Cardiac Glycosides as Novel Inhibitors of Human Ether-a-go-go-Related Gene Channel Trafficking

Lu Wang, Barbara A. Wible, Xiaoping Wan, and Eckhard Ficker

Rammelkamp Center for Education and Research, MetroHealth Campus, Case Western Reserve University, Cleveland, Ohio (L.W., X.W., E.F.); and ChanTest Inc., Cleveland, Ohio (B.A.W.)

Received August 26, 2006; accepted November 8, 2006

ABSTRACT

Direct block of the cardiac potassium channel human ether-a-go-go-related gene (hERG) by a large, structurally diverse group of therapeutic compounds causes drug-induced QT prolongation and torsades de pointes arrhythmias. In addition, several therapeutic compounds have been identified more recently that prolong the QT interval by inhibition of hERG trafficking to the cell surface. We used a surface expression assay to identify novel compounds that interfere with hERG trafficking and found that cardiac glycosides are potent inhibitors of hERG expression at the cell surface. Further investigation of digoxin, ouabain, and digoxin revealed that all three cardiac glycosides reduced expression of the fully glycosylated cell surface form of hERG on Western blots, indicating that channel exit from the endoplasmic reticulum is blocked. Likewise, hERG currents were reduced with nanomolar affinity on long-term exposure. hERG trafficking inhibition was initiated by cardiac glycosides through direct block of Na+/K+ pumps and not via off-target interactions with hERG or another closely associated protein in its processing or export pathway. In isolated guinea pig myocytes, long-term exposure to 30 nM of the clinically used drugs digoxin or digitoxin reduced hERG/rapidly activating delayed rectifier K+ current (I[Kr]) currents by approximately 50%, whereas three other cardiac membrane currents—inward rectifier current, slowly activating delayed rectifier K+ current, and calcium current—were not affected. Importantly, 100 nM digitoxin prolonged action potential duration on long-term exposure consistent with a reduction in hERG/I[Kr] channel number. Thus, cardiac glycosides are able to delay cardiac repolarization at nanomolar concentrations via hERG trafficking inhibition, and this may contribute to the complex electrocardiographic changes seen with compounds such as digitoxin.

The cardiac potassium channel hERG encodes the α-subunit of the rapid delayed rectifier current IKr in the heart that contributes prominently to terminal (phase 3) repolarization in human ventricular myocytes. The strategic position occupied by hERG/IKr channels in cardiac repolarization was uncovered first in patients with familial long QT syndrome (LQTS2) with the identification of inherited mutations in the hERG gene (KCNH2) that were ultimately shown to reduce hERG/IKr currents (Sanguinetti et al., 1995; Sanguinetti and Tristani-Firouzi, 2006). In symptomatic patients, LQTS is characterized by a prolongation of the QT interval on the electrocardiogram, which is considered a surrogate marker for ventricular repolarization at the cellular level, and, as a direct consequence, an increased propensity to develop arrhythmias that may deteriorate into ventricular fibrillation and cause sudden cardiac arrest (Keating and Sanguinetti, 2001). Similar electrocardiographic abnormalities can be induced via direct block of hERG/IKr channels by a large group of structurally diverse therapeutic compounds, including many antiarrhythmics, antiarrhythmics, anti-arrhythmics, and antibiotics (Fermini and Fossa, 2003), a phenomenon known as drug-induced or acquired long QT syndrome (acLQTS). Since acLQTS due to unintended block of hERG channels is largely preventable, regulatory agencies have removed certain drugs such as terfenadine and cisapride from the market place and restricted the use of others. Based on this well recognized susceptibility of hERG for block by small organic molecules, regulatory guidelines are currently being instituted for the development of novel therapeutic agents.

This work was supported by National Institute of Health Grant HL71789 (to E.F.) and CA106028 (to B.A.W.). B.A.W. is an employee of ChanTest Inc., a company that provides hERG trafficking assays as part of their services. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.106.113043.

The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: hERG, human ether a-go-go-related gene, IKr, rapidly activating delayed rectifier K+ current, IKs, slowly activating delayed rectifier K+ current; LQTS, long QT syndrome; acLQTS, acquired long QT syndrome; HEK, human embryonic kidney; WT, wild-type; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; [K+]o, extracellular K+ concentration; MEM, minimum Eagle’s medium; K-sf, serum-free potassium; CHO, Chinese hamster ovary; IKr, inward rectifier current; ER, endoplasmic reticulum; fg, fully glycosylated; cg, core glycosylated; APD, action potential duration; E4031, 1-[2-(6-methyl-2-pyridyl)ethyl]-4-(4-methylsulfonyl aminobenzoyl)pipendine.
compounds that include the assessment of direct hERG block in electrophysiological measurements, ion flux, or binding assays (Fenichel et al., 2004).

More recently, we have described a novel mechanism for acLQTS that goes undetected in conventional safety studies. We have shown that at least two therapeutic compounds, arsenic trioxide, which is used in the treatment of acute promyelocytic leukemia, and the antiprotozoal agent pentamidine, reduce hERG/I_{Ks} currents not by direct block but by inhibition of hERG/I_{Ks} trafficking to the cell surface (Ficker et al., 2004; Kuryshhev et al., 2005). In contrast to direct hERG block where a detailed mechanistic understanding of the "universal" drug binding site of hERG may be exploited in the near future to avoid unintended drug-channel interactions (Sanguinetti and Tristani-Firouzi, 2006), less is known about how small organic molecules impede hERG trafficking and whether certain compound classes exist that are particularly prone to inhibit hERG trafficking. A critical first step depends on the systematic identification of novel compound classes that reduce cell surface expression of hERG with high specificity. To accomplish this, we have recently developed a high-throughput antibody-based chemiluminescent assay to monitor heterologous expression of hERG channels at the cell surface (Wible et al., 2005).

