A Structural View on the Functional Importance of the Sugar Moiety and Steroid Hydroxyls of Cardiotonic Steroids in Binding to Na,K-ATPase

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The Na,K-ATPase is specifically inhibited by cardiotonic steroids (CTs) like digoxin and is of significant therapeutic value in the treatment of congestive heart failure and arrhythmia. Recently, new interest has arisen in developing Na,K-ATPase inhibitors as anticancer agents. In the present study, we compare the potency and rate of inhibition as well as the reactivation of enzyme activity following inhibition by various cardiac glycosides and their aglycones at different pH values using shark Na,K-ATPase stabilized in the E2MgP or in the E2BeF3 conformations. The effects of the number and nature of various sugar residues as well as changes in the positions of hydroxyl groups on the β-side of the steroid core of cardiotonic steroids were investigated by comparing various cardiac glycoside compounds like ouabain, digoxin, digitoxin, and gitoxin with their aglycones. The results confirm our previous hypothesis that CT binding sites primarily to the E2-P ground state through an extracellular access channel and that binding of extracellular Na+ ions to K+ binding sites relieved the CT inhibition. This reactivation depended on the presence or absence of the sugar moiety on the CT, and a single sugar is enough to impede reactivation. Finally, increasing the number of hydroxyl groups of the steroid was sterically unfavorable and was found to decrease the inhibitory potency and to confer high pH sensitivity, depending on their position on the steroid β-face. The results are discussed with reference to the recent crystal structures of Na,K-ATPase in the unbound and ouabain-bound states.

Ouabain was the first cardiotonic steroid (CTS)² shown to be a specific inhibitor of the Na,K-ATPase (1). This is believed to be the basis for the long-known therapeutic effect of CTSs, like digoxin and digitoxin, in treatment of congestive heart failure and arrhythmia. Inhibition of myocardial cell Na,K-ATPase raises intracellular Na⁺ concentration, which suppresses NCX, the 3Na⁺-Ca²⁺ exchanger, thus increasing intracellular Ca²⁺ stores, producing an increase in heart contractility and output (the inotropic effect). Recently, CTS as a mediator of anti-cancer effects has drawn much attention, emphasizing their potential use in oncology (2–4). Indeed, many cancer cells overexpress α₁ (e.g. kidney cancers), or α₃ (e.g. colon cancers) (for a review, see Ref. 2).

The Na,K-ATPase is indispensable in maintaining cellular ion homeostasis in animals. This integral membrane protein is an ion pump fueled by ATP and is responsible for actively maintaining the electrochemical gradients for Na⁺ and K⁺ across the animal cell membrane, which are essential for many physiological processes, like secondary active co- and countertransport and volume regulation, and forms the basis for maintaining the resting membrane potential. Cardiotoxic steroids are composed of three major structural components (Fig. 1): 1) a steroid core, in which rings AB and CD are cis-fused, whereas rings BC are trans-fused; 2) a 5- or 6-membered lactone ring at position 17 (cardenolides or bufadienolides, respectively); and 3) a variable number of sugar residues at position 3. Thus, ouabain and evomoside each contains one α-L-rhamnose residue, whereas digoxin, digitoxin, and gitoxin all contain three β-D-digitoxose molecules. In contrast to the cardiac glycosides, cardiac aglycones lack the sugar moiety. The sugar residues have been demonstrated to stabilize the enzyme-CTS complex through interactions of the 3’-α-hydroxyls with both proton-donating and proton-accepting groups on the enzyme (5).

The CTS binding site of Na,K-ATPase is evolutionarily conserved (6), and it is known that ouabain binds from the extracellular side and mainly to the phosphorylated E2P conformation (7) or more specifically to the E2-P ground state of Na,K-ATPase, as demonstrated by measurement of the binding of the fluorescent anthroyl ouabain to various enzyme phosphoforms stabilized by fluoride analogues of phosphate (8). Indeed, recent investigations strongly indicate that CTSs reach

* This work was supported by The Danish Medical Research Foundation and by an FI/Danish-Japanese cooperation program from the Danish Agency for Science Technology and Innovation (to F. C.); by a Specially Promoted Project Grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and by a Strategic Japanese-Danish Cooperative Program Grant from Japan Science and Technology Agency (to C. T.).

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‡ The abbreviations used are: CTS, cardiotonic steroid; ITC, isothermal titration calorimetry; PDB, Protein Data Bank.

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their binding site through an extracellular access channel structure between M1–M2 and M4–M6 in the E2-P ground state (8, 9). To reach the binding site, ouabain causes rearrangements of the M1–M4 transmembrane helices to create a cavity much like the one observed in crystal structure of SERCA1a in the E2-P ground state stabilized by BeF$_3$/H$_{11002}$ (10, 11). Thus, association and especially dissociation of ouabain are relatively slow processes (12, 13). High concentrations of NaCl reactivate enzyme activity after inhibition by ouabagenin, but not by ouabain, demonstrating the pivotal role of the sugar moiety for closing the extracellular cation pathway (8). In the present study, this mechanism is further investigated by comparing different CTS compounds.

The crystal structure of ouabain-bound Na,K-ATPase in low affinity state (9) demonstrates that ouabain is bound deeply into the transmembrane domain with the lactone ring placed near the K$^+$-binding sites and the sugar exposed to the solvent. A low resolution (4.6 Å) crystal structure of the high affinity E2P/ouabain state confirms this position of bound ouabain (14). The hydrophobic $\alpha$-face of ouabain interacts with three Phe residues (Phe-323 on M4, Phe-790 and Phe-793 on M5; shark enzyme numbering), whereas the hydrophilic $\beta$-face shows little interaction with protein residues (9), which is the reason for the low affinity to ouabain. However, in the homology model of ouabain-bound Na$^+$/K$^+$-ATPase in the high affinity state (9, 15) and in the high affinity E2P-ouabain crystal structure (14), M1/M2 has approached the ouabain $\beta$-face, allowing, for example, Gln-118 and Asn-129 to interact with hydroxyl groups on the CTS steroid core. In a previous paper (16), it was found that ouabain and ouabagenin were the most pH-sensitive inhibitors, with a dramatic decrease in apparent affinity at high pH, indicating that the steroid hydroxyl groups are essential for this pH effect. In the present investigation, we describe the importance of hydroxyl groups at various positions on the $\beta$-side of the steroid core as well as the effects of the nature and number of sugar residues for the decrease in inhibitory potency of various CTSs at high pH, and we attempt to relate this to the structural changes following ouabain binding to Na,K-ATPase as observed in the known crystal structures.

