 in shark) at either ends of the L1/2 loop close to the membrane face with charged ones, which is responsible for the low ouabain affinity of sheep Na,K-ATPase, has been commonly used to induce ouabain-resistant mutants (*36*, *37*).

At high pH values the coordination of the glycoside sugar becomes more unfavorable and the resulting inhibition of Na,K-ATPase slower as shown in Figure 4 and Table 2. This is unlike the binding of the aglycons bufalin and DG, which may bind stronger to the Na,K-ATPase due to the lack of OH side chains on the β face of the steroid moiety. De Pont et al. (*16*) have argued from model considerations that the absence of sugars allows the aglycon to penetrate more deeply into the membrane binding pocket. However, as indicated by the crystal structure (*9*) the lactone moiety is very strongly coordinated in the binding pocket and is probably located similarly for different cardiotonic steroids independently of the presence of a sugar moiety, in agreement with the similar trypsin digestion pattern found for ouabain, DG, and bufalin.

The general effects of increasing inhibitory potency by lowering pH might have an electrostatic component probably related to protonation of carboxyls on amino acid side chains either in the superficial part of the binding cavity or deeper into the TM domain, where the lactone is coordinated. An increase in positive charge between the lactone ring and the steroid C-16 and an increase in negative charge around the carbonyl oxygen of the lactone ring have previously been suggested to favor binding (*10*). As seen from Figure 9, the binding vestibule lined by the L1/2 and the L7/8 loops contains several charged residues, many of which are known from mutagenesis studies to be important for ouabain binding (indicated in red in Figure 9) (*12*−*15*, *38*). Price et al. (*37*) have previously suggested that electrostatic interaction of the M1−M2 loop of such charged border amino acids with other charged residues may prevent the conformational change associated with ouabain binding.

Increasing pH has previously been demonstrated to shift the conformational poise toward the E_1 form (*39*). In the present study pH is demonstrated to shift the E_1P/E_2P poise so that E_1P is stabilized at high pH (Figure 5). This would tend to decrease the availability of the high-affinity E_2P form during preincubation with inhibitors and therefore decrease binding of cardiotonic steroids. Although this may contribute to the lower inhibitory potencies of cardiotonic steroids at high pH, in general it cannot explain the very different effect of pH between the group of glycosides (ouabain and DHO) and the aglycons (bufalin and DG).

The detailed structure of the lactone moiety of cardiotonic steroids seems to be pivotal for the interaction with and binding to Na,K-ATPase. Thus, hydrogen bonding of the lactone carbonyl to Val329 and Arg330 fixes the lactone to the binding site on the enzyme (*9*). Rotation around the $C_{17}-C_{20}$ bond then positions the steroid moiety against the complementary enzyme surface where it is coordinated by Phe323 (M4), Phe790 and Phe793 in M5, and Thr804 of M6. Indeed, previous investigations have demonstrated that the inhibitory potency of genins is a function of the carbonyl oxygen position attached to C_{17} of the lactone ring (*32*, *33*). This is in accordance with the finding in the present investigation of a decrease in the inhibitory potency of DHO with a saturated lactone compared to ouabain. Relative to DG the position of the carbonyl oxygen of bufalin ~ -1.5 Å, resulting in an increased inhibitory potency of a factor of almost 10 (bufalin) relative to DG, at least for binding to phosphoenzyme induced by Mg^{2+}, Na^+, and ATP (*33*, *34*).

As seen from Figures 6 and 7, ouabain binding to Na,K-ATPase was found to induce a conformational change as detected by a change in the trypsin digestion pattern in the higher MW part of the SDS gels. In the presence of Na^+, Mg^{2+}, and P_i or fluoride analogues the Na,K-ATPase is cleaved by trypsin to yield a pronounced ~80 kDa fragment (Figure 6A) plus some lower molecular mass fragments (not shown) including the 19 kDa fragment (*40*). A similar 80 kDa band is absent if cleavage of the Na,K-ATPase dephospho form is performed (Figure 6), indicating that the cleavage of this fragment is retarded by phosphorylation of the enzyme. After ouabain binding the trypsin digestion pattern changes: the 80 kDa band is absent or weakened, and instead an ~95 kDa band appears, the intensity of which depends on the phosphoenzyme species. This shift in tryptic cleavage pattern is particular clearly demonstrated in Figure 7, where the enzyme stabilized in the $E_2MgF_4^{2-}$ state is incubated at increasing ouabain concentration. The same shift in the trypsin digestion pattern was observed if Na^+ was replaced by K^+, but at higher concentrations of ouabain (Figure 6, right panel) in accord with the low-affinity ouabain-bound Na,K-ATPase crystal structure recently published (*9*).

