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Molecular determinants of pentamidine-induced hERG trafficking inhibition

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The abbreviations used are: acLQTS, acquired long QT syndrome; astemizole, 1-[(4-fluorophenyl)methyl]-N-[1-[2-(4-methoxyphenyl)ethyl]-4-piperidyl]-benzimidazol-2-amine; bEAG, bovine ether a-go-go gene; cg, core-glycosylated; ER, endoplasmic reticulum; fg, fully-glycosylated; HEK, human embryonic kidney; hERG, human ether a-go-go-related gene; I_{Kr} , rapidly activating delayed rectifier K current; NRVM, neonatal rat ventricular myocyte; pentamidine, 4,4'-[pentane-1,5-diylbis(oxy)] dibenzenecarboximidamide.

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ABSTRACT

Pentamidine is an anti-protozoical compound that clinically causes acquired long QT syndrome which is associated with prolonged QT intervals, tachycardias and sudden cardiac arrest. Pentamidine delays terminal repolarization in human heart by blocking cardiac inward rectifier currents acutely. At the same time, pentamidine reduces surface expression of the cardiac potassium channel I_{Kr} /hERG. This is unusual in that aCLQTS is caused most often by direct block of the cardiac potassium current I_{Kr} /hERG. The present study was designed to provide a more complete picture of how hERG surface expression is disrupted by pentamidine at the cellular and molecular level. Using biochemical and electrophysiological methods we found that pentamidine exclusively inhibits hERG export from the endoplasmic reticulum to the cell surface in a heterologous expression system as well as in cardiomyocytes. hERG trafficking inhibition could be rescued in the presence of the pharmacological chaperone astemizole. We used rescue experiments in combination with an extensive mutational analysis to locate an interaction site for pentamidine at phenylalanine 656, a crucial residue in the canonical drug binding site of terminally folded hERG. Our data suggest that pentamidine binding to a folding intermediate of hERG arrests channel maturation in a conformational state that cannot be exported from the endoplasmic reticulum. We propose that pentamidine is the founding member of a novel pharmacological entity whose members act as small molecule ‘anti-chaperones’.

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Introduction

Pentamidine is an anti-protozoical compound used in the treatment of trypanosomiasis, leishmaniasis and *Pneumocystis carinii* pneumonia that causes drug-induced or acquired long QT syndrome (acLQTS) in clinical use (Bibler et al., 1988; Burchmore et al., 2002; Girgis et al., 1997; Nacher et al., 2001; Sands et al., 1985; Wharton et al., 1987). AcLQTS produces electrocardiographic abnormalities that have been associated with syncope, torsades de pointes arrhythmias and sudden cardiac death (Kannankeril et al., 2010) and are most often caused by direct block of the cardiac potassium current I_{Kr} /hERG, which is crucial for terminal repolarization in human heart (Sanguinetti and Tristani-Firouzi, 2006). Because hERG block constitutes an adverse side effect of therapeutic compounds as well as a significant hurdle in the development of novel drug compounds, several preclinical assays have been developed for early detection including binding assays and the so-called hERG assay which consists of patch clamp measurements performed on cloned hERG channels (Pollard et al., 2010; Thomsen et al., 2006). Importantly, a number of therapeutic compounds exist that go undetected in conventional assays targeting direct hERG block (Dennis et al., 2007; van der Heyden et al., 2008). For example, we have shown that arsenic trioxide, which is used in the treatment of leukemia (Ficker et al., 2004), reduces the number of hERG channels at the cell surface by inhibiting the maturation of hERG channels in the endoplasmic reticulum (ER) and preventing export from the ER. At the same time, arsenic trioxide increases cardiac calcium currents via oxidative inactivation of the lipid phosphatase PTEN (Wan et al., 2011). Both mechanisms converge to create cardiac repolarization abnormalities which are reflected in a high incidence of adverse cardiac events during therapy (Barbey et al., 2003; Ohnishi et al., 2002; Ohnishi et al., 2000). Similarly, a large number of direct hERG-blockers provide a double hit on cardiac repolarization in that they combine conventional hERG block with unconventional hERG trafficking inhibition (Wible et al., 2005; Rajamani et al., 2006; Takemasa et al., 2008; Obers et al., 2010; Staudacher et al.,

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2011). Unfortunately, few compounds that cause acLQTS by unconventional mechanisms have been fully characterized at the cellular and molecular level.

Dicationic pentamidine is another example of a therapeutic compound that has been associated with a high incidence of cardiac arrhythmias due to a combination of unconventional mechanisms: (1) reduced hERG surface expression and (2) acute block of cardiac inward rectifier channels, which destabilizes the cardiac membrane potential late during repolarization and under resting conditions (Cordes et al., 2005; de Boer et al., 2010; Ficker et al., 2005; Kuryshev et al., 2005). While a mechanistic model for acute block of cardiac inward rectifier channels by pentamidine has been developed (de Boer et al., 2010), the precise mechanisms by which pentamidine interferes with hERG surface expression are unknown. Currently, it is only known that the fully-glycosylated cell surface form of hERG together with the corresponding membrane currents disappear following long-term incubation with pentamidine (Kuryshev et al., 2005). Importantly, pentamidine does not directly block hERG in the therapeutic concentration range (Cordes et al., 2005).

We focus here on how hERG surface expression is disrupted by pentamidine at the cellular and molecular level. We find that pentamidine exclusively inhibits export of hERG from the endoplasmic reticulum (ER). We show for the first time that pentamidine arrests hERG maturation in a non-exportable conformation via binding to a critical amino acid residue, F656, which contributes to the well-characterized canonical drug binding site in the conduction pathway of terminally folded hERG. We propose that pentamidine may inhibit hERG trafficking by acting as a novel pharmacological entity, namely a small molecule ‘anti-chaperone’.