**EXPERIMENTAL PROCEDURES**

**Materials**—The cardiotonic steroids ouabain, ouabagenin, digoxin, digitoxygenin, digoxigenin, strophanthidin, and bufalin were obtained from Sigma-Aldrich. Evomonoside was from ABCR GmbH & Co. (Karlsruhe, Germany). Gamabufotalin was from Faces Biomedical (China). Digoxigenin didigitoxide was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and other digitoxose sugar derivatives of digoxigenin (digoxigenin mono- and tetradigitoxide) were a gift from Drs. Adriana Katz and Steven J. D. Karlish (Weizmann Institute of Science).
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Preparation of Shark Na,K-ATPase—Crude membrane fractions (microsomes) from the rectal gland of the shark *Squalus acanthias* were prepared by homogenization followed by washing and isolation by centrifugation in 30 mM histidine, 1 mM EDTA, 0.25 mM sucrose, pH 6.8. The purified microsomes were activated by a mild deoxycholate treatment (≈0.15% deoxycholate) to extract extrinsic proteins and to open sealed vesicles. After washing and resuspension, the purified membrane preparation is obtained by differential centrifugation essentially as described previously (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 α₁ and β₁ 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—Enzyme was incubated in 4 mM Mg, 1 mM P₅, and 30 mM imidazole to induce an E2P conformation. Then various concentrations of cardiotonic steroid were added at the desired pH and incubated for 1 h at 23 °C. After this preincubation, the enzyme was diluted 10 times in test solution containing 130 mM NaCl, 20 mM KCl, 3 mM ATP, 4 mM MgCl₂, 30 mM imidazole (pH 7.5), and 0.066% albumin, and the residual hydrolytic activity was measured at 23 °C by the method of Baginski et al. (21).

Reactivation of Enzyme Activity after CTS Binding—To induce high affinity binding state for cardiotonic steroids to Na,K-ATPase, the enzyme was initially stabilized in the E2P conformation by incubation in MgP₅ for 10 min as described above. In order to stabilize the enzyme in the E2BeFx ground state, the enzyme was reacted with florides essentially as described by Cornelius et al. (8) as follows. The enzyme was incubated for 10 min in 25 mM Na⁺, 5 μM BeSO₄, 5 mM NaF, and 30 mM imidazole at the desired pH. For both E2P and the E2BeFx₅, CTS was then added, and incubation continued for another 50 min. Reactivation of enzyme activity following the addition of 150 mM NaCl to CTS-inhibited enzyme was followed by activity measurements using the Fiske and SubbaRow method (22) with amidol as the reducing reagent because the florides apparently interfered with the Baginski method.

Isothermal Titration Calorimetry—An iTC200 microcalorimeter from MicroCal (Northampton, MA) was used for the measurement of heat generation associated with CTS binding to Na,K-ATPase phosphorylated by MgP₅. Analysis of the experimental data was performed using the program Origin7 and a one-site binding model. 2.4 μl of digoxin (150 μM) or 2.4 μl of digoxigenin (300 μM) was injected at 250- or 350-s time intervals, respectively, into the cuvette containing 20 μM enzyme in 10 mM imidazole-HCl, pH 7.0, 3 mM MgCl₂, 3 mM P₅, Tris, and 25% glycerol (23). All experiments were performed at 25 °C.

Statistics and Curve Fitting—Results are expressed as mean ± S.E. Inhibition by cardiotonic steroid was evaluated by fitting to a sigmoid dose-response equation,

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y = y_{\text{min}} + \frac{y_{\text{max}} - y_{\text{min}}}{1 + 10^{\text{log}_{10} x}}
\]  

(Eq. 1)

where \( y_{\text{min}} \) and \( y_{\text{max}} \) are base-line and maximum activity. \( K_i \) 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.

Remodeling of the Na,K-ATPase-Ouabain Complex—Because the ouabain-bound model in a high affinity form (PDB entry 3N23) shows steric clashes between ouabain and Thr-797, some remodeling was necessary. For this purpose, 10 atomic models of ouabain with different conformations were generated by molecular dynamics simulation using Sybyl version 7.0 with a Tripos force field (Tripos, Inc., St. Louis, MO). Then 10 models of the Na,K-ATPase-ouabain complex were made by replacing the ouabain in 3N23 with new models generated here.

The position of ouabain was first refined by rigid body refinement using CNS (24) and the structure factor derived from 3N23. CNS parameter files for ouabain were generated with the HIC-Up server (the Hetero-compound Information Centre, Uppsala, Sweden; see Ref. 25) so that the conformation of ouabain was strongly restrained to the initial model. Then simulated annealing and energy minimization using CNS were applied to the models with the x-ray term off and examining the “energy” of the model. In these calculations, ouabain and the side chains of the residues within 5.0 Å from the ouabain were set free to move. The model that showed the lowest energy was adopted as the high affinity model and used for further calculations. The root mean square deviation of the remodeled ouabain from that in 3N23 was 0.31 Å.

Modeling of the Complex of Na,K-ATPase and Digoxin or Gitoxin—Coordinates of digoxin and gitoxin were obtained from the Protein Data Bank (PDB entry 3B0W) and the Cambridge Structural Database (IUCr A19351), respectively, and used for generating 10 atomic models of digoxin or gitoxin using Sybyl version 7.0. The model of digoxin or gitoxin was introduced into the atomic model of the Na,K-ATPase-ouabain complex so that the steroid core matched that of ouabain as closely as possible. Dihedral angles of glycosyl bonds between digitoxose residues were adjusted so that digitoxose residues could form hydrogen bonds with the charged residues on the L1/2, L3/4, and L7/8 loops of Na,K-ATPase. The atomic models were optimized by simulated annealing and energy minimization using CNS. The model that showed the lowest energy was adopted for further analyses. Protein pKₐ values were calculated using PROPKA version 3.1. Structural figures were prepared with PyMOL (Schrödinger, LLC, New York).

RESULTS

The Effects of Sugars on the Inhibitory Potency of Cardiotonic Steroids—To investigate the effects of the sugar moiety of CTS, the inhibitory potency of various cardiac glycosides and their aglycones was compared. The affinity for inhibition of shark Na,K-ATPase by cardiotonic steroids was determined after binding to the high affinity E2P conformation induced by incubation with MgP₅ and P₅, followed by measurement of residual activity. Na,K-ATPase was preincubated for 1 h with different concentrations of inhibitor (10⁻³ to 10⁻⁵ M) in the presence of MgP₅ and imidazole (pH 6.5, 7.5, or 8.5). The 1-h incubation time was necessary to achieve nearly equilibrium conditions.

Statistics and Curve Fitting—Results are expressed as mean ± S.E. Inhibition by cardiotonic steroid was evaluated by fitting to a sigmoid dose-response equation,
with a constant level of inhibition. Following this, the residual Na,K-ATPase activity was determined at optimal turnover conditions (i.e. at 130 mM Na⁺, 20 mM K⁺, 4 mM Mg²⁺, 3 mM ATP, and 30 mM imidazole (pH 7.5)). Preincubation and subsequent activity measurements were performed at 23 °C.