The locations of the trypsin splits before and after ouabain binding to E_2P or its fluoride analogues are within two small helices, M2′ (K161) and A3 (R269), in the $E_2P\cdot K_2$ crystal structure (Figure 9). This is a little unexpected, since primary proteolysis sites are rarely on helical structures. Ouabain binding does not change the A- and N-domain structures, and the M2′ and A3 helices are also present in the ouabain-bound state (*9*). Therefore, the ouabain exposure of the trypsin cleavage sites at K161 giving rise to the pronounced 95 kDa digestion bands does not result from unwinding of the A2′ helix following ouabain binding. Comparing with SERCA1a, the A3 helix is present in all E_2-like conformations but absent in E_1-like conformations. The M2′ helix is present in all E_2-like conformations except in the E_2P ground state but absent in all E_1-like conformations. If a similar arrangement is present in Na,K-ATPase, we should expect to observe a specially strong 95 kDa band in the E_2P ground state with BeF_3^-. As seen from Figure 6, this is not the case. Alternatively, the splits are secondary, and the initial cleavage sites are in the loop regions after M2′ and before A3, i.e., at R156 and R264, which subsequently cause unwinding of the helices and exposing of the trypsin cleavage sites. In both cases, however, the observed shifts in trypsin-exposed splitting sites induced by ouabain binding show that binding of ouabain is accompanied by rearrangements in transmembrane helices M1−M4, as also observed in the crystal structure of the ouabain-bound Na,K-ATPase (*9*).

ACKNOWLEDGMENT

We thank Hanne R. Z. Christensen, Anne Lillevang, and Anne Mette Beck Rasmussen for excellent technical assistance and Dr. Chikashi Toyoshima for valuable discussions.

