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Selectivity of Digitalis Glycosides for Isoforms of Human Na,K-ATPase*

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There are four isoforms of the α subunit (α1–4) and three isoforms of the β subunit (β1–3) of Na,K-ATPase, with distinct tissue-specific distribution and physiological functions. α2 is thought to play a key role in cardiac and smooth muscle contraction and be an important target of cardiac glycosides. An α2-selective cardiac glycoside could provide important insights into physiological and pharmacological properties of α2. The isoform selectivity of a large number of cardiac glycosides has been assessed utilizing $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ isoforms of human Na,K-ATPase expressed in *Pichia pastoris* and the purified detergent-soluble isoform proteins. Binding affinities of the digitalis glycosides, digoxin, β-methyl digoxin, and digitoxin show moderate but highly significant selectivity (up to 4-fold) for $\alpha2/\alpha3$ over $\alpha1$ (K_D $\alpha1 > \alpha2 = \alpha3$). By contrast, ouabain shows moderate selectivity (≈2.5-fold) for $\alpha1$ over $\alpha2$ (K_D $\alpha1 \leq \alpha3 < \alpha2$). Binding affinities for the three isoforms of digoxigenin, digitoxigenin, and all other aglycones tested are indistinguishable (K_D $\alpha1 = \alpha3 = \alpha2$), showing that the sugar determines isoform selectivity. Selectivity patterns for inhibition of Na,K-ATPase activity of the purified isoform proteins are consistent with binding selectivities, modified somewhat by different affinities of K^+ ions for antagonizing cardiac glycoside binding on the three isoforms. The mechanistic insight on the role of the sugars is strongly supported by a recent structure of Na,K-ATPase with bound ouabain, which implies that aglycones of cardiac glycosides cannot discriminate between isoforms. In conclusion, several digitalis glycosides, but not ouabain, are moderately α2-selective. This supports a major role of α2 in cardiac contraction and cardiotonic effects of digitalis glycosides.

For more than 200 years congestive heart failure has been treated with the plant-derived digitalis cardiac glycosides such as digoxin, which increase force of contraction of failing cardiac muscle and reduce cardiac conduction rate. However, digoxin is now less used than in the past due to the narrow therapeutic window and drug toxicity. Cardiac glycosides are produced in mammals as well as plants. As reviewed recently (1) five different cardiac glycosides have been identified in mammalian tissues including the cardenolides ouabain and digoxin and the bufadienolides marinobufagenin, telocinobufagin, and 19-norbufalin. Cardiac glycosides inhibit Na,K-ATPase and control cellular Na^+ and K^+ gradients and also activate cellular signaling pathways that mediate control of gene expression and tissue growth (2, 3). Thus, a multiplicity of physiological functions could be affected by the multiplicity of endogenous cardiac glycosides, but this is not well understood (1, 4, 5).

The Na,K-ATPase consists of both α and β subunits, including four isoforms of α (α1, α2, α3, α4) and three isoforms of β (β1, β2, β3) (6) and FXYD (1–7) proteins, which are accessory subunits (7). α1 is almost ubiquitously distributed, whereas other isoforms are expressed in a tissue- and development-specific fashion. α2 is found mainly in muscle (skeletal, smooth, and cardiac), α3 is found primarily in nervous tissue, and α4 is found only in testicles. Human heart expresses α1, α2, and α3 isoforms and β1 (8). In cardiac myocytes α2 is concentrated in the T-tubules adjacent to the sarcoplasmic reticulum (SR),[2] whereas α1 is more evenly distributed in T-tubules and SR (9). In smooth muscle and brain astrocytes, α1 is uniformly distributed, but α2 and α3 show a punctuated distribution, overlying the SR (10).

Increased force of contraction of cardiac muscle induced by cardiac glycosides is the result of inhibition of Na,K-ATPase. Raised intracellular Na^+ concentration limits Ca^{2+} extrusion via the $3Na^+/Ca^{2+}$ exchanger, leading to enhanced Ca^{2+} uptake into the SR by the Ca-ATPase and increased calcium-induced calcium release during excitation-contraction coupling. Cardiac glycoside-induced toxicity is associated with excessive inhibition of Na,K-ATPase, accumulation of Ca^{2+} ions (*i.e.*"calcium overload"), and "spontaneous" SR Ca^{2+} release that can trigger delayed after-depolarizations and cardiac arrhythmias (11). Experiments with mice engineered to have ouabain-sensitive α1 and insensitive α2 isoforms ($\alpha1^{S/S}\alpha2^{R/R}$) or ouabain-insensitive α1 and α2 ($\alpha1^{R/R}\alpha2^{R/R}$) instead of the wild type with ouabain-insensitive α1 and ouabain-sensitive α2 ($\alpha1^{R/R}\alpha2^{S/S}$) have shown that α2 can play a predominant role in cardiac glycoside-induced positive inotropy (12) and also that α2 carries a large fraction of the Na,K-pump current in T-tubules of cardiac myocytes (13). Very recent work shows that α2 preferentially modulates Ca^{2+} transients and SR Ca^{2+} release in cardiac myocytes compared with α1 (14). Similarly, α2 plays an important role in contractility of vascular

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[2] The abbreviations used are: SR, sarcoplasmic reticulum; $C_{12}E_8$, octaethylene glycerol mondodecyl ether; SOPS, 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phosphor-L-serine]; CG, cardiac glycoside; BP, blood pressure; MOPS, 4-morpholinepropanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine.

smooth muscle and control of blood pressure (15, 16). Experiments with $\alpha1^{S/S}\alpha2^{R/R}$, $\alpha1^{R/R}\alpha2^{S/S}$, and $\alpha1^{R/R}\alpha2^{R/R}$ mice also provide strong evidence for endogenous mammalian cardiac glycosides by showing differential responses to physiological stimuli and states that alter blood pressure (12, 17). In view of the accumulated information on $\alpha1$ and $\alpha2$ isoforms, one could envisage that $\alpha2$-selective cardiac glycosides could provide important insights into the physiological role of $\alpha2$ and, in particular, complement the information obtained from genetically engineered mice. At the pharmacological level an $\alpha2$-selective cardiac glycoside could induce cardiac contraction with minimal Ca^{2+} overload and so act as a safer cardiotonic agent than conventional cardiac glycosides.

Structure-activity analyses of inhibition of Na,K-ATPase by cardiac glycosides or displacement of bound [^{3}H]ouabain have been conducted for many years; see for example Refs. 18–22. As summarized in Ref. 23, essential structural features of active cardiac glycosides include rings A/B and C/D that are cis-fused and B/C rings that are trans-fused, a hydroxyl group at C14, and an unsaturated lactone attached at C17 of the steroid. The sugar at C3 of the steroid is not essential for inhibition but strongly affects binding affinity and rates; see Table 1B and Ref 23. Despite the great potential interest in isoform selectivity of cardiac glycosides, there is little clear-cut information. Variations in potency of digitoxigenin monoglycosides as inhibitors of kidney ($\alpha1\beta1$) and brain ($\alpha1$, $\alpha2$, $\alpha3$) Na,K-ATPases were attributed to isoform selectivity, and a role of the sugar was proposed (24). However, most native tissues except kidney ($\alpha1\beta1$) contain mixtures of isoforms, and it is difficult to differentiate interactions of cardiac glycosides with the individual isoforms and exclude possible complicating factors. Because, for example, human cardiac membranes express $\alpha1$, $\alpha2$, and $\alpha3$ isoforms (8), healthy cardiac tissue would not be useful for this purpose even if it was readily available. Individual human isoforms have been expressed in *Xenopus* oocytes ($\alpha1$–3, $\beta1$–3) (25) and *Saccharomyces cerevisiae* ($\alpha1$–3, $\beta1$) (26, 27) at low levels and used to characterize [^{3}H]ouabain binding. More recently we have expressed human isoforms ($\alpha1$, $\alpha2$, and now also $\alpha3$, with $\beta1$) at high levels in *Pichia pastoris* and purified the $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ protein complexes (28–31). This system permits accurate analysis of cardiac glycoside binding and inhibition of Na,K-ATPase activity of the individual human isoforms, leading to the finding that several digitalis glycosides show moderate but highly significant selectivity for $\alpha2$ (and $\alpha3$) compared with $\alpha1$.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli XL-1 blue strain was used for propagation and preparation of plasmid constructs. Yeast lytic enzyme from ICN Biomedicals Inc (catalog no. 152270) was used for transformation of *P. pastoris* protease-deficient strain SMD1165 (his4, prb1). *n*-Dodecyl-β-D-maltopyranoside (catalog no. D310) and $C_{12}E_8$ (25% w/w, catalog no. O330) were purchased from Anatrace. Synthetic SOPS (sodium salt)) was obtained from Avanti Polar Lipids and stored as a chloroform solution. BD Talon metal affinity resin (catalog no. 635503) was obtained from Clontech. Cholesterol and ouabain (O3125) were obtained from Sigma. [^{3}H]Ouabain and [^{3}H]digoxin were obtained from PerkinElmer Life Sciences. All other materials were of analytical grade.