In order to test the effect of CTS glycosylation, the inhibition curves for glycosylated and unglycosylated CTS were compared for a range of compounds (Table 1 and Fig. 2). The effect of glycosylation on the apparent inhibitor constant, \( K_i \), was variable. As seen, by far the largest effect was observed with the ouabain/ouabagenin pair, where the presence of the sugar \( \alpha \)-rhamnose increased the apparent affinity by a factor of more than 25. Also, the presence of three \( \beta \)-digitoxose sugars in gitoxin increased the \( K_i \) by a factor of almost 3 compared with that of gitoxigenin. In contrast, digitoxin and digoxin only showed moderate increase in inhibitor affinity (6 and 25%, respectively) compared with their aglycones, although they contain the same sugar moiety as gitoxin.

Comparing digitoxin with evomonoiside, where the three digitoxose residues of digitoxin are replaced with a single rhamnose residue, the \( K_i \) value was decreased for evomonoiside, indicating that the nature of the sugar had some effect on the CTS affinity (Fig. 2 and Table 1). This is in accord with previous results demonstrating that the stability of Na,K-ATPase-cardiac monoglycoside complexes is higher for \( \alpha \)-rhamnose than for \( \beta \)-digitoxides (5, 26). The difference in the effect on the \( K_i \) values between the various glycoside/aglycone pairs, however, cannot depend solely on the number and/or nature of the sugar residues but must depend also on the steroid core (see below). Indeed, the same sugar moiety has different effects, depending on the number and positions of \( \text{OH} \) groups present on the steroid core. The calculated apparent inhibitor constants (\( K_i \)) for the CTS compound measured in the present investigation are shown in Table 1. These values are consistent with previous observations, although many measurements were done under conditions that cannot be compared, with enzyme preparations from other species, or using different techniques (2, 5, 16, 26–29).

In order to investigate whether the number of sugar residues on the steroid of CTS affected the inhibitory potency, we compared the inhibitory effects of the aglycone digoxigenin with digoxigenin derivatives containing an increasing number of \( \beta \)-digitoxose molecules from one to four (see structure in Fig. 1) at pH 7.5. As seen from Fig. 3A, the inhibition constants at pH 7.5 were rather similar for the various digoxigenin derivatives, ranging from 110 to 150 nM, and a little lower than for digoxigenin itself, which was about 200 nM.

To test whether the effect of the number of sugar molecules on the steroid core was \( pH \)-dependent, the inhibition by digoxigenin and its sugar derivatives was measured at \( pH \) 6.5 and 8.5. As seen from Fig. 3B, the apparent inhibitor constants of the various digoxigenin compounds were rather similar at the lower \( pH \) of 6.5, and a small increase in affinity compared with \( pH \) 7.5 was observed for all tested compounds. However, increasing \( pH \) to 8.5 increased the \( K_i \) values drastically in a sugar-dependent way. Thus, \( K_i \) for digoxigenin increased more than 10 times, going from \( pH \) 6.5 to 8.5, whereas in the presence of a single sugar residue on digoxigenin, the increase in \( K_i \) was significantly decreased. As the number of digitoxose sugars increased, the effect on \( K_i \) increased proportionally, so that \( K_i \) for digoxigenin tetradigitoxide was almost identical to that for digoxigenin itself.

The effect of the three \( \beta \)-digitoxose sugar substitutions of digoxigenin on the binding to the phosphorylated enzyme was also examined by isothermal titration calorimetry (ITC). To measure the apparent enthalpy change, \( \Delta H_{\text{298K}} \), for the binding of digoxigenin or digoxin to shark Na,K-ATPase phosphorylated by MgP₈, multiple single injections of digoxigenin (300 \( \mu \)M, 2.4-\( \mu \)L injections) or digoxin (150 \( \mu \)M, 2.4-\( \mu \)L injections) into Na,K-ATPase (20 \( \mu \)M) in the presence of 3 mM MgP₈ were performed. As seen from Fig. 4, A and B (top panels), each injection was followed by a negative power signal (\( \mu \)cal/s) representing the power that needs to be applied to the sample cell to maintain isothermal conditions with respect to the reference cell. The negative sign indicates an exothermic binding reaction (i.e. a reduction in power is necessary). After each pulse, the system returned to the base line, indicating that no further CTS binding occurred. The integral under each power peak yields the enthalpy change of the reaction. As seen, the power peaks decreased and became broader with each titration as the concentration of free receptor sites decreased, until they became constant and narrow again. The latter constant peak intensities,
which are considered to represent nonspecific CTS binding to the lipid phase and therefore are highest for the more hydrophobic digoxigenin, were subtracted from the former peak intensities, representing high affinity binding of CTS, to give the concentration ratio (CTS/Na,K-ATPase) dependence of the binding heat changes depicted in the bottom panels of Fig. 4, A and B. Fitting these data using a single-site model yielded apparent enthalpy changes of 

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\Delta H = 45.8 \pm 0.6 \text{ kJ mol}^{-1} (10.96 \text{ kcal mol}^{-1}) \text{ for binding of digoxin and } -27.9 \pm 0.8 \text{ kJ mol}^{-1} (-6.69 \text{ kcal mol}^{-1}) \text{ for binding of digoxigenin. As noted from Fig. 4, A and B,} \\
\text{saturation of the CTS binding sites occurred at a molar ratio of less than 1 for digoxin (} n = 0.60 \pm 0.01 \text{) and close to 1 for digoxigenin (} n = 0.93 \pm 0.03 \text{). The binding constants (} K \text{) and entropy changes (} \Delta S \text{) could not be determined very accurately under these conditions. The binding constants amounted to } 5.3 \times 10^6 \pm 0.7 \times 10^6 \text{ M}^{-1} \text{ for digoxin and } 15.4 \times 10^6 \pm 0.7 \times 10^6 \text{ M}^{-1} \text{ for digoxigenin, giving inhibitor constants (} \text{Ki} \text{) of about } 189 \text{ nM for digoxin and } 65 \text{ nM for digoxigenin, in reasonable agreement with the inhibition measurements (Table 1). The entropy changes were approximately } -25.1 \text{ J K}^{-1} \text{ mol}^{-1} (-6.0 \text{ kcal K}^{-1} \text{ mol}^{-1}) \text{ for digoxin and } 43.5 \text{ J K}^{-1} \text{ mol}^{-1} (10.4 \text{ kcal K}^{-1} \text{ mol}^{-1}) \text{ for digoxigenin. A positive } \Delta S \text{ is a strong indication that water molecules are expelled from the complex interface, whereas ordering of water at the complex interface contributes unfavorably to } \Delta S. \text{ The data were not sufficient yet, however, to conclude whether the difference in } \Delta S \text{ for digoxin...}
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![Figure 4](https://example.com/fig4.png)