REFERENCES

1. Schatzmann, H. J. (1953) Hertzglycoside als hemmstoffe für den aktiven kalium- und natriumtransport durch die erythrocytenmembran. *Helv. Physiol. Pharmacol. Acta 11*, 346–354.
2. Li, Z., and Xie, Z. (2009) The Na/K-ATPase/Src complex and cardiotonic steroid-activated protein kinase cascade. *Pfluegers Arch. 457*, 635–644.
3. Soltoff, S. P., and Hedden, L. (2008) Regulation of ERK1/2 by ouabain and Na-K-ATPase-dependent energy utilization and AMPK activation in parotid acinar cells. *Am. J. Physiol. Cell Physiol. 295*, C590–C599.
4. Yoda, A., and Yoda, S. (1982) Interaction between ouabain and the phosphorylated intermediate of Na,K-ATPase. *Mol. Pharmacol. 22*, 700–705.
5. Akera, T., and Brody, T. M. (1971) Membrane adenosine triphosphatase. The effect of potassium on the formation and dissociation of the ouabain-enzyme complex. *J. Pharmacol. Exp. Ther. 176*, 545–557.
6. Tobin, T., and Brody, T. M. (1972) Rates of dissociation of enzyme-ouabain complexes and $K_{0.5}$ values in ($Na^+ + K^+$) adenosine triphosphatase from different species. *Biochem. Pharmacol. 21*, 1553–1560.
7. Yoda, A. (1974) Association and dissociation rate constants of the complex between various cardiac monoglycosides and Na,K-ATPase. *Ann. N.Y. Acad. Sci. 242*, 598–616.
8. Shinoda, T., Ogawa, H., Cornelius, F., and Toyoshima, C. (2009) Crystal structure of the sodium-potassium pump at 2.4 Å resolution. *Nature 459*, 446–451.
9. Ogawa, H., Shinoda, T., Cornelius, F., and Toyoshima, C. (2009) Crystal structure of the sodium-potassium pump (Na^+,K^+-ATPase) with bound potassium and ouabain. *Proc. Natl. Acad. Sci. U.S.A. 106*, 13742–13747.
10. Paula, S., Tabet, M. R., and Ball, W. J., Jr. (2005) Interactions between cardiac glycosides and sodium/potassium-ATPase: three-dimensional structure-activity relationship models for ligand binding to the E2-Pi form of the enzyme versus activity inhibition. *Biochemistry 44*, 498–510.
11. Toyoshima, C., Norimatsu, Y., Iwasawa, S., Tsuda, T., and Ogawa, H. (2007) How processing of aspartylphosphate is coupled to lumenal gating of the ion pathway in the calcium pump. *Proc. Natl. Acad. Sci. U.S.A. 104*, 19831–19836.
12. Olesen, C., Picard, M., Winther, A.-M. L., Gyrup, C., Morth, P., Oxvig, C., Møller, J. V., and Nissen, P. (2007) The structural basis of calcium transport by the calcium pump. *Nature 450*, 1036–1044.
13. Qiu, L. Y., Koenderink, J. B., Swarts, H. G. P., Willems, P. H. G. M., and De Pont, J. J. H. H. M. (2003) Phe783, Thr797, and Asp804 in transmembrane hairpin M5-M6 of Na^+, K^+-ATPase play a key role in ouabain binding. *J. Biol. Chem. 278*, 47240–47244.
14. Qiu, L. Y., Krieger, E., Schaftenaar, G., Swarts, H. G. P., Willems, P. H. G. M., De Pont, J. J. H. H. M., and Koenderink, J. B. (2005) 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*, 32349–32355.
15. Qiu, L. Y., Swarts, H. G. P., Tonk, E. C., Willems, P. H. G. M., Koenderink, J. B., and De Pont, J. J. H. H. M. (2006) 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*, 13533–13539.
16. De Pont, J. J. H. H. M., Swarts, H. G. P., Karawajczyk, A., Schaftenaar, G., Willems, P. H. G. M., and Koenderink, J. B. (2009) The non-gastric H,K-ATPase as a tool to study the ouabain-binding site in Na,K-ATPase. *Pfluegers Arch.—Eur. J. Physiol. 457*, 623–634.
17. Skou, J. C., and Esmann, M. (1988) Preparation of membrane Na^+, K^+-ATPase from rectal glands of *Squalus acanthias*. *Methods Enzymol. 156*, 43–46.
18. Mahmmoud, Y. A., Vorum, H., and Cornelius, F. (2000) Identification of a phospholemman-like protein from shark rectal glands. Evidence for indirect regulation of Na,K-ATPase by protein kinase C via a novel member of the FXYDY family. *J. Biol. Chem. 275*, 35969–35977.
19. Peterson, G. L. (1983) Determination of total protein. *Methods Enzymol. 91*, 95–106.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193*, 265–275.
21. Baginski, E. S., Foa, P. P., and Zak, B. (1967) Determination of phosphate: study of labile organic phosphate interference. *Clin. Chim. Acta 14*, 155–158.
22. Mahmmoud, Y. A., and Cornelius, F. (2002) Protein kinase C phosphorylation of purified Na,K-ATPase: C-terminal phosphorylation sites at the alpha- and gamma-subunits close to the inner face of the plasma membrane. *Biophys. J. 82*, 1907–1919.