Yeast Transformation; Expression of Human Isoforms α1β1, α2β1, and α3β1—The pHIL-D2 (α/His$_{10}$-β_1) vectors contained cDNAs encoding human $\alpha1$, $\alpha2$, and $\alpha3$ (Swiss-Prot accession numbers: $\alpha1$, P05023; $\alpha2$, P050993; $\alpha3$, p13637) and human His$_{10}$-β_1 (accession number P05026) were constructed by replacing the porcine $\alpha1$ and His$_{10}$-$\beta1$ described in Refs. 28, 30 with the appropriate human $\alpha1$, $\alpha2$, and $\alpha3$ and $\beta1$ cDNA. Linear DNA, obtained by digestion of the pHIL-D2 with NotI, was used to transform spheroplasts of *P. pastoris* SMD1165, and His$^+$Mut^s transformants were selected (31). Mut^s clones of the $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ isoforms were grown in small-scale cultures (5 ml). Protein expression was induced with 0.5% methanol for 5 days, and membrane preparations were then screened for expression by Western blotting using anti-KETYY. Large scale growth of the optimally expressing clones was done in Bellco Spinner Flasks^{TM} in 10-liter volumes of growth medium (28–31). Expression of the Na,K-ATPase was induced by adding 0.5% methanol daily for 5 days at 25 °C for a1β1 and a3β1 and at 20 °C for a2β1 clones (30). Cells were collected, washed, and broken with glass beads, and membranes were prepared as described (31). Membranes were stored at −80 °C in a solution containing 10 mM MOPS-Tris, pH 7.4, and 25% glycerol with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, chemostatin, and leupeptin). About 2 g of membrane protein was obtained per 100 g of cells.

Solubilization and Purification of Isoforms of Na,K-ATPase—Procedures for solubilization of membranes in *n*-dodecyl-β-D-maltopyranoside, binding to BD-Talon beads, and washing and elution of purified Na,K-ATPase have been described in detail (28–30). Purification of $\alpha3\beta1$, which has not been described previously, follows exactly the same procedure. The three purified isoform complexes (0.3–0.5 mg/ml) were eluted from the BD-Talon beads in a solution containing 170 mM imidazole, 100 mM NaCl, 20 mM Tricine·HCl, pH 7.4, 0.1 mg/ml $C_{12}E_8$, 0.05 mg/ml SOPS, 0.01 mg/ml cholesterol, 25% glycerol. The proteins were stored at −80 °C. Protein concentration was determined with BCA (B9643 Sigma).

Cardiac Glycoside Binding to P. pastoris Membranes—[^{3}H]Ouabain binding to yeast membranes (200–300 μg of protein) was assayed at 37 °C for 1 h in medium containing 10 mM MOPS-Tris, pH 7.2, 3 mM $MgCl_2$, 1 mM vanadate-Tris, 1 mM EGTA-Tris (32). Binding of ouabain or competitive displacement by other cardiac glycosides was assessed by varying the total concentrations of ouabain or other cardiac glycosides at constant [^{3}H]ouabain (between 1 and 2 nM; specific activity 30–40 Ci/mmol). Full curves of bound *versus* free ouabain were generated, and K_D values were estimated using a one-site model $B = B\text{max}\,[\text{Ou}_f]/([\text{Ou}_f] + K_D)$, where $[\text{Ou}_f]$ refers to the concentration of free ouabain, and B and Bmax have their usual meaning. Fitted dissociation constants, K_D, were obtained using Kaleidagraph. For other inhibitors $K_{0.5}$ was calculated using a one-site inhibition model, $B/B_{CG=0} = K_{0.5}/([CG] + K_{0.5})$. B refers to the [^{3}H]ouabain bound at a particular concentration of the cardiac glycoside (CG), and $B_{CG=0}$ refers to the [^{3}H]ouabain bound at 1–2 nM [^{3}H]ouabain in the absence of

other cardiac glycosides. The $K_{D\ CG}$ was calculated from $K_{0.5}$ by taking into account ouabain-CG competition as $K_{D\ CG} = K_{0.5}/(1+[Ou_f]/K_{D\ Ou})$. With $K_{D\ Ou}$ values $\alpha1\beta1$ 9.2 nM, $\alpha2\beta1$ 21.5 nM, and $\alpha3\beta1$ 11 nM, respectively (see Table 1), and 1 nM total ouabain, the values of $1 + [Ou_f]/K_{D\ Ou}$ were 1.06, 1.03, and 1.06, respectively. Binding of each cardiac glycoside was estimated in 3–5 separate experiments. Average K_D values ± S.E. for each isoform were calculated, and statistical significance was calculated by the unpaired Student's *t* test. *p* values <0.05 were considered significant. The selectivity ratios $K_D\ \alpha1/K_D\ \alpha2$ and $K_D\ \alpha1/K_D\ \alpha3$ were calculated as the quotient of the individual K_D values. The error of the ratios of $K_D\ \alpha1/K_D\ \alpha2$ and $K_D\ \alpha1/K_D\ \alpha3$ are calculated from the S.E. K_D values using the formula

$$\text{S.E.}\frac{K_D\,\alpha1}{K_D\,\alpha2/3} = \frac{\sqrt{\left(\frac{K_D\,\alpha1}{K_D\,\alpha2/3}\cdot\text{S.E.}\,\alpha2/3\right)^2 + \text{S.E.}\,\alpha1^2}}{K_D\,\alpha2/3} \quad \text{(Eq. 1)}$$

Digoxin binding was measured in the same conditions as ouabain ([^{3}H]digoxin ~7 nM stock, specific activity 40 Ci/mmol).

Assay of Na,K-ATPase Activity of Purified Isoform Complexes—Inhibition of Na,K-ATPase activity of the detergent-soluble $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ complexes by cardiac glycosides was done as described (29, 30). Before assay, the proteins were incubated at 25 °C for 30 min. The inhibitors were added to the recombinant enzyme (≈0.08–0.2 μg of protein) in 400 μl of reaction medium containing 130 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 25 mM histidine, pH 7.4, 1 mM EGTA, 0.01 mg/ml SOPS, 0.001 mg/ml cholesterol, and 0.005 mg/ml $C_{12}E_8$ in 48-well plates. The reaction (37 °C for 1 h) was started by the addition of 1 mM ATP. P_i release was measured with a malachite green dye to detect the phosphomolybdate (P_i Color Lock, Innova Biosciences). The percent inhibition V_{CG}/V_0 was calculated for each cardiac glycoside concentration, and K_i values were obtained by fitting the data to the function $V_{CG}/V_0 = K_i/([CG] + K_i) + c$ (using Kaleidagraph). V_0 and V_{CG} represent the control rate and rate of Na,K-ATPase activity at particular concentrations of cardiac glycosides, [CG], respectively. The constant c represents a small fraction of uninhibited activity (range 0–10%). Including c improves the fit. Inhibition was estimated in 3–5 separate experiments, average K_i values ± S.E. were calculated, and the significance of differences was calculated by the unpaired Student's *t* test. *p* values <0.05 were considered significant.

Inhibitors—Most cardiac glycosides were dissolved in 70-100% ethanol. Bufalin, marinobufagenin, and gitoxin were dissolved in DMSO. Stock solutions were diluted in 50% ethanol or 50% DMSO. Inhibitors were added directly to the reaction medium containing membranes ([^{3}H]ouabain binding) or pure enzymes (Na,K-ATPase activity), so that the final ethanol or DMSO concentration was 1%. Didigitoxosyl digoxigenin was prepared by selective degradation of digoxin as described (33). The product was analyzed by high performance liquid chromatography and shown to be close to 80% pure.

RESULTS

Expression and Purification of Human $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ Isoforms—The three isoform complexes were expressed in *P. pastoris*, yeast were grown in large quantities, membranes were prepared, and the isoform proteins were purified (see "Experimental Procedures"). Ouabain binding capacities of the membranes expressing $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ were 25–30, 20–25, and 20 pmol/mg of protein, respectively. Fig. 1 presents a gel of a typical purification experiment. The $\beta1$ subunit appears as two bands that are glycosylated to different degrees and are easily deglycosylated by endoglycosidase H to produce a single band of 37 kDa (28, 29). Between 1 and 2 mg of purified proteins were obtained per gram of membranes, with Na,K-ATPase activities of $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$, respectively, of 12–15, 6–8, and ≈10 μmol of Pi/mg/min.

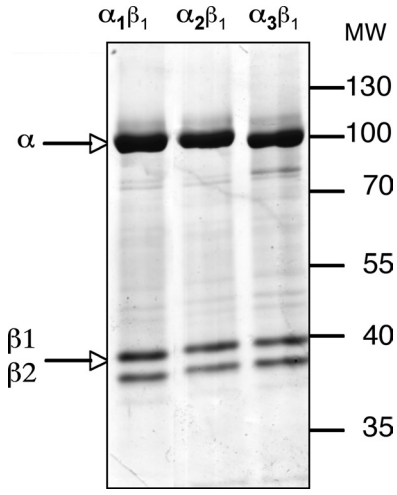


FIGURE 1. **Purification of human $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ isoform complexes.** Coomassie-stained Laemmli SDS-PAGE of purified isoform complexes at 5 μg of protein per *lane* is shown.