**Figure 4. ITC of digoxin and digoxigenin binding to phosphorylated Na,K-ATPase.** Shown is a series of 2.4-µl sequential single injections of digoxin (150 µM) (A) and digoxigenin (300 µM) (B) into shark Na,K-ATPase (20 µl). The experiments were conducted at 25 °C in a medium containing 10 mM imidazole-HCl (pH 7.0), 3 mM MgCl₂, 3 mM Pi-Tris, and 25% glycerol. The top panels show the power (µcal/s⁻¹) that needs to be applied to the sample cell to maintain isothermal conditions relative to the reference cell. Integrations of the individual peaks yield the apparent enthalpy change of the binding reaction, which is depicted in the bottom panels as a function of the CTS/Na,K-ATPase molar ratio. Fitting of the data (curves in the bottom panels) with a one-site binding model gave the following parameters. A, digoxin: ΔH₂₉₈ = -45.8 ± 0.6 kJ/mol⁻¹, K = 5.3·10⁸ ± 0.7·10⁸ M⁻¹, ΔS₂₉₈ = -25.4 ± 0.6 kJ·mol⁻¹, and n = 0.60 ± 0.01. B, digoxigenin, ΔH₂₉₈ = -28.0 ± 0.7 kJ/mol⁻¹, K = 15.4·10⁶ ± 0.7·10⁶ M⁻¹, ΔS₂₉₈ = 44 ± 0.9 kJ·mol⁻¹, and n = 0.93 ± 0.03.

and digoxigenin was significant. A previous investigation using ITC to determine the heat change of binding of ouabain to shark Na,K-ATPase under very similar conditions found an apparent enthalpy change of −85 ± 5 kJ/mol⁻¹ and a stoichiometric coefficient of 0.62 ± 0.05 (30), in reasonable agreement with the data for digoxin binding in the present investigation.

**Reactivation following Inhibition by CTS—** We have previously demonstrated that CTS inhibition of Na,K-ATPase is partially relieved by the addition of Na⁺ from the extracellular side and that the effect of the sugar moiety on the reactivation is quite dramatic, as demonstrated by comparing reactivation of enzyme inhibited by either ouabain or ouabagenin (8). Thus, the reactivation of enzyme inhibited by ouabain or ouabagenin in the E₂-P ground state stabilized by BeFx is significant only in the ouabagenin-inhibited enzyme (i.e. in the absence of a sugar moiety) (see Fig. 10C in Ref. 8). Here we demonstrate that this is a general finding by comparing reactivation of inhibited Na,K-ATPase by high Na⁺ for a range of glycosylated and non-glycosylated CTSs bound to either the E₂P formed by phosphorylation of the enzyme with MgP₂, which produces a mixture of E₂P phosphoforms (here termed E₂MgP₂), or to the E₂-P ground state analog stabilized by BeFx. The enzyme was first reacted with MgP₂ or BeFx for 10 min, and then the CTS was added, and incubation continued for a further 50 min at 23 °C. At time 0, 150 mM NaCl was added, and the activity was measured at optimal conditions (i.e. at 130 mM Na⁺, 20 mM K⁺, 4 mM Mg²⁺, 3 mM ATP, and 30 mM imidazole, pH 7.5) with time. Dilution of enzyme with bound aglycones may cause instability of the complex and dissociation of the aglycone (31); therefore, the final CTS concentration after dilution with NaCl was kept high enough (>10 times K, at all pH values) to ensure maximum inhibition. Under these conditions, measurements in the presence or absence of aglycone in the test solution at a concentration identical to that in the preincubation solution were identical. Fig. 5A shows reactivation of enzyme activity after inhibition by glycosides or aglycones to the MgP₂-reacted enzyme. In Fig. 5B, the same is shown for reactivation of enzyme activity after CTS binding to the BeFx-activated enzyme. The same general pattern was observed in the two cases; whereas the reactivation of enzyme activity after cardiac glycoside binding by 150 mM NaCl was almost absent, there was a considerable, but slow reactivation for all five of the different aglycones used. The degree of reactivation obtained after binding of the various cardiac aglycones to the E₂-P ground state stabilized by BeFx, at concentrations of ∼100 × K, was between 30 and 100% of the fully active enzyme in the following ranking: digoxigenin (100%) > ouabagenin (66%) > digitoxigenin = gitoxigenin (52%) > bufalin (30%). In the case of E₂MgP₂, the reactivation rate of gitoxigenin-bound enzyme was considerably faster than those for the other CTSs in the order (Fig. 5A): gitoxigenin >> digitoxigenin ≥ ouabagenin ≥ digoxigenin >> bufalin. In the case of E₂-P ground state stabilized by BeFx (Fig. 5B), the rates were more alike, but again the fastest reactivation was obtained with the gitoxigenin-reacted enzyme. For both E₂MgP₂ and E₂BeFx, the reactivation was faster at lower pH, and, for both conformations, the reactivation rate constant was...
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The lowest for bufalin and the highest for gitoxigenin (not shown). It was also noted that the rates of reactivation following cardiac aglycone inhibition were generally higher (about 30–40%) for CTS-bound enzyme in the E2MgP₁ conformation than for CTS-bound enzyme in the E2BeFₓ conformation. Fig. 6 further demonstrates the difference in the levels of reactivation obtained following inhibition by either digitoxigenin or digitoxin, which have similar Kᵢ values (Table 1), and whether the inhibitor is bound to enzyme phosphorylated by MgP₁ or to the E2-P ground state stabilized by BeFₓ. As seen in Fig. 6, the reactivation level was significantly higher for the cardiac aglycone digitoxigenin than for the cardiac glycoside digitoxin. Thus, reactivation was absent above 10 μM digitoxin and was half-maximum at about 4 μM, whereas about 50% reactivation was present at 10 μM digitoxigenin. Reactivation of digitoxitin-bound enzyme was similar for MgP₁ or BeFₓ-reacted enzyme, whereas a notable difference in reactivation was observed in the case of digitoxigenin, where the reactivation of MgP₁-reacted enzyme was significantly larger than that of BeFₓ-reacted enzyme. This indicates that the complex of cardiac aglycones bound to other E2P conformations present in the MgP₁-reacted enzyme (i.e. E2-P product state and E2–P transition state) are more unstable than that of digitoxigenin bound to the E2-P 2 ground state stabilized by BeFₓ. This difference in stability of the inhibitor–enzyme complexes was absent in the presence of a sugar moiety.