Using this assay, we have discovered that cardiac glycosides constitute a novel compound class that decreases hERG surface trafficking with nanomolar affinity. Cardiac glycosides are well known specific inhibitors of Na^+/K^-ATPases, the molecular pumps that maintain the steep Na^+ and K^+ gradients at the plasma membrane, and they have been used for many years in the treatment of congestive heart failure and/or atrial fibrillation (Hauptman and Kelly, 1999; Gheorghiade et al., 2004; Wasserstrom and Aistrop, 2005). However, due to a narrow therapeutic window their clinical use has always been troubled by cardiac toxicity involving lethal arrhythmias (Ma et al., 2001; Grimard et al., 2005). The identification of cardiac glycosides as potent hERG trafficking inhibitors will further our understanding of the mechanisms that may underlie adverse cardiac events triggered by cardiac glycosides.

**Materials and Methods**

**Cell Culture.** Human embryonic kidney (HEK)293 cells stably expressing hERG WT or hERG WT HA_{ex} with an extracellular hemagglutinin (HA) tag (Ficker et al., 2003, 2004) were maintained at 37°C, 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, l-glutamine, penicillin/streptomycin, and Geneticin/zeocin for selection.

Human embryonic kidney (HEK)293 cells stably transfected with pcDNA3-RCNQ1(KvLQT1)/pZEOSV2-RCNE1 (minK) and maintained in MEM-α supplemented with 10% fetal bovine serum, l-glutamine, penicillin/streptomycin, and Geneticin/zeocin for selection.

**Chemiluminescence Detection of Cell Surface hERG Protein.** Stably transfected HEK/hERG WT HA_{ex} cells were plated at 40,000 cells/well in a 96-well plate. After overnight incubation with test compounds, cells were fixed, blocked, and incubated for 1 h with anti-h-ERG monoclonal antibody (Roche Diagnostics). After washing, horseradish peroxidase-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and the double-stranded DNA stain SYBR Green (Invitrogen) were added for 1 h (Wible et al., 2005). SYBR Green fluorescence was measured to determine cell numbers. Chemiluminescent signals were developed using SuperSignal (Pierce Chemical, Rockford, IL) and captured in a Beckman Coulter DTX 880 plate reader (Beckman Coulter, Fullerton, CA). Luminescence signals were corrected for cell loss as measured by SYBR Green fluorescence with the data presented as normalized surface expression relative to control.

**Western Blot Analysis.** Antibodies used in the present study have been described previously (Ficker et al., 2003, 2004). In brief, HEK/hERG and L/hKv1.5 cells were solubilized for 1 h at 4°C in a lysis buffer containing 1% Triton X-100 and protease inhibitors (Complete; Roche Diagnostics). Protein concentrations were determined by the BCA method (Pierce Chemical). Proteins were separated on SDS polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and developed using either rabbit anti-hERG polyclonal antibody (hERG 519) or rabbit anti-h-Kv1.5 polyclonal antibody followed by ECL Plus (GE Healthcare, Piscataway, NJ). For quantitative analysis, chemiluminescent signals were captured directly on a Storm PhosphorImager (GE Healthcare) (Ficker et al., 2003).

**Cellular Electrophysiology.** HEK/hERG, L/hKv1.5, and CHO/KCNQ1-KCNE1 cells were recorded using patch pipettes filled with 100 mM K^-aspartate, 20 mM KCl, 2 mM MgCl_2, 1 mM CaCl_2, 10 mM EGTA, and 10 mM HEPES, pH 7.2. The extracellular solution was 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 1.8 mM CaCl_2, 10 mM HEPES, and 10 mM glucose, pH 7.4. To study long-term drug effects on currents, cardiac glycosides were added for 16 to 20 h (overnight) before recording. Stock solutions for all compounds were prepared in dimethyl sulfoxide. Final dimethyl sulfoxide concentrations in drug-containing solutions did not exceed 0.1%. The cardiac membrane currents I_{Ks}, I_{Kr}, and I_{K1} were recorded in freshly isolated ventricular guinea pig myocytes to study short-term effects of cardiac glycosides or in guinea pig myocytes cultured overnight in M199 medium at 37°C in the presence of 5% CO_2 to study long-term effects (Ficker et al., 2003) using the following intracellular solution: 119 mM K^-gluconate, 15 mM KCl, 3.75 mM MgCl_2, 5 mM EGTA, 5 mM HEPES, 4 mM K^-ATP, 14 mM phosphocreatine, 0.3 mM Tris-GTP, and 50 U/ml creatine phosphokinase, pH 7.2. The extracellular solution was 132 mM NaCl, 4 mM KCl, 1.2 mM MgCl_2, 1.8 mM CaCl_2, 5 mM HEPES, and 5 mM glucose, pH 7.4. In recordings of cardiac potassium currents, Ca^{2+} currents were blocked with 1 μM nisoldipine. The specific hERG/I_{Ks} blocker E4031 was used to isolate I_{Ks} currents. Cardiac calcium currents were recorded using the following extracellular solution: 137 mM NaCl, 5.4 mM CsCl, 1.8 mM MgCl_2, 1.8 mM CaCl_2, 10 mM glucose, and 10 mM HEPES, pH 7.4. The intracellular solution was 130 mM CsMeSO_4, 20 mM tetraethylammonium chloride, 1 mM MgCl_2, 10 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, 14 mM Tris-phosphocreatine, 0.3 mM Tris-GTP, and 50 U/ml creatine phosphokinase, pH 7.2. Action potentials were recorded either in freshly isolated ventricular guinea pig myocytes or in myocytes kept in short-term (overnight) culture using the perforated patch technique to preserve the intracellular milieu of the intact cardiomyocyte. In brief, patch pipettes were back-filled with 120 mM K^-aspartate, 20 mM KCl, 10 mM NaCl, 2 mM MgCl_2, and 5 mM HEPES, pH 7.3, supplemented with 240 μg/ml amphotericin-B (Sig-
ma-Aldrich, St. Louis, MO). The extracellular solution was Tyrode's solution (137 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 10 mM glucose, and 10 mM HEPES, pH 7.3). Following seal formation, access resistance was continuously monitored. Experimental protocols were initiated after the access resistance dropped below 40 MΩ. Action potentials were elicited in current clamp mode at a stimulation frequency of 0.1 Hz. pClamp software and an Axon 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA) were used for the generation of voltage/current-clamp protocols and data acquisition. To analyze current densities, membrane capacitance was measured using the analog compensation circuit of the patch-clamp amplifier. All current recordings were performed at room temperature (20–22°C). Cardiac action potentials, however, were recorded at near physiological temperatures of 30–32°C.