Cardiac Glycoside Binding to P. pastoris Membranes Expressing $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$—The high level isoform expression permits accurate determination of [^{3}H]ouabain binding and competitive displacement of [^{3}H]ouabain by many cardiac glycosides (Fig. 2, Table 1).[3] The ligand conditions (3 mM with $MgCl_2$ and 1 mM vanadate-Tris) are optimal for high affinity ouabain binding to an E_2-vanadate conformation (32), vanadate serving as a transition-state analogue of inorganic phosphate. Full binding curves of [^{3}H]ouabain yielded dissociation constants K_D (see Table 1, line 1) with values in the tens of nM range and order $\alpha1 < \alpha3 < \alpha2$. These values are consistent with previous observations on [^{3}H]ouabain binding to human isoforms expressed in *S. cerevisiae* (26, 27) or *Xenopus* oocytes (25). Fig. 2 depicts typical displacement curves for three digitalis glycosides, digoxin, digitoxin, and a semisynthetic derivative, β-methyl digoxin as well as three aglycones, digoxigenin, digitoxigenin, and bufalin, a bufadienolide (see Table 1 for structures). For digoxin, digitoxin, and β-methyl digoxin, the curves for $\alpha2$ and $\alpha3$ are shifted to the left of the curves for $\alpha1$, with little or no difference between $\alpha2$ and $\alpha3$. By contrast, for all three aglycones the curves are superimposable. A single-site

[3] The accuracy of these measurements crucially depends on two factors. The first is a high signal to background ratio. At 1 nM [^{3}H]ouabain, 50–70% of the total radioactivity is bound (about 10,000 cpm per sample) and is displaced by increasing concentrations of other cardiac glycosides to a very low background level (about 200 cpm per sample), i.e. roughly a 50-fold ratio. Second, at 1 nM ouabain, corrections of the $K_{0.5}$ for [^{3}H]ouabain-CG competition to calculate K_D values are very small.

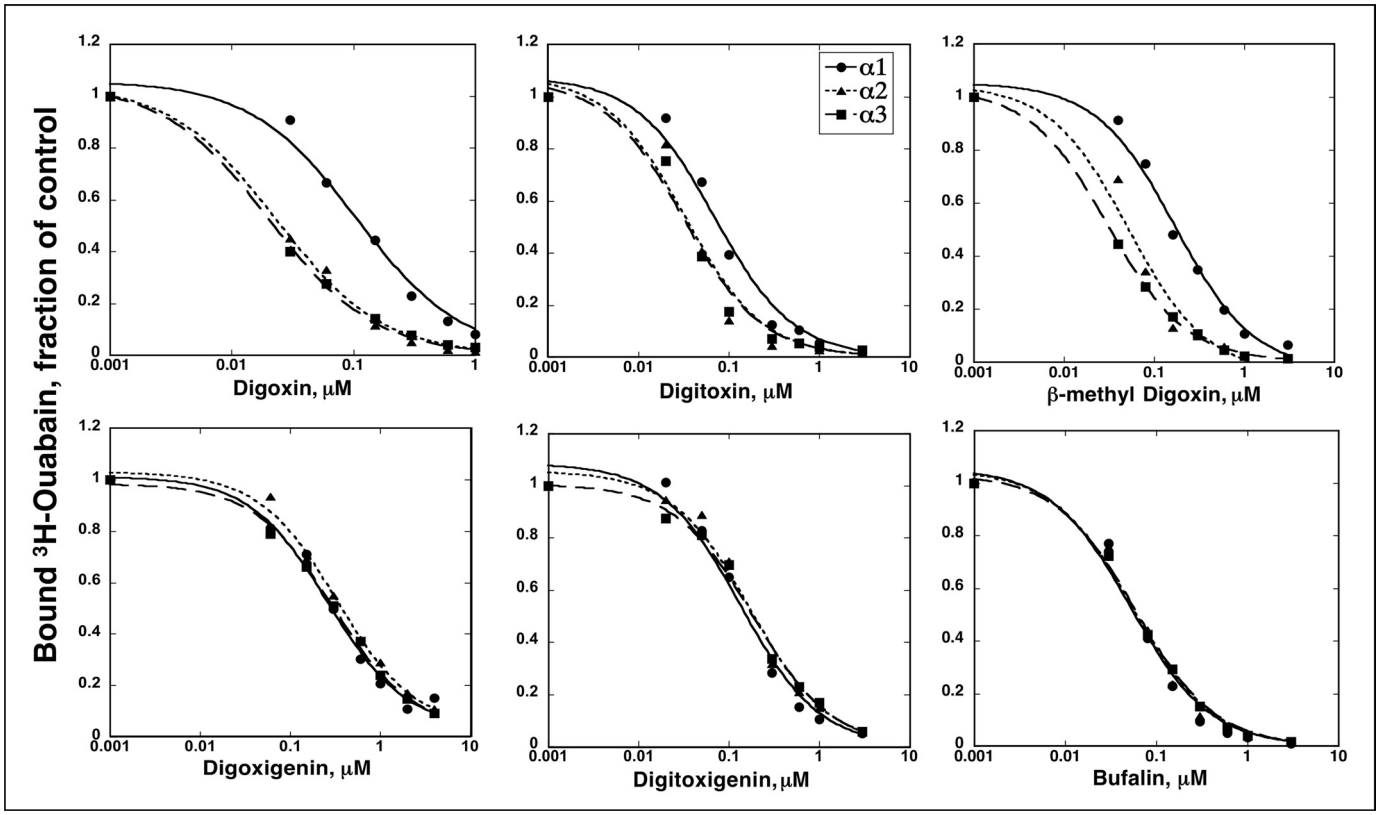


FIGURE 2. **Competitive displacement of [^{3}H]ouabain by cardiac glycosides and aglycones.** Representative experiments for displacement of [^{3}H]ouabain by digoxin (*upper left*), digitoxin (*upper middle*), β-methyl digoxin (*upper right*), digoxigenin (*lower left*), digitoxigenin (*lower middle*), and bufalin (*lower right*) are shown. ●, α1; ▲, α2; ■, α3. The concentration of [^{3}H]ouabain was 1 nM. *Solid lines* are the fitted curves for a one-site competitive displacement model (see "Experimental Procedures").

displacement model was used to calculate values of K_D, compiled in Table 1. Digoxin, β-methyl digoxin, and digitoxin indeed show a moderate but highly significant selectivity for both α2 and α3 over α1, up to 4-fold (see also Table 1, Group B). The three aglycones digoxigenin, digitoxigenin (see also Table 1, Group B), and bufalin (see also Table 1, Group F) show essentially identical affinity for all three isoforms, *i.e.* they show no isoform selectivity. Experiments like those in Table 1 were carried out on four different preparations of membranes expressing the different isoforms, with essentially similar results.

The observation that the aglycones do not discriminate between the isoforms implies that α2/α3:α1 selectivity depends on the sugar residues. To test this hypothesis more systematically as well as the effect of the number and nature of the sugar substituents and the possible influence of the steroid and lactone moieties, we have performed similar experiments on a large number of glycosides and aglycones. These results are collected together in Table 1 into five main groups, B, C, D, E, and F. Group B consists of the digoxigenin and digitoxigenin glycosides with one, two, three, or four digitoxose substituents (see also Fig. 3). The addition of a single digitoxose to digoxigenin strongly increases the affinity for all isoforms but even more for α2 and α3 compared with α1, leading to a significant α2/α3:α1 selectivity (~2-fold). The addition of the two and then three sugars to digoxigenin increases α2/α3:α1 selectivity further, to more than 3-fold in the case of digoxin, whereas the addition of a fourth sugar slightly reduces the affinity for all isoforms but does not affect the α2/α3:α1 selectivity. Fig. 3 illustrates these features and confirms that α2 and α3 show the same profile compared with α1. In the case of digitoxigenin glycosides the addition of either two or three digitoxoses to digitoxigenin has a similar effect on affinity and α2/a3:α1-selectivity. Group C consists of nine synthetic monoglycosyl derivatives of digitoxigenin (20). Several of these derivatives show a higher affinity for all three isoforms compared with the parent digitoxigenin, but only the β-L-rhamnosyl derivative, evomonoside, seems to show significant selectivity, in this case for α3:α1. Group D consists of natural or semisynthetic 16-OH steroid and formylated or acetylated derivatives of gitoxin (19) and oleandrin. The aglycone gitoxigenin has a low affinity, whereas the tridigitoxosyl gitoxigenin (gitoxin) binds much better to all isoforms, but in neither case is there significant isoform selectivity. The 16-formylated gitoxigenin has a much higher affinity compared with the 16-OH gitoxigenin, but no isoform selectivity. By contrast, the 3β-digitoxosyl derivatives of 16-formyl and 16-acetyl gitoxigenin both show significant α2/α3:α1 selectivity. Similarly, oleandrin, with a 16-acetyl modification and a single oleandrose sugar, shows very high affinity and significant α2/α3:α1-selectivity. Group E consists of uzarin derivatives, which are rare cardenolides with planar steroid moieties. The aglycone shows low affinity and no selectivity, whereas the uzarigenin diglucoside shows even lower affinity and significant selectivity, at least for α2:α1. The rhamnoside shows a much higher affinity for all three isoforms but no selectivity. Group F consists of two bufadienolide

TABLE 1

Binding constants of Cardiac Glycosides to P. Pastoris membranes expressing $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$

Each value of K_D ± S.E. represents the average of 3–5 separate estimates calculated as described under "Experimental Procedures." p-Values are quoted only for K_D values that are significantly different between $\alpha2$ and $\alpha1$ or $\alpha3$ and $\alpha1$ ($p < 0.05$).