23. Danko, S., Yamasaki, K., Daiho, T., and Suzuki, H. (2004) Distinct natures of beryllium fluoride-bound, aluminum fluoride-bound, and magnesium fluoride-bound stable analogues of an ADP-insensitive phosphoenzyme intermediate of sarcoplasmic reticulum Ca^{2+}-ATPase. *J. Biol. Chem. 279*, 14991–14998.
24. Cornelius, F. (1995) Phosphorylation/dephosphorylation of reconstituted shark Na^+,K^+-ATPase: one phosphorylation site per alpha beta protomer. *Biochim. Biophys. Acta 1235*, 197–204.
25. Yoda, A., and Yoda, S. (1978) Influence of pH on the interaction of cardiotonic steroids with sodium- and potassium-dependent adenosine triphosphatase. *Mol. Pharmacol. 14*, 624–632.
26. Hansen, O. (1999) Heterogeneity of Na^+/K^+-ATPase from rectal gland of *Squalus acanthias* is not due to α isoforms diversity. *Pfluegers Arch.—Eur. J. Physiol. 437*, 517–522.
27. Moczydlowski, E. G., and Fortes, P. A. G. (1980) Kinetics of cardiac glycoside binding to sodium, potassium adenosine triphosphatase studied with a fluorescent derivative of ouabain. *Biochemistry 19*, 969–977.
28. Keenan, S. M., DeLisle, R. K., Welsh, W. J., Paula, S., and Ball, W. J., Jr. (2005) Elucidation of the Na^+,K^+-ATPase digitalis binding site. *J. Mol. Graphics Modell. 23*, 465–475.
29. Yatime, L., Buch-Pedersen, M. J., Musgaard, M., Morth, J. P., Lund Winther, A. M., Pedersen, B. P., Olesen, C., Andersen, J. P., Vilsen, B., Schiøtt, B., Palmgren, M. G., Møller, J. V., Nissen, P., and Fedosova, N. (2009) P-type ATPases as drug targets: tools for medicine and science. *Biochim. Biophys. Acta 1787*, 207–220.
30. Munson, K., Law, R. J., and Sachs, G. (2007) Analysis of the gastric H,K ATPase for ion pathways and inhibitor binding sites. *Biochemistry 46*, 5398–5417.
31. Yoda, A., Yoda, S., and Sharif, A. M. (1973) Structure-activity relationship of cardiotonic steroids for the inhibition of sodium- and potassium-dependent adenosine triphosphate. II. Association rate constant of various enzyme-cardiac glycoside complexes. *Mol. Pharmacol. 9*, 766–773.
32. Fullerton, D. S., Yoshioka, K., Rohrer, D., From, A. H. L., and Ahmed, K. (1979) Digitalis genin activity: side-group carbonyl oxygen position is a major determinant. *Science 205*, 917–919.
33. Ahmed, K., Roher, D. C., Fullerton, D. S., Deffo, T., Kitatsuji, E., and From, A. H. L. (1983) Interaction of (Na^+, K^+)-ATPase and digitalis genins. A general model for inhibitory activity. *J. Biol. Chem. 258*, 8092–8097.
34. Fullerton, D. S., Kitatsuji, E., Deffo, T., Roher, D. C., Ahmed, K., and From, A. H. L. (1983) Use of prophet and MMS-X computer graphics in the study of the cardiac steroid receptor site of Na, K-ATPase. *Current Top. Membr. Transp. 19*, 257–264.
35. Yoda, A. (1973) Structure-activity relationship of cardiotonic steroids for the inhibition of sodium- and potassium-dependent adenosine triphosphatase. I. Dissociation rate constants of various enzyme-cardiac glycoside complexes formed in the presence of magnesium and phosphate. *Mol. Pharmacol. 9*, 51–60.
36. Lingrel, J. B., Argüello, J. M., Van Huysse, J., and Kuntzweiler, T. A. (1997) Cation and cardiac glycoside binding sites of the Na, K-ATPase. *Ann. N.Y. Acad. Sci. 834*, 194–206.
37. Price, E. M., Rice, D. A., and Lingrel, J. B. (1990) Structure-function studies of Na,K-ATPase. Site-directed mutagenesis of the border residues from the H1-H2 extracellular domain of the alpha subunit. *J. Biol. Chem. 265*, 6638–6641.
38. Radkov, R., Kharoubi-Hess, S., Schaer, D., Modyanov, N. N., Geering, K., and Horisberger, J. D. (2007) Role of homologous ASP334 and GLU319 in human non-gastric H,K- and Na,K-ATPases in cardiac glycoside binding. *Biochem. Biophys. Res. Commun. 356*, 142–146.
39. Skou, J. C. (1982) The effect of pH, of ATP and of modification with pyridoxal 5-phosphate on the conformational transition between the Na^+-form and the K^+-form of the ($Na^+ + K^+$)-ATPase. *Biochim. Biophys. Acta 688*, 369–380.
40. Karlish, S. J. D., Goldshleger, R., and Stein, W. D. (1991) A 19-kDa C-terminal tryptic fragment of the α chain of Na,K-ATPase is essential for occlusion and transport of cations. *Proc. Natl. Acad. Sci. U.S.A. 87*, 4566–4570.