Data Analysis. Data are expressed as mean ± S.E.M. of n experiments or cells studied. Differences between means were usually tested using either a two-tailed Student's t test or single-factor analysis of variance followed by a two-tailed Dunnett's test to determine whether multiple treatment groups were significantly different from control. P values <0.05 were considered statistically significant. Concentration-response relationships were fitted to a Hill equation of the following form: $I_{\text{drug}}/I_{\text{control}} = 1/(1 + [D/IC_{50}]^nH)$, where $I$ indicates current or image densities, [D] is the drug concentration, nH is the Hill coefficient, and IC$_{50}$ is the drug concentration necessary for half-maximal inhibition.

Results

Identification of Cardiac Glycosides as a Compound Class That Reduces Cell Surface Expression of hERG.

To identify therapeutic compounds that may increase cardiac risk by reducing cell surface expression of hERG/I$_{Kr}$, we have developed a quantitative antibody-based chemiluminescent assay to monitor the heterologous expression of hERG channels at the cell surface using an HA epitope tag engineered into the extracellular loop spanning transmembrane domains S1 and S2 (hERG WT HAex; Wible et al., 2005). Using this assay, we exposed HEK293 cells stably expressing hERG WT HAex overnight in a 96-well format to increasing concentrations of 14 different cardiac glycosides. These included two clinically relevant drugs, digoxin and digitoxin, both of which are prototypical cardiac glycosides characterized by a steroid body, terminal sugar residues and an unsaturated lactone ring at position C17. In addition, we tested cardiac glycosides without carbohydrate moieties (so-called "genins"); the compound pair ouabain/dihydro-ouabain, which differs only in saturation of the lactone ring; and several natural glycosides, including bufalin, proscillaridin A, and peruvoside. We found that 11 of 14 cardiac glycosides used in our cell-based assay reduced cell surface expression of hERG with nanomolar affinity (Fig. 1; Table 1).

Evaluation of Cardiac Glycosides as hERG Trafficking Inhibitors Using Western Blots and Current Recordings. Arsenic trioxide and pentamidine both reduce hERG surface expression by inhibition of channel trafficking from the endoplasmic reticulum (ER) to the cell surface. To determine whether this is the case with cardiac glycosides, we performed Western blot experiments to test for changes at the protein level using three compounds: the two in clinical use today, digoxin and digitoxin, which in patients reach plasma concentrations of 1 to 2 and 20 to 30 nM, respectively (Smith and Haber, 1970; Lehmann et al., 2000), as well as ouabain, which is used extensively as a research tool. In these experiments, we exposed stably transfected HEK/hERG WT cells overnight to increasing concentrations of digoxin, digitoxin, or ouabain. hERG is synthesized as an immature, ER-resident protein of 135 kDa and as a fully glycosylated protein of 155 kDa that is found at the cell surface (Zhou et al., 1998). In our experiments, we show that overnight incubation with all three cardiac glycosides produced a concentration-dependent decrease in the amount of mature, fully glycosylated cell surface protein, whereas the amount of ER-resident hERG protein was either unaltered or increased at higher drug concentrations as a consequence of hERG trafficking inhibition (Fig. 2A). Production of the fully glycosylated 155-kDa form of hERG was suppressed with IC$_{50}$ values of 18.1 ± 1.6, 27.8 ± 2.1, and 60.8 ± 6.2 nM for digitoxin, ouabain, and digitoxin, respectively (n = 3; Fig. 2B).