In the following, it was tested whether the number of sugar residues on the steroid moiety of the aglycone was important for the reactivation of enzyme activity. Fig. 7 compares the reactivation of enzyme activity after binding of digitoxigenin or its glycosides with one to four digitoxose residues to either E2MgP₁ or E2BeFₓ. As seen in Fig. 7, a single sugar residue was enough to seriously decrease reactivation of enzyme activity. Indeed, the reactivation of enzyme activity of digoxigenin monodigitoxide-bound enzyme was only 10% complete after 90 min, whereas digoxigenin-bound enzyme was fully reactivated after 60 min. Furthermore, the rate of the reactivation was found to be inversely proportional to the number of sugar residues on digoxigenin, as shown in Fig. 8, and saturated at about 5·10⁻⁶ s⁻¹ for digoxin. The rate of reactivation for digoxigenin tetradigitoxide was too low to be measured accurately. Thus, increasing the number of digitoxose residues on digoxigenin increased the stability of the CTS complex with the enzyme, as also previously demonstrated by Yoda et al. (32).

Steroid Core Hydroxyl Groups and pH Sensitivity—In the following, the effects of –OH groups at different positions on the steroid core of a range of CTS compounds, as detailed in Fig. 1 (table), were compared at different pH values. Digitoxigenin and bufalin contain only the universally present –OH groups at C3 and C14, whereas digoxigenin and gitoxigenin have an additional –OH group at C12 and C16, respectively. Ouabagenin has four additional –OH groups at positions C1, C5, C11, and C19. All of these hydroxyl groups, except that at C11, are exposed to the β-side of the steroid. When the inhibitory potency of the aglycones tested in this investigation was compared, the following ranking of Kᵢ values was obtained: bufalin < digitoxigenin < strophanthinid < strophanthidol < digoxigenin < gitoxigenin < ouabagenin. This order was identical at high pH (Fig. 9A). This indicates that steroid –OH groups are sterically unfavorable for the inhibitory potency of cardiac aglycones. The difference between bufalin and digitoxigenin indicates a slightly larger inhibitory effect when the lactone ring is six-membered (16).

We have previously demonstrated that the very drastic effect of high pH (pH 8.5) on inhibitory potency of ouabain and ouabagenin could not be explained by the shift in the poise of the E1P/E2P equilibrium toward E1P and hypothesized that it was related to the presence of hydroxyl groups on the β-side of the steroid core (16). As can be seen from Fig. 5B, also digoxigenin and its glycosides with 1–4 sugar residues had this characteristic pH-induced shift in inhibitor potency, probably related in this case to the presence of an –OH group at C12. In Fig. 9B, we compared the shift of apparent inhibitory potency caused by increasing pH from 7.5 to 8.5 for cardiac glycosides and their aglycones with –OH groups at various positions on the steroid core of a range of CTS compounds, as detailed in Fig. 1 (table), were compared at different pH values. Digitoxigenin and bufalin contain only the universally present –OH groups at C3 and C14, whereas digoxigenin and gitoxigenin have an additional –OH group at C12 and C16, respectively. Ouabagenin has four additional –OH groups at positions C1, C5, C11, and C19. All of these hydroxyl groups, except that at C11, are exposed to the β-side of the steroid. When the inhibitory potency of the aglycones tested in this investigation was compared, the following ranking of Kᵢ values was obtained: bufalin < digitoxigenin < strophanthinid < strophanthidol < digoxigenin < gitoxigenin < ouabagenin. This order was identical at high pH (Fig. 9A). This indicates that steroid –OH groups are sterically unfavorable for the inhibitory potency of cardiac aglycones. The difference between bufalin and digitoxigenin indicates a slightly larger inhibitory effect when the lactone ring is six-membered (16).

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the steroid core. It is clear that the pH sensitivity, measured as the ratio of $K_r$ values measured at pH 8.5 and 7.5, was independent of the presence of a sugar moiety, except for the gitoxin/gitoxigenin pair. However, as seen by comparing the $K_r$ values for ouabain and ouabagenin in Fig. 10, A and B, and Table 1, the presence of a sugar moiety was critical for the absolute value of $K_r$. Comparison of evomonomide and ouabain, both with an α-L-rhamnose residue but different steroid hydroxyls, showed very different $K_r$ sensitivity to high pH (Fig. 10A), confirming the role of steroid hydroxyls as modulators of pH sensitivity. Interestingly, if the Mg$^{2+}$ concentration was increased from 5 to 10 mM during the 1-h incubation of Na,K-ATPase with CTS, the $K_r$ value was significantly increased, especially at high pH values, both for the ouabain, ouabagenin, and dihydro-ouabain (Fig. 10, A and C, filled symbols). The pH sensitivity of dihydro-ouabagenin with a C20-C22-saturated lactone ring was higher than for ouabain, as also previously demonstrated (16), and still affected by increasing Mg$^{2+}$ (Fig. 10A). The Mg$^{2+}$ effect was low affinity and did not saturate within the concentration range investigated (Fig. 10B). Because a similar increase in the $K_r$ for ouabagenin was obtained by including 10 mM Na$^+$ or 0.5 mM K$^+$ in the CTS preincubation (Fig. 10C), this effect is caused by binding to the cation binding sites, which affects the interaction with the lactone ring, similar to the effect of binding K$^+$ ions that prevents closure of the CTS binding cavity, as described previously (9).

In Fig. 11, A and B, the time course of inhibition of digitoxigenin and ouabagenin (10$^{-3}$ M) is shown at pH 6.5, 7.5, and 8.5, using either 5 mM Mg$^{2+}$ (black symbols) or 10 mM Mg$^{2+}$ (red symbols) in the CTS incubation medium. The binding rate decreased by increasing pH from 6.5 to pH 7.5 by a factor of ~2 but decreased more significantly at pH 8.5 (by a factor of ~10), and the inhibition tended to become biphasic, with a slow fraction comprising ~75%. If the change in observed rate constants for inhibition measured at increasing pH from 6.5 to 8.5 for the various aglycones was compared (Fig. 11C), it was clear that ouabagenin ($\triangle$) was the compound with the lowest association rate. After ouabagenin followed digoxigenin ($\bigcirc$), strophanthidol ($\bigotimes$), gamabufotalin ($\bigodot$), and gitoxigenin ($\bigtriangledown$), with –OH substitutions at C12, C5/C19, C11, and C16, respectively. Digitoxigenin ($\square$) and bufalin ($\diamond$) with no additional –OH groups, except for the one universally present at C14β showed the fastest rate of inhibition. Even at pH 8.5, where the rate constants were more alike, this relation holds. The variations in the rates of inhibition versus pH were rather small for ouabagenin and digitoxigenin in accordance with Yoda and Yoda (31), who previously found that the binding constant for digitoxigenin (and ouabain) changes only slightly between pH 6.5 and 8.5. Increasing the Mg$^{2+}$ concentration during CTS preincubation from 5 to 10 mM decreased the rate of inhibition ($k_{obs}$) for all aglycones except ouabagenin, where Mg$^{2+}$ had a slightly stimulating effect (Fig. 11C), which is also the case for ouabain (not shown). The fact that increasing Mg$^{2+}$ affected most the CTSs with the fewest steroid –OH substitutions indicates that the Mg$^{2+}$ effects are not related to the steroid hydroxyls but more likely to interactions with the lactone ring. Also, the effects of steroid...
The hydroxyls on the CTS binding rates may be indirect through their effects on the lactone position.