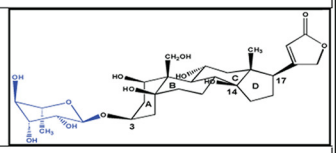
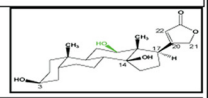
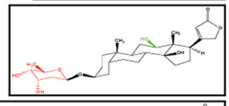
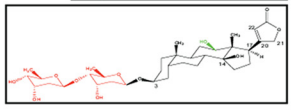
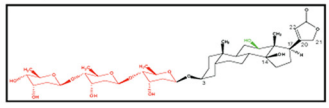
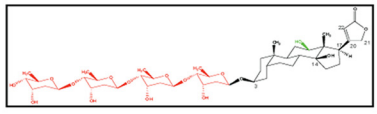
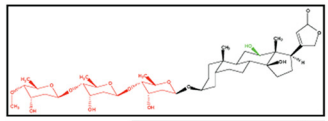
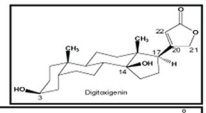
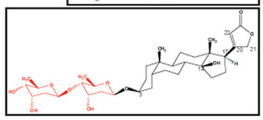
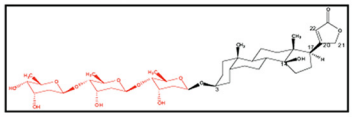
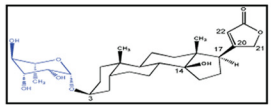
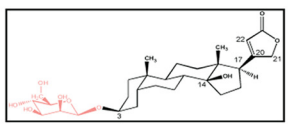
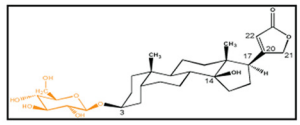
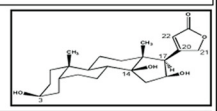
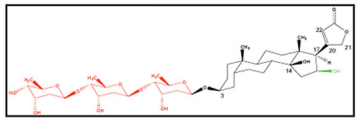
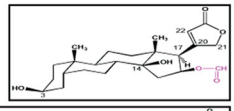
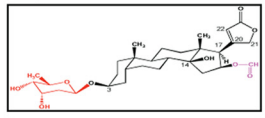
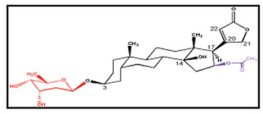
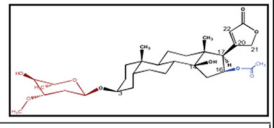
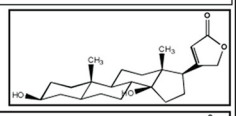
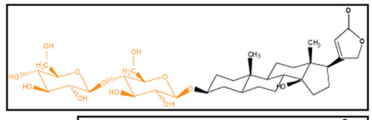
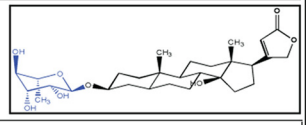
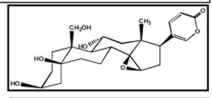
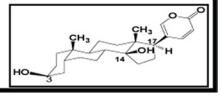
Cardiac glycosides	K_D, nM±SEM p-value			Selectivity ratio±SE		CG structure
	α1	α2	α3	α1/α2	α1/α3	
A. Ouabain	9.8±0.33	21.9±0.56 p=0.0001	11.1±1.3	**0.44±0.01**	**0.88±0.1**	
B. Digitalis glycosides.						
Digoxigenin	270±21	332±11	310±27	**0.81±0.06**	**0.88±0.1**	
Monodigitoxosyl-digoxigenin	65±5.7	35±6.2 p=0.02	31.8±5.8 p=0.01	**1.87±0.37**	**2.06±0.41**	
Didigitoxosyl-digoxigenin	69±3	27±2.5 p=0.008	24.4±2 p=0.006	**2.55±0.24**	**2.82±0.17**	
Tridigitoxosyl-digoxigenin (Digoxin)	87±6	25.6±2.8 p=0.001	25±2.4 p=0.001	**3.39±0.43**	**3.48±0.41**	
Tetradigitoxosyl-digoxigenin	200±35	66±12 p=0.023	74±13 p=0.029	**3.0±0.76**	**2.7±0.66**	
β-Methyl digoxin	129±20	43±7 p=0.018	31±4 p=0.009	**3±0.67**	**4.1±0.84**	
Digitoxigenin	101±13.4	125±13.6	134±15.6	**0.8±0.13**	**0.75±0.13**	
Didigitoxosyl-digitoxigenin	52±4	22±5 p=0.017	23±2 p=0.006	**2.36±0.58**	**2.26±0.29**	
Tridigitoxosyl-digitoxigenin (Digitoxin)	38±3	18.3±4 p=0.02	14±3.9 p=0.008	**2.07±0.48**	**2.7±0.78**	
C. Monoglycosyl derivatives of Digitoxigenin*						
α-L-rhamnosyl	27±5	23±3	19±3	**1.17±0.26**	**1.42±0.35**	
β-L-rhamnosyl (Evomonoside)	25±2.2	20±4	11.4±2.3 p=0.012	**1.25±0.27**	**2.19±0.48**	
α-D-mannosyl	81±13	93±20	122±20	**0.87±0.13**	**0.66±0.15**	
β-D-mannosyl	56±7	61±10	58±10	**0.92±018**	**0.97±0.20**	
α-L-mannosyl	94±18	135±30	101±10	**0.7±0.20**	**0.93±0.20**	
β-L-mannosyl	23±4	24±10	19±3	**0.96±0.43**	**1.21±0.28**	

TABLE 1—*continued*

α-D-glucosyl	22±4	24±10	19±2	**0.92±0.41**	**1.16±0.02**	
β-D-glucosyl	46±13	35±10	32±10	**1.31±0.35**	**1.44±0.42**	
α-L-glucosyl	43±9	28±10	35±10	**1.54±0.63**	**1.23±0.43**	
* = For the different isomers of the same glycoside, only one configuration is represented.						
D. 16-OH steroid derivatives						
Gitoxigenin	500±90	1180±190	1050±100	**0.42±0.10**	**0.48±0.9**	
Gitoxin	123±8	85±3 p=0.047	108±17	**1.45±0.10**	**1.14±0.19**	
16-Formate gitoxigenin	37±4	34±5	30±1.5	**1.09±0.19**	**1.23±0.14**	
16-Formate 3β-digitoxosyl-gitoxigenin	65±9.3	27.7±6.8 p=0.018	23±4.7 p=0.007	**2.35±0.66**	**2.83±0.70**	
16-Acetate 3β-digitoxosyl-gitoxigenin	53±10	34±12	27±10	**1.56±0.62**	**1.96±0.81**	
Oleandrin	35±4.5	13±3 p=0.016	11±2.5 p=0.009	**2.7±0.71**	**3.18±0.83**	
E. Planar steroid derivatives						
Uzarigenin	1100±120	883±56	1278±142	**1.25±0.15**	**0.86±0.13**	
Uzarin	6080±1090	1500±60 p=0.05	3400±260	**4.04±0.74**	**1.8±0.34**	
β-Rhamnosyl-uzarigenin	90±20	96±7.1	122±8.6	**0.93±0.21**	**0.73±0.17**	
F. Bufodienolide aglycones						
Marinobufagenin	2240±137	2470±45	2430±205	**0.91±0.05**	**0.92±0.09**	
Bufalin	42.5±6.5	45±8	40±15	**0.94±0.22**	**1.06±0.33**	

aglycones, marinobufagenin and bufalin. Bufalin was discussed above. Marinobufagenin, which is reported to have α1 selectivity in some systems (for review, see Ref. 34), shows low affinity in this assay and, like bufalin, shows no isoform selectivity. These data confirm the importance of the sugars in α2/α3:α1 selectivity and provide several other conclusions on structural requirements of the sugar and steroid moieties (Fig. 3 and "Discussion").