To examine the functional consequences of hERG trafficking inhibition in HEK/hERG WT cells more directly, we recorded hERG currents from cells after overnight exposure to increasing concentrations of either digitoxin, digitoxin, or ouabain. As expected from the Western blot data, we found

<table>
<thead>
<tr>
<th>Cardiac Glycosides</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bufalin</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Proscillaridin A</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Peruvoside</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>Nerifolin</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>Ouabain, 1</td>
<td>9.8 ± 0.3</td>
</tr>
<tr>
<td>Digitoxin, 2</td>
<td>13.9 ± 2.5</td>
</tr>
<tr>
<td>Lanatoside C</td>
<td>60.2 ± 4.1</td>
</tr>
<tr>
<td>Digitoxigenin, 3</td>
<td>62.8 ± 9.4</td>
</tr>
<tr>
<td>Digoxin, 4</td>
<td>73.1 ± 11.4</td>
</tr>
<tr>
<td>Dihydroouabain, 5</td>
<td>194.6 ± 28.0</td>
</tr>
<tr>
<td>Digoxigenin, 6</td>
<td>206.3 ± 30.8</td>
</tr>
<tr>
<td>Strophanthidol</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Gitoxigenin</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>Ouabagenin, 7</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A., ouabagenin did not affect hERG surface expression in the concentration range studied.

Fig. 1. Effects of cardiac glycosides on surface expression of hERG. Overnight treatment with cardiac glycosides reduces surface expression of HAex-tagged hERG protein stably expressed in HEK293 cell in a concentration-dependent manner. Surface expression levels were determined by chemiluminescence measurements and normalized relative to untreated controls. Shown are concentration-response curves for seven different cardiac glycosides. Concentration-response data were prepared from at least three independent assays and fitted to Hill equations with the Hill coefficient kept constant at 3. IC$_{50}$ values for all cardiac glycosides tested in the hERG surface expression assay are shown in Table 1.
that hERG currents were reduced by analyzing tail current amplitudes on return to −50 mV after maximal activation at +60 mV (Fig. 3A). The IC_{50} values measured were 11.1 ± 1.4 nM for digitoxin, 15.2 ± 0.9 nM for ouabain, and 53.9 ± 14.3 nM for digoxin (n = 3–11; Fig. 3B), which is similar to the dose-dependent reductions in fully glycosylated, mature hERG protein determined from Western blots. To exclude that hERG currents are directly blocked by cardiac glycosides, we examined the effects of short-term (acute) extracellular application of digitoxin and digoxigenin on hERG currents. hERG currents were activated by depolarizing pulses to +20 mV from a holding potential of −80 mV and tail currents were measured on return to −50 mV in the absence and presence of 500 nM digoxin, which is approximately 5 times the concentration needed for half-maximal block of channel trafficking. With 500 nM digoxin in the extracellular perfusate, we observed after 5 min, on average, an inhibition of 12.7 ± 5.8% (n = 5), which was not different from current rundown observed over the same time period under control conditions (5 min; 8.4 ± 3.3%; n = 5; Fig. 3C). With the aglycon digoxigenin at 1000 nM, hERG tail currents decreased on average by 18.1 ± 2.5% (n = 4), which was also not significantly different from control.

Is Na⁺/K⁺-ATPase Inhibition Required for hERG Trafficking Inhibition? As a class, cardiac glycosides are generally thought to bind to and inhibit Na⁺/K⁺-ATPase function with high potency and specificity. Nevertheless, considerable evidence exists that cardiac glycosides may also target sites other than the Na⁺/K⁺-ATPase. For example, cardiac glycosides directly activate calcium release channels in the sarcoplasmic reticulum of the heart (Sagawa et al., 2002; Nishio et al., 2004) and modulate proteins in the nuclear factor-kB signaling pathway (Srivastava et al., 2004). Given this multiplicity of effects, we designed two experiments to directly address possible off-target effects. In a first set of experiments, we took advantage of an ouabain-resistant rat α1-subunit encoded by the pCMV Ouabainr vector, which we transiently transfection together with hERG WT cDNA into HEK293 cells. We then incubated cotransfected HEK293 cells overnight with increasing concentrations of ouabain and compared those transfections with similarly treated cells, which had been transfected with hERG WT and a pcDNA3 vector control. We found that cotransfection of the ouabain-resistant Na⁺/K⁺-ATPase subunit attenuated the reduction of the fully, glycosylated cell surface form of hERG observed with ouabain (Fig. 4A). In a second set of experiments, we inhibited the Na⁺/K⁺ pump of HEK293 cells by using a potassium-free extracellular solution (Orlov et al., 1999). In these experiments, we incubated stably transfected HEK/hERG WT cells overnight in defined serum-free salt solutions with 5K-sf (physiological control) and 2K-sf or in a solution that was nominally potassium-free (0K-sf). On Western blots, we found that overnight incubation with 0K-sf reduced the amount of mature, fully glycosylated cell surface protein by 90%, whereas 2 mM [K⁺]_o did not alter expression of the cell surface form of hERG compared with controls incubated with 5 mM [K⁺]_o (Fig. 4, B and C; n = 3). The data from Western blots were closely mirrored in electrophysiological current recordings performed in the presence of 5 mM [K⁺]_o to test for the functional effects of overnight incubation with 0K-sf. In these experiments, maximal hERG tail current densities were significantly reduced from 49.3 ± 6.8 pA/pF after overnight incubation in 5 mM [K⁺]_o to 2.5 ± 0.8 pA/pF after incubation in nominally potassium-free solution.
Cardiac Glycosides Do Not Affect hKv1.5 and KCNQ1/KCNE1 Trafficking. Since the Na+/K+ pump is critical for maintaining intracellular ion homeostasis, inhibition of the pump will alter intracellular Na+ and Ca2+ concentrations, which could affect the processing and trafficking of many different ion channel proteins in a nonspecific manner. To address the specificity of cardiac glycoside effects on hERG trafficking, we exposed stable cell lines constitutively expressing either hKv1.5, a cardiac potassium channel expressed prominently in the atrium, or KCNQ1/KCNE1 heteromultimers, encoding the cardiac slow delayed rectifier current I_{Kr}, to increasing concentrations of digoxin, and we performed Western blots and/or electrophysiological current recordings. After overnight incubation with 10, 100, or 300 nM digoxin, hKv1.5 was detected on Western blots both as a core-glycosylated, immature ER form of approximately 68 kDa and as a mature, fully glycosylated cell surface form of approximately 75 kDa (Fig. 5A), suggesting that long-term exposure to digoxin did not alter the expression pattern of hKv1.5 (Fig. 5B). In line with our biochemical data, we found that hKv1.5 current densities were not significantly reduced upon overnight incubation with 100 nM digoxin (Fig. 4C). We determined current densities at the end of depolarizing pulses to +20 mV and measured 738.9 ± 40.3 pA/pF under control conditions and 740.9 ± 59.9 pA/pF on overnight exposure to 100 nM digoxin (n = 10–11; Dunnett’s t test; p < 0.05). Taken together, our experiments suggest that hERG trafficking inhibition is initiated by functional inhibition of plasmalemmal Na+/K+ ATPases and not another cellular protein critically involved in hERG processing and trafficking.