**Modeling of the Complex of Na,K-ATPase and Digoxin or Gitoxin**—Because the high affinity model of the Na,K-ATPase/ouabain complex (PDB entry 3N23) shows steric clashes around ouabain and is derived from low (4.6 Å) resolution data, the model was first optimized by energy minimization. In this model, in which ouabain was shifted by 0.3 Å but still nicely fitted to the omit-annealed electron density map (Fig. 13B), there was no steric clash around ouabain. In modeling of the complex of digoxin or gitoxin, CTSs were initially placed so that the steroid core matched that of ouabain. In the energetically optimized model, the steroid core of digoxin or gitoxin was shifted by 0.88 or 1.1 Å, respectively, toward M4 (Fig. 13C), and the hydroxyl at the C14 came at 2.5 Å distance from the Thr-797 hydroxyl, similarly to ouabain.

In CTS binding to Na,K-ATPase, the position of the carbonyl group on the lactone ring plays an important role (33). In the optimized model, the lactone carbonyl of ouabain forms hydrogen bonds with the backbone amides of Val-322 and Ala-323. In the models of digoxin and gitoxin, the lactone carbonyl was shifted away from M4 by 1.5 or 2.3 Å, respectively. As a result,
with digoxin, those hydrogen bonds appear to be somewhat reduced but more severely so with gitoxin, explaining the difference in affinity to Na,K-ATPase.

DISCUSSION

In the present study, the structural and functional features of CTS binding to Na,K-ATPase were investigated in relation to the crystal structure of Na,K-ATPase with bound ouabain (Fig 12). We investigated the functional effects of the sugar moiety as well as hydroxyl groups at various positions on the β-face of the steroid core of cardiotonic steroids (cf. Fig. 1) on the inhibitory potency and the inhibition rates of CTS using shark Na,K-ATPase. Furthermore, the rate of reactivation of shark Na,K-ATPase in E2P or E2-P ground conformation stabilized with MgP or BeF$_\text{v}$, respectively, following inhibition by the various cardiotonic steroids (comparing cardiac glycoside ouabain, digoxin, digitoxin, and gitoxin with their aglycones) was investigated.

Generally, the presence of a sugar moiety in CTS increases the inhibitory potency, but the effect varies considerably in relation to the number of –OH substitutions on the steroid core, being highest for the ouabagenin/ouabain pair, where the ratio of $K_i$ values is $\sim 25$ (Fig. 2). The effect of the sugar moiety in stabilizing the CTS binding is in accordance with the finding that variations in $K_i$ depend primarily on variations in the dissociation rate constant (5, 13). This effect has been ascribed mainly to the 3′-α-hydroxyls of the sugars, which have been postulated to interact with both proton-accepting and proton-donating residues on the enzyme (5). As seen from the structures in Fig. 12, possible candidate residues for such interac-

FIGURE 12. The Na,K-ATPase binding cavity for ouabain. Residues of the Na,K-ATPase α subunit important for coordination and ouabain are shown in sticks. In A, residues important for ouabain interaction in the low affinity ouabain-bound crystal structure are shown together with the two bound K$^+$ ions represented by purple spheres (PDB 3A3Y); in B, the original high affinity ouabain-bound structure is shown (PDB entry 3N23). The arrows in A indicate how M1/M2 moves in the transition to the high affinity state. Residues (shark Na,K-ATPase numbering in A, pig kidney Na,K-ATPase numbering in B) within hydrogen bond distance are connected by yellow dotted lines. The steric clash between the hydroxyl at C14 and Thr-797 (distance 2.2 Å) is circled in red. In A and B, the α-face of the ouabain steroid is stacked with aromatic residues (Ile-322, Phe-323, Phe-790, Phe-793). In C, digoxin (PDB entry 3B0W) is shown docked onto the shark Na,K-ATPase with the steroid core A- and B-rings matching that for ouabain found in the 3A3Y crystal structure. The Na,K-ATPase residues Glu-122, Glu-319, and Arg-887 that are within hydrogen bond distance from hydroxyls of α-, β-, and γ-sugar residues are indicated by dotted lines. In the structures, the β and FXYD subunits are omitted for clarity. The structures were drawn with PyMOL.
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tions could be Glu-319 and Arg-887 (shark enzyme numbering). Thus, coordination of the sugar moiety by Na,K-ATPase residues compensates for the sterically unfavorable steroid hydroxyls. The very large difference in $K_i$ ratio for ouabagenin/ouabain compared with the other cardiac aglycone/glycoside pairs could in principle also be caused by the presence of only a single sugar residue (l-rhamnose) in ouabain, whereas the other cardiac glycosides contain three sugar residues (d-digitoxose). However, such a hypothesis is clearly not in accord with the low $K_i$ value found for evomoside with a single l-rhamnose residue. In addition, the results shown in Fig. 3A, where the inhibitory potency is measured for digoxigenin derivatives with 0–4 digitoxose residues, contradict such an explanation because the ratio of $K_i$ values for digoxigenin/digoxigenin monodigitoxide is only $\sim$1.5. Also, the nature of the sugar residues seems less important, indicating a large degree in conformational adaptability at the sugar-binding site of the protein (34).

Our previous results demonstrated that the reactivation of the CTS-bound enzyme in E2BeFx state by high NaCl concentration was almost abolished by the presence of a rhamnose residue on ouabain (8), and we suggested that, in contrast to the cardiac glycosides, the dissociation reaction of the cardiac aglycones is fast enough to allow Na$^+$ ions to access the ion-binding sites open to the extracellular side. These observations have been substantiated and considerably extended in the present investigation. In general, it is found that glycosylation of CTS almost completely abolishes reactivation of CTS-bound enzyme either in the E2MgPi or in the E2BeFx state (Figs. 5 and 6). The sugar moiety is, therefore, important for closing and locking the ouabain binding cavity and the extracellular access channel through which Na$^+$ enters to the binding sites and causes CTS release (8). Therefore, the CTS off-rate is the pivotal parameter here. This is in accordance with the demonstration that the dissociation rate constants of ouabagenin and digoxigenin are 75 and 38 times larger than for ouabain and digoxigenin monodigitoxide (35). A single sugar residue is enough to induce this closing and locking of the binding cavity in the transition to the high affinity CTS-bound state (Fig. 7), although additional sugar residues further decrease the rates of reactivation (Fig. 8). Among the tested cardiac aglycones, reactivation of gitoxigenin-bound enzyme was the fastest, and bufalin-bound enzyme was the slowest (Fig. 5). The unfavorable $\beta$-OH group at C16 of gitoxigenin will probably accelerate its dissociation, which gives a faster reactivation, whereas the six-membered lactone of bufalin results in a low off-rate and a dissociation, which gives a faster reactivation, whereas the six-membered lactone of bufalin results in a low off-rate and a dissociation, which gives a faster reactivation, whereas the six-membered lactone of bufalin results in a low off-rate and a slower reactivation.