We have also looked directly at [^{3}H]digoxin binding to the three isoforms. Estimates of the K_D values based on full binding curves up to μM concentrations are unsatisfactory due to a high background count (about 10-fold higher for [^{3}H]digoxin com-

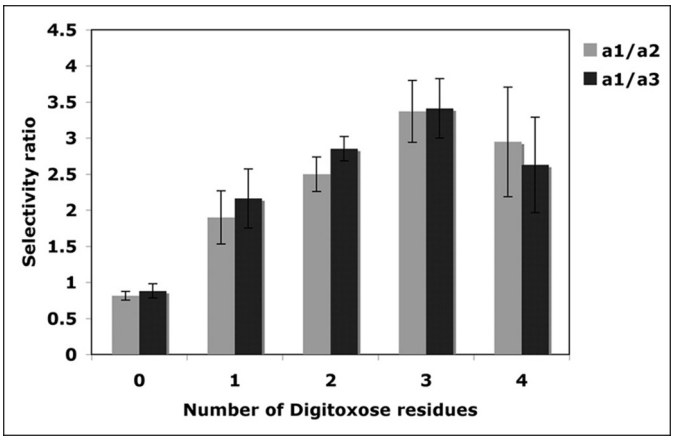


FIGURE 3. **Dependence of isoform selectivity on the number of digitoxose residues.** The ratios of K_D ±S.E. for $\alpha1/\alpha2$ and $\alpha1/\alpha3$ are taken from Table 1 (Group B).

pared with [^{3}H]ouabain). At low concentrations of [^{3}H]digoxin (nM), specific binding is easily detected and has shown two interesting features (Fig. 4). Fig. 4*A* depicts the well known [^{3}H]digoxin-K^+ antagonism at increasing concentrations of K^+. The striking point is that $K_{0.5\ K^+}$ for displacing [^{3}H]digoxin is 5–6-fold higher for $\alpha2$ compared with $\alpha1$, whereas that for $\alpha3$ is closer to that for $\alpha1$ (see Fig. 4*A*). Fig. 4*B* shows the rate of dissociation of [^{3}H]digoxin. The rate is about 5-fold faster for $\alpha2$ compared with $\alpha1$, and that for $\alpha3$ is closer to $\alpha2$. The order of the isoform-dependent differences in $K_{0.5\ K^+}$ ions and dissociation rates is similar to properties of [^{3}H]ouabain binding to human isoforms expressed in *Xenopus* oocytes (25). Thus, these features are intrinsic properties of the isoforms, and all other cardiac glycosides should show the same features.

Inhibition of Na,K-ATPase Activity of Purified α1β1, α2β1, and α3β1 Complexes—As an independent test of isoform selectivity, we have compared inhibition by the different cardiac glycosides of Na,K-ATPase activities of the three purified detergent-soluble isoform complexes.[4] Representative inhibition experiments are shown in Fig. 5 for digoxin, digoxigenin, and β-methyl digoxin, and fitted K_i values for a selection of glycosides and aglycones are presented in Table 2. An obvious feature in Fig. 5 is that the curves for inhibition of $\alpha2$ by digoxin and β-methyl digoxin lies to the left of that for $\alpha1$, and those for $\alpha3$ lie between those for $\alpha1$ and $\alpha2$. The curves for inhibition by digoxigenin are similar for all three isoforms. The fitted K_i values with statistical tests for significance in Table 2 confirm these observations and also illustrate an important additional property. The ratio of K_i for $\alpha1/\alpha2$ is 3–5-fold in the case of digoxin, digitoxin, β-methyl digoxin, oleandrin, and 16-formate 3-β-digitoxose gitoxigenin. By contrast, the ratio K_i $\alpha1/\alpha3$ for all these glycosides is lower than K_i $\alpha1/\alpha2$, and in most cases it is nearer to 1. Again, ouabain is different in that it is the only glycoside for which K_i $\alpha1 \approx \alpha2 \approx \alpha3$. For all the aglycones, ouabagenin, digoxigenin, and digitoxigenin, there are no or only minor differences between the isoforms. By comparison with K_D values for cardiac glycoside binding in Table 1, the K_i values in Table 2 are all higher, which is expected on account of the presence of K^+ and the K^+-cardiac glycoside antagonism. Because, however, $K_{0.5\ K^+}$ values for antagonism of digoxin binding lie on the order $\alpha2 > \alpha3 > \alpha1$ (Fig. 4*A*), CG binding could be antagonized by the K^+ to different extents in the order $\alpha1 > \alpha3 > \alpha2$. Differential K^+-cardiac glycoside antagonism on $\alpha1$, $\alpha3$, and $\alpha2$, respectively, could explain the observed order of inhibitory potency of β-methyl digoxin or digoxin (K_i $\alpha2 < \alpha3 < \alpha1$) and also the finding that for ouabain K_i $\alpha1 \approx \alpha2 \approx \alpha3$ (Table 2). To test this notion we have looked at the inhibition by β-methyl digoxin at 20 mM K^+ rather than the usual 5 mM K^+. Under this condition, the K_i for $\alpha1$ and $\alpha3$ was substantially raised, whereas the K_i for $\alpha2$ was raised only slightly (see Fig. 5 and Table 2). As a result the apparent selectivity of $\alpha2$:$\alpha1$ increased further to 7.3-fold, compared with 4.5-fold at 5 mM K^+, and the order is clear-cut, K_i $\alpha2 < \alpha3 \leq \alpha1$. Experiments like those in Fig. 5 were carried on three different preparations of the purified isoform complexes and also complexes of $\alpha1\beta1$ purified from membranes prepared from yeast grown at 20 °C as well as 25 °C, with essentially similar results. Furthermore, reconstitution of $\alpha1\beta1$ and $\alpha2\beta1$ with FXYD1 (see Ref. 30) does not significantly affect the K_i values for inhibition by digoxin or the $\alpha1/\alpha2$ selectivity ratio (unpublished result, to be presented in detail in a separate paper).

DISCUSSION

Isoform Selectivity and Structures of Cardiac Glycosides—The central finding in this work is that digoxin, digitoxin, β-methyl digoxin, and some other glycosides show a partial but highly significant selectivity for binding to $\alpha2$ and $\alpha3$ compared with $\alpha1$. By contrast, $\alpha2/\alpha3$:$\alpha1$ selectivity was not detected for any aglycone tested, indicating the essential role of the sugar. Ouabain behaves differently to the digitalis glycosides, showing some selectivity for $\alpha1$:$\alpha2$ and little difference between $\alpha1$ and $\alpha3$, confirming the findings in Refs. 25–27. The pattern of inhibition of Na,K-ATPase activity by the digitalis glycosides is consistent with the binding data but is modified by a large difference in apparent affinity of K^+ for antagonizing cardiac glycoside binding. As a result, the order of potency of the digitalis glycosides as inhibitors of Na,K-ATPase is K_i $\alpha2 < \alpha3 \leq \alpha1$. Again, ouabain stands out in that K_i $\alpha2 \approx \alpha3 \approx \alpha1$. It is of course significant that the selectivities observed for cardiac glycoside binding and inhibition of Na,K-ATPase activities are mutually consistent. Furthermore, similar results were obtained on several independent membrane preparations and purified $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ complexes and for the purified $\alpha1\beta1$ prepared from cells grown at 20 °C as well as the usual 25 °C and also for the $\alpha1\beta1$FXYD1 and $\alpha2\beta1$FXYD1 complexes. These findings all suggest that the moderate $\alpha2$ selectivity of the digitalis glycosides is a robust result and cannot be attributed to the type and conditions of the assays, the state of the protein (intact membranes *versus*

[4] An important methodological consideration is that the reaction time must be long enough to ensure full equilibration of all cardiac glycosides with all three isoforms throughout the assay. It is known, for example, that binding of ouabain to $\alpha1$ is much slower than to $\alpha2$ and $\alpha3$ (25). In practice, for assay times ≥40 min at 37 °C, the K_i values for inhibition of all three isoforms were constant. For reaction times that are too short (e.g. 5–10 min), K_i values for $\alpha1$, but not for $\alpha2$, were significantly raised, strongly distorting the selectivity patterns.