Cardiac Glycosides and hERG Trafficking 529

In addition, we recorded KCNQ1/KCNE1 currents in CHO cells under control conditions and after overnight exposure to 100 and 300 nM digoxin (Fig. 6A). At the end of 2-s depolarizing pulses to +40 mV, we measured current densities of 138.1 ± 25.5 pA/pF (n = 10) under control conditions and 97.2 ± 13.3 and 107.3 ± 22.5 pA/pF (n = 9–10) after overnight exposure to 100 and 300 nM digoxin, respectively (Fig. 6B). In a second set of experiments, we inhibited the Na+/K+ pump by using a potassium-free extracellular solution, and we tested for effects on KCNQ1/KCNE1 current amplitudes. At the end of 2-s depolarizing pulses to +40 mV, we measured electrophysiological recordings performed in the presence of 5 mM [K+]o with KCNQ1/KCNE1 current densities of 123.0 ± 13.5 pA/pF after overnight incubation in 5 mM [K+]o and 114.8 ± 18.3 pA/pF after incubation in nominally potassium-free solution (Fig. 6, C and D; n = 9–14). Thus, neither hKv1.5 nor KCNQ1/KCNE1 was significantly affected by long-term exposure to digoxin or potassium-free extracellular solution, supporting specificity of the cardiac glycoside-induced hERG trafficking block.
Evaluation of Cardiac Glycoside Effects in Guinea Pig Ventricular Myocytes. Cardiac glycosides play an important role in the treatment of patients with congestive heart failure and/or atrial fibrillation (Gheorghiade et al., 2004), but their therapeutic use is associated with an increased susceptibility to cardiac arrhythmias (Ma et al., 2001; Grimard et al., 2005). To determine whether a reduction in cardiac hERG/IKr currents after long-term exposure to cardiac glycosides may contribute to the adverse cardiac events observed during therapy, especially as result of intoxication (Roden, 1998), we cultured isolated guinea pig ventricular myocytes overnight in the presence of increasing concentrations of digoxin. hERG/IKr is expressed as a cg protein of 68 kDa and an fg mature protein of 75 kDa. B, image densities of fully glycosylated and core-glycosylated hKv1.5 protein bands quantified as function of digoxin concentration using a PhosphorImager. All image densities were normalized to the fully glycosylated 75-kDa protein form of hKv1.5 measured under control conditions (n = 3). C, representative hKv1.5 currents recorded after overnight culture under control conditions or after long-term exposure to 100 nM digoxin. Cells were held at −80 mV, test pulses were applied from −70 to +40 mV in increments of 10 mV. Tail currents were recorded on return to −100 mV. Dash to left of current traces indicates zero current level. D, quantification of hKv1.5 current densities measured after overnight culture under control conditions or in the presence of 100 nM digoxin. Current densities were measured at the end of a depolarizing tail pulse to +20 mV and normalized to cell capacitance. Current densities were not significantly different between control and digoxin-treated cells (n = 10–11).

Fig. 5. Digoxin does not alter expression of hKv1.5. A, Western blot showing hKv1.5 protein stably expressed in L-cells after overnight exposure to increasing concentrations of digoxin. Hk1.5 is expressed as a cg protein of 68 kDa and an fg mature protein of 75 kDa. B, image densities of fully glycosylated and core-glycosylated hKv1.5 protein bands quantified as function of digoxin concentration using a PhosphorImager. All image densities were normalized to the fully glycosylated 75-kDa protein form of hKv1.5 measured under control conditions (n = 3). C, representative hKv1.5 currents recorded after overnight culture under control conditions or after long-term exposure to 100 nM digoxin. Cells were held at −80 mV, test pulses were applied from −70 to +40 mV in increments of 10 mV. Tail currents were recorded on return to −100 mV. Dash to left of current traces indicates zero current level. D, quantification of hKv1.5 current densities measured after overnight culture under control conditions or in the presence of 100 nM digoxin. Current densities were measured at the end of a depolarizing test pulse to +20 mV and normalized to cell capacitance. Current densities were not significantly different between control and digoxin-treated cells (n = 10–11).
similar reductions in hERG/I\textsubscript{Kc} current density from 0.68 ± 0.08 pA/pF in control myocytes to 0.42 ± 0.03 pA/pF at 30 nM and 0.21 ± 0.02 pA/pF at 100 nM digitoxin (n = 5–6; Dunnett’s test; p < 0.05; Fig. 7C). In marked contrast, two additional cardiac potassium currents, the slow delayed rectifier current I\textsubscript{Ks} and the inward rectifier current I\textsubscript{K1}, as well as cardiac calcium currents, were not affected by long-term exposure to 100 nM digitoxin (Fig. 7, D–F).