It is very likely that residues on the L1/2, L3/4, and L7/8 loops around the entrance to the CTS binding cavity make additional interactions with the $\beta$- and $\gamma$-sugars in digitoxin, gitoxin, and digoxin and thereby further slow down reactivation (Fig. 10). Moreover, the $\beta$- and $\gamma$-sugars in digitoxin and digoxin are important for isofrom selectivity of Na,K-ATPase inhibition, which seems to rely on their coordination with isofrom-specific amino acid residues at the entrance to the CTS binding cavity (27). Interestingly, two of these, Gln-126 and Glu-314 of human $\alpha_1$, corresponding to Ser and Gly in $\alpha_2$ and $\alpha_3$, are also different in the shark enzyme, where they correspond to Ala-126 and Gly-314. Comparative molecular similarity index analysis indicates interactions of the CTS binding site with all major components of digoxin (26). If a digoxin molecule with an approximate extended full length of 27 Å is docked onto shark Na,K-ATPase where ouabain (approximate full length of 16 Å) is located in the low affinity crystal structure (9) so that their steroid core matches, several charged amino acid residues of the L1/2, L3/4, and L7/8 loops that line the ouabain binding cavity, like Glu-122, Arg-887, Arg-889, Asp-892, and Arg-893, are within 28 Å from the lactone carbonyl (Fig. 12C), and many of these residues are known from mutagenesis studies to be important for CTS binding (36–39). Moreover, the sugar residues can rotate around the glycosyl bonds and generate several sites of interaction. Thus, it is easy to imagine that if the coordination between the sugar C4$^\beta$–OH and Glu-319 is absent, as in the case of ouabagenin, the hydrogen bond network between these residues and the $\beta$-OH groups on the steroid $\beta$-face could be disturbed (Fig. 12B), explaining the very low affinity of ouabagenin. Price et al. (40) have previously suggested that electrostatic interaction of the L1/2 loop of such charged border amino acids with other charged residues may prevent the conformational change associated with ouabain binding. The significant increase in the negative apparent enthalpy change from $\sim$28 kJ mol$^{-1}$ to $\sim$46 kJ mol$^{-1}$ in the ITC measurements given in Fig. 4 upon the addition of three $\beta$-digitoxose sugars to digoxigenin is consistent with an increase in interactions between the ligand and the receptor site, such as formation of hydrogen bonds between the sugar hydroxyls and Na,K-ATPase residues (Fig. 12C).

OH groups on the steroid core in excess of the one at C14 are sterically unfavorable for rapid CTS binding to Na,K-ATPase (Fig. 11B), as suggested previously (16). Also, Yoda et al. (41) have previously shown that the association rate constant depends primarily on the steroid moiety and not on the sugar moiety. This is understandable from the crystal structures where the $\beta$-face of ouabain shows little interaction with protein residues in the low affinity structure, except for the hydrogen bond between Thr-804 and the C14$^\beta$–OH group between the C14$^\beta$–OH group between the steroid C and D rings (9). However, in the transition to the high affinity state, a structural rearrangement must take place in which M1/M2 swings in and forms a complementary surface to the CTS $\beta$-side stabilized by several hydrogen bonds to steroid hydroxyls, as shown in Fig. 12B. Thus, the two essential residues Gln-118 and Asn-129, the mutations of which confer low ouabain affinity in rodent (40, 42), come within hydrogen bond distance to hydroxy groups at C1, C14, and C19 (Table 2), emphasizing the importance of the ouabain-specific $\beta$-OH groups at C1 and C19. Asp-128, which has previously been shown to be important for ouabain binding (43), is also within hydrogen bond distance of C14$^\beta$–OH. The Phe-790, which has been demonstrated by mutagenesis to be very important for ouabain binding because substitution by a Leu reduces ouabain affinity by a factor of 60 (44), seems to be flipped and clashes with the steroid ring D of ouabain, pushing it toward M1/M2 (Fig. 13A). Actually, ouabain in the high affinity complex structure at 4.6 Å resolution, which is shifted by about 2 Å toward M1/M2 from that in the low affinity complex structure at 2.8 Å resolution, is in apparent steric clash. This is because Thr-797 (pig kidney numbering), a critical residue for ouabain binding.
sensitivity (36, 45), comes too close to the C14–OH in the model (only 2.2 Å distance; Table 2, PDB code 3N23). Indeed, Phe-790/Thr-804 (Phe-783/Thr-797 in pig kidney) form a bottleneck restricting a deeper access of ouabain. Therefore, we first energy-minimized the high affinity model, positioning ouabain slightly differently (Fig. 13B), and then used it for docking of gitoxin (see below).

Comparison of the $K_i$ values for the various cardiac aglycone compounds investigated indicates that the hydroxyls at C5β (strophantidin) and at C11α (gambubofotalin) are of minor importance for CTS binding to Na,K-ATPase. In fact, neither of the hydroxyls at C5β or at C11α forms hydrogen bonds in the high affinity state (Fig. 12B). Furthermore, gambubofotalin has the same affinity as bufalin (Table 1), and its growth-inhibitory

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**TABLE 2**

Distance between steroid hydroxyls and nearest residues in the high affinity ouabain-bound pig kidney Na,K-ATPase model (3N23) and in the docking models for digoxin and gitoxin. Numbering refers to pig kidney enzyme with shark numbers in parenthesis.

| –OH position | Residues | 3N23 model | Digoxin and gitoxin models$^a$ |
|--------------|----------|------------|-------------------------------|
| C1β          | Gln-111 (Gln-118) | 3.7 | 4.3 |
| C5β          | Gln-111 (Gln-118) | 5.3 | 4.3 |
| C11α         | Ile-315, Asn-122, Gln-111 (Ile-322, Asn-129, Gln-118) | 4.2, 4.5, 5.4 | 3.9, 4.5, 2.5 |
| C12β         | Ile-315 (Ile-322) | 4.3 | 3.8, 4.5, 4.4 |
| C14β         | Asp-121, Asn-122, Thr-797 (Asp-128, Asn-129, Thr-804) | 2.7, 4.1, 2.2(!) | 3.9, 4.5, 2.5 |
| C16β         | Phe-783, Leu-795, Thr-797 (Phe-790, Leu-802, Thr-804) | 4.2, 4.5, 2.2(!) | 3.9, 4.5, 2.5 |
| C19β         | Gln-111, Asn-122 (Gln-118, Asn-129) | 3.3, 4.1 | 3.3, 4.1 |

$^a$ Estimated from docking of digoxin and gitoxin structures onto the repositioned high affinity ouabain-bound state. For details, see Fig. 13C. The steric clash is indicated (!).
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activity is even higher than that of the unsubstituted bufalin (46). Substitutions at C12β (digoxigenin) and at C19β (strophanthidol) are more important in decreasing the inhibitory potency, especially at high pH (Table 1 and Fig. 9). The closest Na,K-ATPase residue in the shark to the C12β hydroxyl is Ile-322, which is 3.9 Å away (Fig. 12C) (4.3 Å in pig kidney; Fig. 13C).