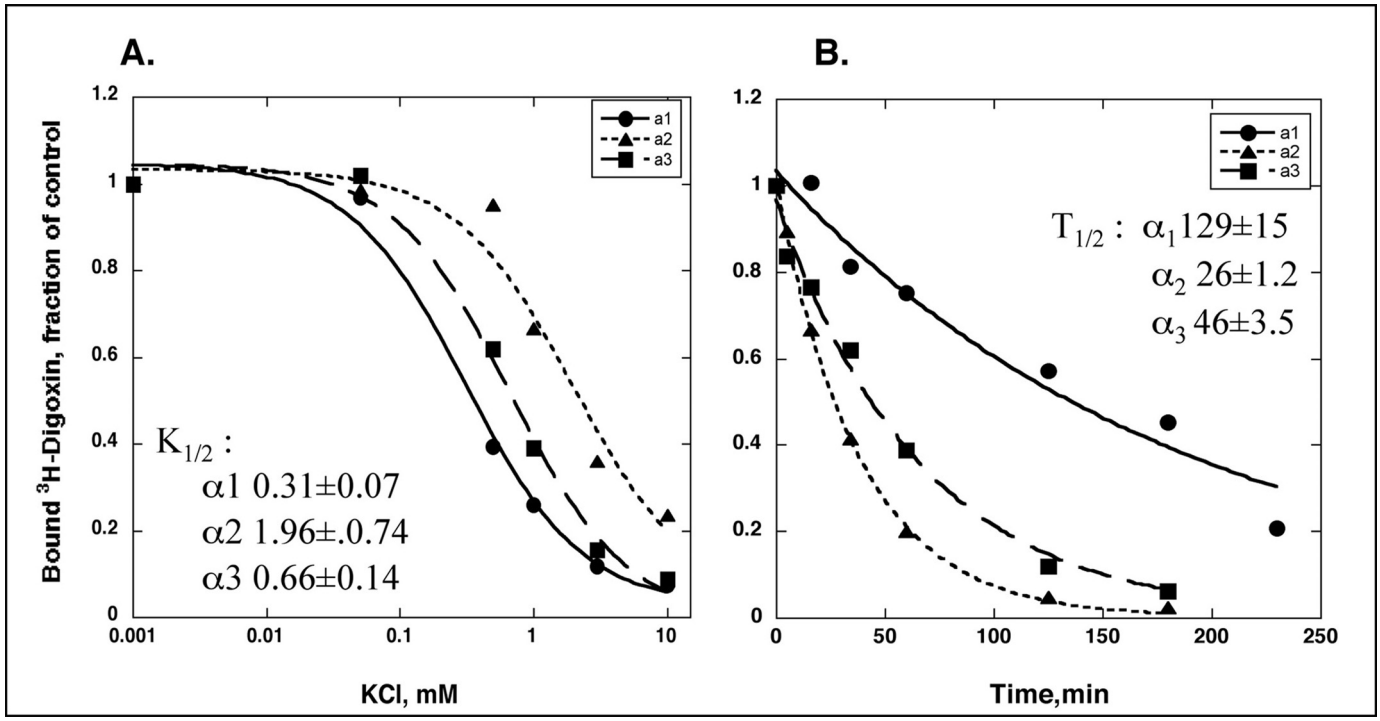


FIGURE 4. **[^{3}H]Digoxin-K^+ antagonism (*A*) and [^{3}H]digoxin dissociation rates (*B*).** ●, α1; ▲, α2; ■, α3. *A*, [^{3}H]digoxin binding in the presence of 0–10 mM K^+ is shown. Average $K_{0.5\,K}$ values based on single site inhibition for two separate experiments are shown. *B*, digoxin 1 mM was added to the membranes pre-bound with [^{3}H]digoxin, and the remaining bound [^{3}H]digoxin was measured at 37 °C at the indicated times.

detergent-soluble proteins), type of the purified complex, or mere chance.

As mentioned in the Introduction, extensive structure-activity relationships for binding of many cardiac glycosides and inhibition of Na,K-ATPase activities, utilizing native enzyme preparations, have been published previously (see for example Refs. 18–22 and 35). These have established the basic structural features necessary for cardiac glycoside action. Overly detailed comparisons between the different studies or with the current study using recombinant human proteins are not worthwhile because of wide differences in experimental conditions, species differences, and enzyme sources with mixed isoform content. Results for the recombinant human α1β1 and native sheep and pig kidney Na,K-ATPase (α1β1) (19, 20, 22), the only native source of the enzyme with the defined isoform content, do appear in general to be consistent despite the species difference.

An attempt to measure competition of various cardiac glycosides with [^{3}H]ouabain for binding to human α1, α2, and α3 expressed in *S. cerevisiae* was reported recently (36). With the exception of [^{3}H]ouabain binding data, the results for other cardiac glycosides were quite different from the present findings. In particular, in comparable conditions of high affinity binding, no difference was detected for digoxin or digitoxin binding to α1, α2, and α3, and β-methyl digoxin was stated to be partially selective for α1 over α2 and α3. We believe that the most likely explanation of the inconsistency is that very low levels of expression of the isoforms in *S. cerevisiae* (α1, 4.95; α2, 1.32; α3, 4.51 pmol/mg) compared with *P. pastoris* (α1, 25–30; α2, 20–25; α3, 20 pmol/mg) compromised the accuracy of the measurements and the ability to detect moderate isoform-dependent differences.[5]

The data in Table 1 provide several additional conclusions on the role of the number and nature of the sugars. First, compared with digoxigenin and digitoxigenin, the addition of only one digitoxose residue raises affinity for all isoforms and produces some α2/α3:α1 selectivity, whereas two or more digitoxoses added to digoxigenin or digitoxigenin induce the maximal affinity and α2/α3:α1 selectivity ratio (see also Fig. 3). Second, only cardiac glycosides with sugars in β-glycosidic links display α2/α3:α1 selectivity. Third, only sugars having 5-methyl groups (digitoxose, oleandrose and to some extent L-rhamnose) display α2/α3:α1 selectivity. Fourth, the nature of the steroid is crucial as well as the sugar (as seen for groups D (gitoxin) and E (uzarin) derivatives in Table 1). The latter observations make it less surprising that ouabain behaves differently to digoxin and digitoxin. Presumably the hydrophilic steroid and α-L-rhamnoside of ouabain interact better with α1 than α2 compared with the hydrophobic steroid and β-digitoxose in digoxin and digitoxin, which bind better to α2 and α3 than to α1.

Compatibility with the Structure of Na,K-ATPase with Bound Ouabain—The structures of shark rectal gland Na,K-ATPase (37), especially a structure with bound ouabain (38), provide an interesting insight into the proposed role of the sugars in iso-

[5] Low expression levels in *S. cerevisiae* necessitate use of much higher [^{3}H]ouabain concentrations (20 nM) compared to the 1–2 nM used with *P. pastoris* membranes. This reduces signal to background ratio and also entails large corrections for ouabain-CG competition to convert the $K_{0.5}$ to K_D values. Both factors must decrease the accuracy of the experiments (see also footnote 3).

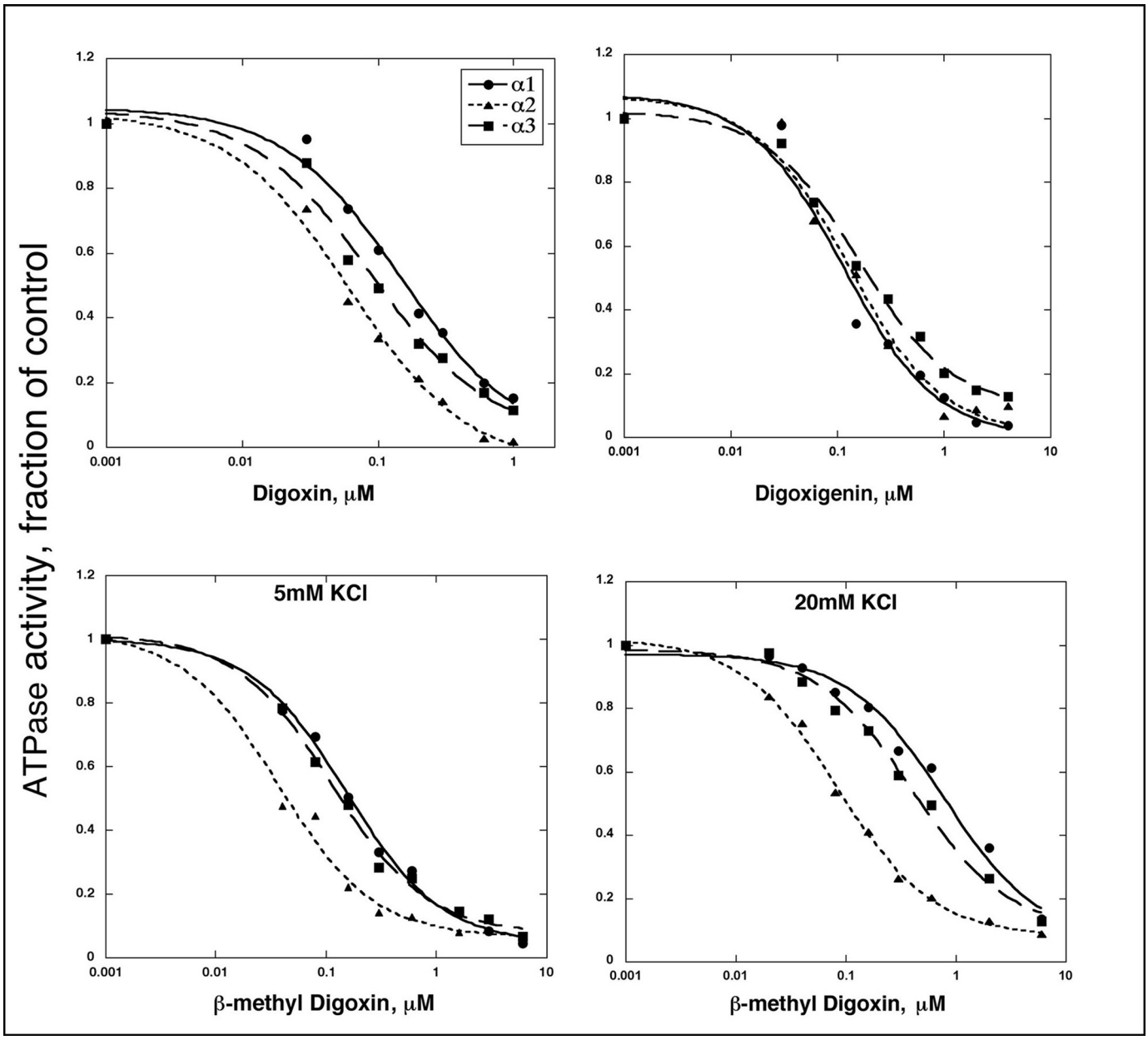


FIGURE 5. **Inhibition of Na,K-ATPase activity of purified isoform complexes.** Shown are representative experiments for inhibition of Na,K-ATPase activity by digoxin (*upper left*), digoxigenin (*upper right*), and β-methyl digoxin (*lower left*, 5 mM K^+; *lower right*, 20 mM K^+). ●, α1; ▲, α2■, α3. *Solid lines* are the fitted curves for a one-site inhibition model (see "Experimental Procedures").