Based on the long-term effects exerted by cardiac glycosides on hERG/I\textsubscript{Kc} currents, we hypothesized that cardiac action potentials may similarly be prolonged after long-term but not short-term (acute) exposure to digitoxin. We focused on digitoxin because it reaches much higher plasma levels in patients (20–30 nM) than digoxin (1–2 nM). We studied short-term effects of extracellularly applied digitoxin on cardiac action potentials evoked in freshly isolated guinea pig ventricular myocytes at a stimulation frequency of 0.1 Hz. Current-clamp experiments were performed at 30–32°C in the perforated patch configuration to preserve the intracellular milieu of native cardiomyocytes as much as possible since cardiac glycosides are known to induce complex changes in intracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} concentrations, which depend critically on intracellular buffer capacities as well as the unimpeded function of several ion channel and ion transport proteins. We found that action potential duration was not affected significantly by rapid application of 10 and 100 nM digitoxin with the extracellular perfusate. Action potential duration was 367.4 ± 36.8 ms under control conditions and 374.2 ± 37.3 and 385.0 ± 44.7 ms when measured 5 min after start of perfusion with 10 or 100 nM digitoxin in the bath (n = 5; Fig. 8A). Furthermore, we found that none of three major cardiac membrane currents, I\textsubscript{K1}, I\textsubscript{Ks}, and I\textsubscript{Ca}, were blocked by fast extracellular application of 500 nM digitoxin, which was consistent not only with our action potential recordings but also with experimental data reported by others (e.g., Rocchetti et al., 2003; Supplemental Table 1). To study long-term effects of cardiac glycosides, we cultured guinea pig ventricular myocytes overnight in the presence of either 10 or 100 nM digitoxin and evoked action potentials using the perforated patch-clamp technique (Fig. 8B). In these experiments action potential duration was significantly prolonged from 296.3 ± 27.9 to 360.3 ± 47.6 and 607.2 ± 50.0 ms on incubation with 10 and 100 nM digitoxin, respectively (n = 4–6, Dunnett’s; p < 0.05; Fig. 8C). Thus, the possibility exists that hERG trafficking inhibition may contribute to the increase in cardiac excitability seen with certain cardiac glycosides in the clinic.
that changes in intracellular calcium handling not only are responsible for the inotropic effect produced by cardiac glycosides but also contribute to their considerable arrhythmogenic potential. In line with this hypothesis, ECG manifestations of digoxin toxicity include premature ventricular contractions most probably triggered by spontaneous sarcoplasmic reticulum Ca\(^{2+}\) release events due to an increased Ca\(^{2+}\) load of the sarcoplasmic reticulum (Hauptman and Kelly, 1999). Examples of cardiac arrhythmias precipitated by digoxin include ventricular bigeminy, atrial tachycardia, and varying degrees of altered sinus and atrioventricular node function (Ma et al., 2001; Grimard et al., 2005) but typically not torsade de pointes arrhythmias (Fenichel et al., 2004), which together with QT prolongation are indicative for acLQTS. On the contrary, a shortening of the QT interval seems to be characteristic for patients with symptoms of cardiac glycoside toxicity (Cheng, 2004).

Therefore, we were surprised to find that a large set of 14 different cardiac glycosides reduced surface expression of hERG with high affinity in HEK293 cells, many at nanomolar concentrations. Western blots using clinically relevant digoxin and digitoxin as well as ouabain revealed that reduced surface expression was associated with a decrease in the fully glycosylated cell surface form of hERG. Thus, the dose-dependent reduction in surface expression of hERG protein and of hERG currents on long-term exposure to cardiac glycosides is the result of a drug-induced trafficking block. The effect was specific since neither heterologously expressed hK\(_{1.5}\) nor KCNQ1/KCNE1 channels/currents were affected.

Interestingly, three different experimental approaches suggested that hERG trafficking inhibition was initiated by functional inhibition of plasmalemmal Na\(^+/K\^-\)ATPases and not another cellular protein. First, overexpression of ouabain-insensitive rat Na\(^+/K\^-\)ATPase subunits attenuated the effect of ouabain as a hERG trafficking inhibitor. Second, inhibition of Na/K pumps with nominally potassium free extracellular solution mimicked the effects of cardiac glycosides on hERG trafficking. Finally, we observed a good correlation between hERG trafficking inhibition and the published inhibition of the enzymatic Na\(^+/K\^-\)ATPase activity by a diverse set of cardiac glycosides. Proscillaridin A, peruvoside, nerifollin, ouabain, and digitoxin reduce hERG cell surface expression and bind to/inhibit Na\(^+/K\^-\)ATPases with similar affinity (Paula et al., 2005). Bufalin, however, the most potent inhibitor of hERG surface expression (Table 1), binds to Na\(^+/K\^-\)ATPase with only moderate affinity. This disconnect may be resolved by the observation that bufalin shows much higher potency in assays that monitor the functional inhibition of Na\(^+/K\^-\)ATPase activity rather than drug binding (Paula et al., 2005). A close correlation can also be seen in cardiac glycosides without carbohydrate moieties. Loss of sugar residues not only reduces the binding affinity of the respective molecules by 1 order of magnitude but also decreases their potency as inhibitors of hERG surface expression. Likewise, saturation of the lactone ring in the compound pair ouabain/dihydro-ouabain causes a reduced potency in our cell-based surface expression assay as well as in Na\(^+/K\^-\)ATPase binding/inhibition assays. Overall, most major trends that have been observed with respect to binding affinity and inhibitory potency as a consequence of structural modification of cardiac glycosides seem to be mirrored in our cell surface expression assay. This suggests that hERG traf-