The effects of increasing pH on CTS inhibition of Na,K-ATPase activity and rate of inhibition are very complex and include effects on the levels of the lactone ring, the steroid hydroxyls, and the sugar moiety, making it extremely difficult to pinpoint the primary site of action. The inhibitory potency of CTS generally decreases at increasing pH. Comparison of the $K_i$ ratios for the aglycones and glycosides at pH 7.5 and 8.5 (Fig. 9B) shows that the increase of the $K_i$ ratio caused by changing pH 7.5 to 8.5 is about 14 for ouabain and ouabagenin, about 8 for digoxin and digoxigenin, and only about 2 for digitoxin, digitoxigenin, bufalin, and evomoside. Although the relative pH effect is identical for the aglycone and the glycoside (with a notable exception for gitoxigenin, which has a large pH-dependent increase in the $K_i$ value of about 15, whereas the glycoside gitoxin has a much lower pH-dependent shift in $K_i$ by a factor of only ~3) the absolute value of $K_i$ is heavily dependent on the presence of a sugar moiety, as discussed above. The large difference between the relative pH sensitivity of gitoxigenin and digoxigenin is surprising, given their close structural similarity (47), but may result from change in the orientation of the 17β-lactone ring caused by the C16β–OH substitution (33).

In the gitoxin-docked model of Na,K-ATPase, the same two residues (Phe-783/Thr-797) that formed a bottleneck in ouabain binding also influence the gitoxin binding. For a CTS to bind to Na,K-ATPase with high affinity, it has to fit in the narrow space delimited by Phe-783 and Thr-797 and form hydrogen bonds with Val-322 and Ala-323 in M4. This in turn requires that the CTS changes the conformation of the steroid core and the orientation of the lactone. These structural changes are observed in the high affinity model of the ouabain-Na,K-ATPase complex and to a slightly lesser extent in the energy-minimized model of the digoxin-Na,K-ATPase complex (Fig. 13C). In contrast, these changes are not observed in the model of the gitoxin-Na,K-ATPase, suggesting that the hydroxyl at C16β prevents such conformational changes in gitoxin. This observation will explain the lower inhibitory potency of gitoxin compared with that of digoxin and also the much lower inhibitory potency of gitoxigenin. This idea is supported by previous findings that the C16β–OH substitution decreases the inhibitory potency of digitoxigenin and bufadienolides (27, 33, 45, 48), which is also found in the present investigation (Table 1). Previous studies using different C16 substitutions indicated that they have important structural implications, including the important change in the 17β-lactone carbonyl oxygen position (49–53).

Except for the gitoxigenin/gitoxin pair, the relative pH sensitivity of $K_i$ is not very dependent on the presence of a sugar moiety but seems to rely on the presence of hydroxyls at various positions on the steroid core, as previously hypothesized (16). Thus, digitoxin, digitoxigenin, bufalin, and evomoside, which have the lowest pH dependence, also have only the –OH group universally present at C14, whereas the other CTS compounds have one or more additional –OH groups present on the steroid core (cf. Fig. 1). If the relative sensitivity of cardiac aglycones to high pH is ranked according to the positions of the steroid hydroxyl groups (Fig. 9A and Table 1), the following order is observed: C1, C5, C11, C19 (ouabagenin) > C16 (gitoxigenin) > C12 (digoxigenin) > C5 (strophanthidin) > C11 (gambabufotalin) = none (bufalin, digitoxigenin). Thus, the most pH-sensitive –OH position seems to be at C1β, or the high pH sensitivity is a combined effect of several hydroxyls at the steroid core. Apparently, ouabain and ouabagenin are the only CTSs with a C1β–OH substitution.

The effect of high pH on the CTS inhibitory potency could be indirect through changes in the protonation state either of Na,K-ATPase residues important for hydrogen bonding of steroid hydroxyls, like the C1β-, C14β-, and C19β-hydroxyls of ouabain/ouabagenin (Fig. 12, A and B), or of carboxyls in the cation binding site. Several Asp and Glu residues near the cation binding sites of shark Na,K-ATPase (e.g. Glu-334, Glu-786, Asp-815, and Asp-933) have unusually high pKₐ values, indicating that they could be protonated (54). The observation that the decreasing inhibitory potency induced by increasing pH is further decreased by increasing the Mg²⁺ concentration during CTS incubation for ouabain, ouabagenin, and dihydro-ouabain (Fig. 10) and that this pH effect is also observed by including small concentrations of Na⁺ or K⁺ seems to indicate that the cation binding sites are involved. Mg²⁺ binding to or near the cation binding sites has previously been suggested (55) and could change the lactone–protein interactions, as earlier described for the binding of K⁺ ions in the low affinity ouabain-bound Na,K-ATPase structure (9). This would lead to repositioning of CTS in the binding cavity, which is most challenging for CTS with multiple steroid hydroxyls. It has previously been suggested that in CTS binding to Na,K-ATPase, the lactone ring, apart from being hydrogen-bonded, is also attached via electrostatic interactions (56). The effects of Mg²⁺ could therefore be due to an electrostatic screening effect. However, Mg²⁺ also increased $K_i$ for dihydro-ouabain (Fig. 10A), although ionic interactions are not possible for dihydro-ouabain with a C20-C22-saturated lactone.

In conclusion, the rate of reactivation of CTS-bound Na,K-ATPase, which is proportional to the rate of CTS dissociation, is strongly dependent on the presence of sugar, whereas steroid core hydroxyls seem less important. The rate of inhibition, which is proportional to the rate of CTS binding, is strongly dependent on the presence of –OH groups on the steroid core. $K_i$ is therefore a complex function of both the presence of sugars and steroid –OH groups. Increasing pH decreased inhibitory potency, depending both on the number of sugar residues and on the number of –OH groups on the β-side of the steroid core. This effect is enhanced by increasing the Mg²⁺ concentrations or by adding small concentrations of Na⁺ during CTS binding, indicating that they affect the steroid lactone–protein interactions near the cation binding sites.

Acknowledgments—We thank Anne Mette Beck Sørensen and Bianca Franchi for excellent technical assistance and Ayami Hirata for performing the ITC experiments.
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