form selectivity. Fig. 6 shows a detail of the structure of Na,K-ATPase with two occluded K^+ ions and ouabain bound in a deep cavity facing the external surface. Because the digitalis glycoside binding affinities are the same and higher for both human α2 and α3 compared with α1, one could assume that the higher affinity is due to interactions with residues that are the same in α2 and α3 but differ in α1. All such residues at the extracellular surface and trans-membrane segments have been mapped onto the structure (α1 residues). The interesting feature is that all these residues are located in the extracellular loops in proximity to the rhamnose moiety of ouabain, but within the steroid-lactone binding cavity there are no such isoform-specific differences. This structure is not an ideal model because ouabain is bound with low affinity due to the presence of occluded Rb^+, but as discussed in Ref. 38, it explains most effects of mutations on high affinity ouabain binding. In addition, the high affinity state was modeled by movement of transmembrane segments TM1/TM2 to close off the binding cavity, with ouabain bound in an almost identical position (38). With this caveat, the structure predicts quite naturally that no aglycones can show isoform selectivity, because the differences between the isoforms are found only in proximity to the sugar. This prediction is exactly consistent with all our observations. Conversely, our observations imply that the steroid-lactone moieties of all cardiac glycosides should be bound similarly to that of ouabain. By comparison with this structure, prior models of cardiac glycoside binding have assumed that the cardiac glycoside molecule lies more or less horizontally across the

TABLE 2

Inhibition of Na,K-ATPase activity of $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ complexes

Each value of $K_i \pm$ S.E. represents the average of 3–5 separate estimates calculated as described under "Experimental Procedures." *p* values are quoted only for K_i values that are significantly different between $\alpha2$ and $\alpha1$ or $\alpha3$ and $\alpha1$ ($p < 0.05$).

CG	$K_i \pm$ S.E., *p* value			Ratio of K_i values ± S.E.	
	α_1	α_2	α_3	α_1/α_2	α_1/α_3
		nM			
Ouabain	97 ± 4.6	94 ± 16	97 ± 13	1.03 ± 0.18	1.0 ± 01.4
Ouabagenin	721 ± 70	739 ± 89	592 ± 3	0.97 ± 0.15	1.21 ± 0.11
Digitoxin	72 ± 8	23 ± 4, $p = 0.006$	52 ± 11	3.13 ± 0.64	1.38 ± 0.33
Digitoxigenin	76 ± 18	45 ± 6.7	72.7 ± 7	1.69 ± 0.47	1.04 ± 0.26
Digoxin	250 ± 44	63 ± 10, $p = 0.003$	136 ± 24	3.96 ± 0.93	1.83 ± 0.45
β-Methyl digoxin, 5 mM K^+	253 ± 57	56 ± 13, $p = 0.015$	151 ± 24	4.51 ± 1.4	1.67 ± 0.46
β-Methyl digoxin, 20 mM K^+	655 ± 110	89 ± 27, $p = 0.008$	540 ± 14	7.36 ± 2.55	1.21 ± 0.20
Digoxigenin	139 ± 17	130 ± 13.5	144 ± 15	1.07 ± 0.17	0.96 ± 0.15
Oleandrin	58 ± 4	20 ± 3, $p = 0.0028$	46 ± 1	2.90 ± 0.47	1.26 ± 0.09
16-Formate 3β-digitoxin gitoxigenin	80 ± 2	19.5 ± 1.1, $p = 0.001$	73 ± 6	4.10 ± 0.25	1.09 ± 0.09

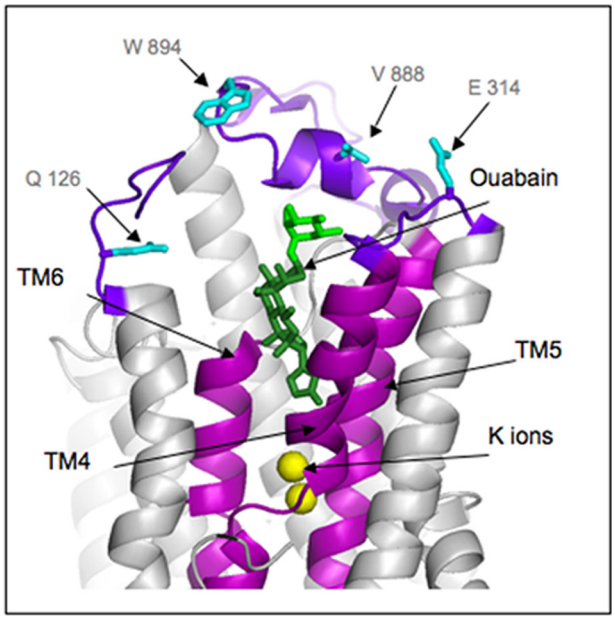


FIGURE 6. **Structure of Na,K-ATPase with bound ouabain.** The figure shows the Protein Data Bank codes 3A3Y structure (38). Residues identical in human $\alpha2$ and $\alpha3$ and different in $\alpha1$ are shown in *cyan*. Residues and numbering are those for human $\alpha1$. *Dark green*, ouabain, steroid-lactone; *light green*, rhamnose. The figure was drawn by PYMOL.

extracellular surface and do not explain our results. Of course, the structure cannot explain why ouabain somewhat favors $\alpha1$ or why the digitalis glycosides somewhat favor $\alpha2/\alpha3$, but it confirms that the key lies in interactions of the sugars near the extracellular loops. This structural insight fits very well with a two-step binding model proposed by Yoda (39) to explain the fact that the sugar is not essential for inhibition but strongly affects the binding affinity and rates (23). On the Yoda model (39), the steroid-lactone and sugar moieties occupy specific subsites. An independent kinetic contribution of the sugar to binding is consistent with the proposed role in isoform selectivity, assuming that only the sugar subsite differs in $\alpha1$ compared with $\alpha2/\alpha3$ isoforms.

Pharmacological and Physiological Implications—Of all the cardiac glycosides we have tested, those that show the greatest, even if partial selectivity for $\alpha2$:$\alpha1$, digoxin, and β-methyl digoxin are the ones in most extensive clinical use. This supports the evidence mentioned in the Introduction that $\alpha2$ is the major player in cardiac contractility and primary target for cardiac glycosides such as digoxin. By contrast, ouabain, which shows no selectivity for $\alpha2$:$\alpha1$, is not used clinically. Although in a comprehensive clinical trial (DIG trial), digoxin did not reduce overall mortality (40), a subsequent post hoc analysis found that a lower serum concentration (0.5–0.9 compared with >1 ng·ml) has overall benefit and reduces mortality (41). This could fit a hypothesis that therapeutic ($\alpha2$?) and toxic ($\alpha1$?) effects are separable, and in principle, a more $\alpha2$-selective cardiac glycoside may increase the therapeutic window and reduce toxicity. Expression of Na,K-ATPase in human cardiac muscle is lowered up to 40% in congestive heart failure, especially $\alpha1$, whereas $\alpha3$ or $\alpha2$ levels may be unchanged or lower depending on the region of the heart (42). Therefore, a more $\alpha2$-selective cardiac glycoside than digoxin could be considered to be tailored to the failing heart.