**Fig. 8.** Long-term exposure to digitoxin prolongs the cardiac action potential in isolated guinea pig ventricular myocytes. A, quantitative analysis of action potential duration (APD) on short-term exposure of freshly isolated guinea pig ventricular myocytes to 10 or 100 nM digitoxin. APD was not significantly different in digitoxin-treated and control myocytes (n = 5). B, representative current clamp recordings of action potentials elicited in guinea pig ventricular myocytes cultured overnight under control conditions or in the presence of 10 and 100 nM digitoxin. C, quantitative analysis of APD measured in guinea pig ventricular myocytes cultured overnight under control conditions and in the presence of either 10 or 100 nM digitoxin. APD was significantly prolonged in myocytes cultured overnight in the presence of 100 nM digitoxin (n = 4–6; Dunnett’s test; p < 0.05). All action potentials were recorded at 30–32°C using the perforated patch-clamp technique.

**Discussion**

The cardiac glycoside digoxin is an old and inexpensive medication frequently used in the treatment of congestive heart failure with a major therapeutic benefit thought to be a positive inotropic effect, i.e., an increase in contractility of the failing heart. The mechanism underlying this inotropic effect is directly related to inhibition of Na\(^+/K\^-\)ATPase function by cardiac glycosides that serves to increase intracellular [Na\(^+\)], and it is subsequently translated into elevated intracellular Ca\(^{2+}\) concentrations via the Na\(^+/Ca\(^{2+}\) exchanger (Altamirano et al., 2006). It is now thought
ficking inhibition by cardiac glycosides does not occur via direct interaction with hERG or any closely associated protein necessary for folding or export of hERG from the ER. The specific mechanism coupling inhibition of the Na⁺/K⁺ pump to hERG processing and trafficking in the ER, however, is currently unknown.

Since Na⁺/K⁺-ATPases are considered the main physiological target for the clinically important cardiac glycoside digoxin, which inhibits hERG trafficking with nanomolar affinity, it is puzzling why toxicity in patients has rarely been associated with QT prolongation and/or torsade de pointes arrhythmias (Tan et al., 1998). The simplest explanation may be that plasma concentrations of digoxin typically do not reach levels high enough to inhibit hERG trafficking in patients. We found in guinea pig ventricular myocytes that hERG/Iᵦₑ current density was half-maximally reduced by approximately 30 nM digoxin, whereas three other major cardiac membrane currents were not affected by up to 100 nM. Moreover, digoxin plasma concentrations measured in patients range only from 1 to 2 nM and rarely exceed 3 to 5 nM, even under conditions of severe intoxication (Smith and Haber, 1970). Thus, clinically relevant digoxin concentrations are not likely to affect hERG trafficking.

The situation is different for digitoxin, another cardiac glycoside in use for the treatment of heart failure patients in Europe (Lehmann et al., 2000; Belz et al., 2001; Haux et al., 2001). Digitoxin has a much longer half-life than digoxin, and much higher plasma concentrations, in the range of 20 to 30 nM, are reached during therapy and exceeded under conditions of intoxication (Lehmann et al., 2000). In our experiments, 30 nM digitoxin reduced hERG/Iᵦₑ current density in guinea pig myocytes by half and prolonged cardiac action potential duration. That digitoxin concentrations for half-maximal inhibition of hERG/Iᵦₑ currents are somewhat higher in cardiomyocytes than in HEK/hERG cells (30 versus 11 nM, respectively) may be explained best by expression of different Na⁺/K⁺ pump isoforms or isoform ratios in these two preparations and possibly also by the specific subcellular localization of different Na⁺/K⁺ isoforms in cardiomyocytes (Wasserstrom and Astrup, 2005). Regardless, there is the distinct possibility that hERG trafficking is inhibited by therapeutic concentrations of digitoxin used in patients. Just like digoxin, however, digitoxin has usually not been associated with QT prolongation and torsade de pointes arrhythmias, the electrocardiographic hallmarks of aCLQTS in the clinic. The reason may be that digitoxin exerts multiple, additional effects on heart function, including changes in intracellular calcium handling, as well as neurohumoral changes that alter the functional status of the autonomic nervous system, all of which may contribute to a more complex set of electrical changes and arrhythmias than expected for a simple reduction of hERG currents.

In addition to their value as cardiac drugs, cardiac glycosides have well known antiproliferative properties. In particular, digitoxin is considered a potent anticancer drug effective not only in tumor cell lines (Kometiani et al., 2005; Lopez-Lazaro et al., 2005) but also possibly in patients where antiproliferative effects of cardiac glycosides have been reported for breast cancer, leukemia/lymphomas, and cancer of the kidney/urinary tract (Haux et al., 2001). Because hERG channels are highly expressed in many tumor cells and are thought to control cell proliferation as well as invasiveness, down-regulation of hERG surface expression may facilitate the antiproliferative actions described for cardiac glycosides (Arcangeli, 2005). Furthermore, digitoxin may also be useful to suppress inflammation in cystic fibrosis via inhibition of the nuclear factor-κB pathway (Srivastava et al., 2004) or the cytotoxicity of polyglutamine-expanded proteins (Piccioni et al., 2004). Consequently, digitoxin has been used more recently as a lead compound for drug development (Langenhahn et al., 2005). In addition, natural glycoside compounds such as bufalin or resibufogenin, which are major components of the traditional Chinese medicine chan’su, are being explored as potential drugs, all of which may be burdened by ab initio cardiotoxicity due to hERG trafficking inhibition (Kawazoe et al., 1999; Xie et al., 2001).

In summary, cardiac glycosides, together with two previously described hERG trafficking inhibitors, arsenic trioxide and pentamidine (Ficker et al., 2004; Cordes et al., 2005; Kuryshiev et al., 2005), make up a growing group of compounds that increase cardiac risk not via direct hERG block but indirectly via trafficking inhibition. The significance of drug-induced trafficking inhibition for the development of novel therapeutic compounds is further highlighted by drugs such as celastrol, which belongs to an even larger group of compounds that both block hERG directly and at the same time inhibit its processing and trafficking (Wible et al., 2005; Sun et al., 2006). Even though our observations have no immediate impact on the use of digoxin in the treatment of heart failure and atrial fibrillation, the therapeutic use of digitoxin may be more problematic since hERG trafficking inhibition can contribute to the proarrhythmogenic potential of this particular cardiac glycoside. Finally, the cardiotoxicity of digitalis glycosides contained in natural compounds such as bufalin that inhibit hERG trafficking with very high potency has to be of some concern since natural compounds are regulated less stringently on the market place than “classical” drugs developed by pharmaceutical companies. Overall, the growing number of hERG trafficking inhibitors poses a novel problem for the identification of compounds that impair hERG channel function in preclinical safety studies that are geared toward detection of acute channel block.

References
Kawazoe N, Aiuchi T, Masuda Y, Nakajo S, and Nakaya K (1999) Induction of
Kuryshev YA, Ficker E, Wang L, Hawryluk P, Dennis AT, Wible BA, Brown AM,
Langenhan JM, Peters NR, Guzei IA, Hoffmann FM, and Thorson JS (2005) En-
Paula S, Tabet RMR, and Ball WJ (2005) Interactions between cardiac glycosides
case control and internal dose-response studies. BMC Cancer 11:11.
Kawaroe N, Aiuchi T, Masuda Y, Nakajo S, and Nakaya K (1999) Induction of
apoptosis by bufalin in human tumor cells is associated with a change of intracel-
Keating MT and Sanguinetti MC (2001) Molecular and cellular mechanisms of
Kometiani P, Liu L, and Askari A (2005) Digitalis-induced signaling by Na⁺/K⁺-
Kuryshev YA, Ficker E, Wang L, Hawryluk P, Dennis AT, Wible BA, Brown AM,
Langenhan JM, Peters NR, Guzei IA, Hoffmann FM, and Thorson JS (2005) En-
hancing the anticancer properties of cardiac glycosides by neoglycorandomization.
Proc Natl Acad Sci USA 102:12305–12310.
old patient. A description of a case and review of the literature. Int J Cardiol
Digitoxin inhibits the growth of cancer cell lines at concentrations commonly found
Osborne increases sarcoplasmic reticulum calcium release in cardiac myocytes.
J Pharmacol Exp Ther 306:1181–1190.
Inversion of the intracellular Na⁺/K⁺ ratio blocks apoptosis in vascular smooth
Paula S, Tabet RMR, and Ball WJ (2005) Interactions between cardiac glycosides
and sodium/potassium-ATPase: three-dimensional structure-activity relation-
ships models for ligand binding to the E2-Pi form of the enzyme versus activity
protect against the cytotoxicity of polyglutamine-expanded androgen receptor.
Diverse toxicity associated with cardiac Na⁺/K⁺ pump inhibition: evaluation of
electrophysiological mechanisms. J Pharmac Exp Ther 305:765–771.
82:491–571.
Sagawa T, Sagawa K, Kelly JE, Tsushima RG, and Wasserstrom JA (2002) Activa-
tion of cardiac ryanodine receptors by cardiac glycosides. Am J Physiol
282:H1118–H1126.
between an inherited and an acquired cardiac arrhythmia: HERG encodes the I_{kr}
Sanguinetti MC and Tristani-Firouzi M (2006) hERG potassium channels and car-
Smith TW and Haber E (1970) Digoxin intoxication: the relationship of clinical
Srivastava M, Eidelberg O, Zhang J, Pawelez C, Cashu H, Yang QF, Jacobson KA,
CFTR and suppresses hypersecretion of IL-8 from cystic fibrosis lung epithelial
Kir2.1 and hERG potassium channels by celandrol with dual effects on both ion
Physiol 290:H11781–H11793.
HERG-Lite: a novel comprehensive high-throughput screen for drug-induced
Xie JT, Dey L, Wu JA, Lowell TK, and Yuan CS (2001) Cardiac toxicity of residu-
Zhou Z, Gong Q, Epstein ML, and January CT (1998) HERG channel dysfunction in

Address correspondence to: Dr. Eckhard Ficker, Rammelkamp Center,
MetroHealth Medical Center, 2500 MetroHealth Dr., Cleveland, OH 44109.
E-mail: eficker@metrohealth.org