The different selectivity of digoxin and ouabain for human $\alpha1$ and $\alpha2$ at the protein level was unexpected and may be relevant to differences between ouabain and digoxin observed *in vivo*. For example, injection of rats with low doses of ouabain over several days raise BP, but paradoxically, digoxin does not have this effect and even protects against the ouabain (43). The effect of ouabain derivatives, modified in the lactone ring, on BP does not correlate directly with inhibition of dog kidney Na,K-ATPase ($\alpha1\beta1$) (44). Thus, the mechanism, especially the difference between ouabain and digoxin, is not well understood. The inability of digoxin to raise BP suggests that inhibition of $\alpha2$ may not be involved. Furthermore, digoxin is not known to raise BP in man. Both ouabain and digoxin have been detected in mammalian tissues and fluids (1). Taken together, the selectivity differences of ouabain and digoxin and their different effects *in vivo* support the notion that these two cardiac glycosides have specific but distinct physiological roles (for reviews, see also Refs. 1 and 5). For example, digoxin might affect primarily $\alpha2$-dependent and ouabain primarily $\alpha1$-dependent functions. Furthermore, ouabain induces Ca^{2+} oscillations (45), and long-term effects of ouabain on cell Ca^{2+} and BP might be mediated via $\alpha1$ and the cellular signaling pathways, as suggested in Ref. 2. The first step in ouabain-mediated signaling *in vivo* is tyrosine phosphorylation of the soluble tyrosine kinase, Src (3). In this respect, it is of interest that, unlike

ouabain, digoxin does not activate tyrosine phosphorylation of Src in an *in vitro* assay.[6]

Perspective—The observations that several digital glycosides are partially selective for $\alpha 2$:$\alpha 1$ and the mechanistic insight that aglycones show no isoform selectivity suggest that improved $\alpha 2$ selectivity might be achieved by modifying the digitoxose. A more $\alpha 2$-selective cardiac glycoside could have important applications as a tool to analyze toxicity of cardiac glycosides, physiological functions of $\alpha 2$, and different endogenous cardiac glycosides and possibly as a safer cardiotonic agent compared with digoxin. Isoform-selective cardiac glycosides could also be important in recently discussed applications of cardiac glycosides such as treatment of cancer (46).

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REFERENCES

1. Schoner, W., and Scheiner-Bobis, G. (2007) *Am. J. Physiol. Cell Physiol.* **293,** C509–C536
2. Xie, Z., and Askari, A. (2002) *Eur. J. Biochem.* **269,** 2434–2439
3. Li, Z., and Xie, Z. (2009) *Pflugers Arch.* **457,** 635–644
4. Blaustein, M. P., Zhang, J., Chen, L., Song, H., Raina, H., Kinsey, S. P., Izuka, M., Iwamoto, T., Kotlikoff, M. I., Lingrel, J. B., Philipson, K. D., Wier, W. G., and Hamlyn, J. M. (2009) *Hypertension* **53,** 291–298
5. Dvela, M., Rosen, H., Feldmann, T., Nesher, M., and Lichtstein, D. (2007) *Pathophysiology* **14,** 159–166
6. Blanco, G., and Mercer, R. W. (1998) *Am. J. Physiol.* **275,** F633–F650
7. Garty, H., and Karlish, S. J. (2006) *Annu. Rev. Physiol.* **68,** 431–459
8. Sweadner, K. J., Herrera, V. L., Amato, S., Moellmann, A., Gibbons, D. K., and Repke, K. R. (1994) *Circ. Res.* **74,** 669–678
9. Berry, R. G., Despa, S., Fuller, W., Bers, D. M., and Shattock, M. J. (2007) *Cardiovasc. Res.* **73,** 92–100
10. Juhaszova, M., and Blaustein, M. P. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 1800–1805
11. Bers, D. M. (2008) *Annu. Rev. Physiol.* **70,** 23–49
12. Dostanic-Larson, I., Lorenz, J. N., Van Huysse, J. W., Neumann, J. C., Moseley, A. E., and Lingrel, J. B. (2006) *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290,** R524–R528
13. Despa, S., and Bers, D. M. (2007) *Am. J. Physiol. Cell Physiol.* **293,** C321–C327
14. Despa, S., Wu, Y., Lingrel, J. B., Stefani, E., and Bers, D. M. (2010) *Biophys. J.* **98,**
15. Blaustein, M. P., Zhang, J., Chen, L., and Hamilton, B. P. (2006) *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290,** R514–R523
16. Zhang, J., Lee, M. Y., Cavalli, M., Chen, L., Berra-Romani, R., Balke, C. W., Bianchi, G., Ferrari, P., Hamlyn, J. M., Iwamoto, T., Lingrel, J. B., Matteson, D. R., Wier, W. G., and Blaustein, M. P. (2005) *J. Physiol.* **569,** 243–256
17. Lingrel, J. B. (2010) *Annu. Rev. Physiol.* **72,** 395–412
18. Schönfeld, W., Weiland, J., Lindig, C., Masnyk, M., Kabat, M. M., Kurek, A., Wicha, J., and Repke, K. R. (1985) *Naunyn-Schmiedebergs Arch. Pharmacol.* **329,** 414–426
19. Hashimoto, T., Rathore, H., Satoh, D., Hong, G., Griffin, J. F., From, A. H., Ahmed, K., and Fullerton, D. S. (1986) *J. Med. Chem.* **29,** 997–1003
20. Rathore, H., From, A. H., Ahmed, K., and Fullerton, D. S. (1986) *J. Med. Chem.* **29,** 1945–1952
21. Schön, R., Weiland, J., Megges, R., and Repke, K. R. (1995) *Naunyn Schmiedebergs Arch. Pharmacol.* **351,** 282–292
22. Paula, S., Tabet, M. R., and Ball, W. J., Jr. (2005) *Biochemistry* **44,** 498–510
23. Forbush, B. (1983) *Curr. Top. Membr. Transp.* **19,** 167–201
24. From, A. H., Fullerton, D. S., and Ahmed, K. (1990) *Mol. Cell. Biochem.* **94,** 157–165
25. Crambert, G., Hasler, U., Beggah, A. T., Yu, C., Modyanov, N. N., Horisberger, J. D., Lelièvre, L., and Geering, K. (2000) *J. Biol. Chem.* **275,** 1976–1986
26. Wang, J., Velotta, J. B., McDonough, A. A., and Farley, R. A. (2001) *Am. J. Physiol. Cell Physiol.* **281,** C1336–C1343
27. Müller-Ehmsen, J., Juvvadi, P., Thompson, C. B., Tumyan, L., Croyle, M., Lingrel, J. B., Schwinger, R. H., McDonough, A. A., and Farley, R. A. (2001) *Am. J. Physiol. Cell Physiol.* **281,** C1355–1364
28. Cohen, E., Goldshleger, R., Shainskaya, A., Tal, D. M., Ebel, C., le Maire, M., and Karlish, S. J. (2005) *J. Biol. Chem.* **280,** 16610–16618
29. Haviv, H., Cohen, E., Lifshitz, Y., Tal, D. M., Goldshleger, R., and Karlish, S. J. (2007) *Biochemistry* **46,** 12855–12867
30. Lifshitz, Y., Petrovich, E., Haviv, H., Goldshleger, R., Tal, D. M., Garty, H., and Karlish, S. J. (2007) *Biochemistry* **46,** 14937–14950
31. Strugatsky, D., Gottschalk, K. E., Goldshleger, R., Bibi, E., and Karlish, S. J. (2003) *J. Biol. Chem.* **278,** 46064–46073
32. Pedersen, P. A., Rasmussen, J. H., and Jørgensen, P. L. (1996) *Biochemistry* **35,** 16085–16093
33. Satoh, D., and Aoyama, K. (1970) *Chem. Pharm. Bull.* **18,** 94–99
34. Bagrov, A. Y., Shapiro, J. I., and Fedorova, O. V. (2009) *Pharmacol. Rev.* **61,** 9–38
35. Ahmed, K., Rohrer, D. C., Fullerton, D. S., Deffo, T., Kitatsuji, E., and From, A. H. (1983) *J. Biol. Chem.* **258,** 8092–8097
36. Hauck, C., Potter, T., Bartz, M., Wittwer, T., Wahlers, T., Mehlhorn, U., Scheiner-Bobis, G., McDonough, A. A., Bloch, W., Schwinger, R. H., and Müller-Ehmsen, J. (2009) *Eur. J. Pharmacol.* **622,** 7–14
37. Shinoda, T., Ogawa, H., Cornelius, F., and Toyoshima, C. (2009) *Nature* **459,** 446–450
38. Ogawa, H., Shinoda, T., Cornelius, F., and Toyoshima, C. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 13742–13747
39. Yoda, A. (1973) *Mol. Pharmacol.* **9,** 51–60
40. (1997) *N. Engl. J. Med.* **336,** 525–533
41. Ahmed, A., Rich, M. W., Love, T. E., Lloyd-Jones, D. M., Aban, I. B., Colucci, W. S., Adams, K. F., and Gheorghiade, M. (2006) *Eur. Heart J.* **27,** 178–186
42. Müller-Ehmsen, J., Wang, J., Schwinger, R. H., and McDonough, A. A. (2001) *Cell. Mol. Biol.* **47,** 373–381
43. Manunta, P., Rogowski, A. C., Hamilton, B. P., and Hamlyn, J. M. (1994) *J. Hypertens.* **12,** 549–560
44. Manunta, P., Hamilton, B. P., and Hamlyn, J. M. (2001) *Hypertension* **37,** 472–477
45. Aizman, O., Uhlén, P., Lal, M., Brismar, H., and Aperia, A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98,** 13420–13424
46. Mijatovic, T., Ingrassia, L., Facchini, V., and Kiss, R. (2008) *Expe. Opin. Ther. Targets* **12,** 1403–1417

[6] M. Ferrandi, personal communication.