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Materials and Methods

Cell culture

Human embryonic kidney (HEK) 293 cells stably expressing hERG WT (HEK/hERG WT), were maintained at 37°C, 5% CO₂ in DMEM supplemented with 10% fetal bovine serum, L-glutamine, penicillin/streptomycin (complete DMEM) and G418. Point mutations of hERG were engineered either by overlap extension PCR or using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All mutant constructs were verified by sequencing. Several different mutant hERG channel cDNAs were gifts from M. Sanguinetti (University of Utah, Salt Lake City, UT) and S. Zhang (Queen's University, Kingston, ON, Canada). Mutant hERG as well as bEAG WT (bovine ether-à-go-go) were transiently expressed in HEK293 cells using FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), and compared to transiently transfected hERG WT.

HL-1 cardiomyocytes were maintained as described by Claycomb et al., 1998. Briefly, HL-1 cells were plated on culture dishes coated with gelatin and fibronectin and maintained at 37°C in an atmosphere of 95 % humidified air and 5 % CO₂ in Claycomb Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10 % FBS, 2 mM L-glutamine, 0.1 mM norepinephrine (Sigma-Aldrich), penicillin, and streptomycin. The medium was changed approximately every 24-48 h. When confluent, cells were dissociated using trypsin/EDTA (Invitrogen, Carlsbad, CA) and re-suspended in complete Claycomb Medium at a density of 25 x 10⁶ cells/60 mm dish for Western blotting, and at a density of 25,000 cells/gelatin-fibronectin coated coverslip for electrophysiological recordings.

Neonatal rat ventricular myocytes (NRVM) were isolated from dissected hearts of 1-2 day old rat pups as described previously. All procedures conformed to institutional guidelines for the care and use of animals in research. Briefly, hearts were minced in HBSS and tissue fragments were digested overnight with trypsin at 4°C. Trypsinized tissue fragments were treated

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repeatedly for short periods of time with collagenase at 37°C followed by trituration. Dissociated cells were filtered, collected via centrifugation and pre-plated for 2 h to remove fibroblasts. Supernatants from 'pre-plating' dishes were re-plated in DMEM/5% FBS/PS at a density of 4×10^6 NRVMs/60-mm culture dish. NRVM cultures were maintained at 37°C, 5% CO₂ with bromodeoxyuridine added to suppress fibroblast growth. For electrophysiological studies, NRVMs were grown on collagen-coated glass coverslips. Experiments were typically performed 2-4 days after initial plating.

Pentamidine (Sigma-Aldrich) was added to cell cultures for either 0.5-6 h (short-term) or 16-24 h (overnight) prior to Western blot analysis or current recordings. Pentamidine stock solutions were prepared in H₂O. A stock solution of astemizole was prepared in DMSO. Final DMSO concentrations of drug-containing solutions did not exceed 0.1 %.

Cellular electrophysiology

Current recordings from HEK cells and HL-1 cardiomyocytes were performed using patch pipettes filled with: 100 mM K-aspartate, 20 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA, and 10 mM HEPES (pH 7.2). The extracellular recording solution was: 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES, and 10 mM glucose (pH 7.4). Some hERG mutants with ultrafast current inactivation were recorded in the presence of 140 mM KCl as an equimolar substituent for NaCl in the extracellular solution. In NRVMs hERG/I_{Kr} currents were recorded in isotonic Cs⁺-solutions (pipette: 135 mM CsCl, 1 mM MgCl₂, 10 mM EGTA, 10mM HEPES, pH 7.2; extracellular recording solution: 135 mM CsCl, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, 1 μM nisoldipine, pH 7.4; Zhang, 2006). PCLAMP software and an Axon 200B patch clamp amplifier (Molecular Devices, Sunnyvale, CA) were used for the generation of voltage-clamp protocols and data acquisition. To analyze current densities

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membrane capacitance was measured using the analogue compensation circuit of the patch clamp amplifier. All current recordings were performed at room temperature (20-22°C).

Western blot analysis

A previously described polyclonal anti-hERG antibody, rabbit hERG 519, was used to analyze hERG expression in HEK cells (Ficker et al., 2003). In HL-1 cardiomyocytes and NRVMs hERG/I_{Kr} expression was analyzed using a polyclonal anti-hERG-GST antibody from Alomone Labs, Jerusalem, Israel. Briefly, HEK/hERG cells or cardiomyocytes were solubilized for 1 h at 4°C in lysis buffer containing 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.5, 1% Triton X-100 and protease inhibitors (Complete, Roche Diagnostics). Protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Proteins were separated on SDS polyacrylamide gels, transferred to polyvinylidene difluoride membranes and developed using the appropriate anti-hERG antibody followed by horseradish peroxidase-conjugated secondary antibody and ECL Plus (GE Healthcare, Piscataway, NJ). For quantitative analysis, signals were captured directly on a Kodak Imager R4000 (Carestream Health, Rochester, NY).

hERG ubiquitination

In ubiquitination studies, stable HEK/hERG WT cells were transiently transfected with either HA-ubiquitin or His₆-ubiquitin cDNA using Fugene (Roche Diagnostics). Cells were harvested 2 days after transfection. In experiments with pentamidine, cells were treated overnight on the second day following transfection. Whole cell lysates were immunoprecipitated with anti-hERG antibody. Immunoprecipitates were analyzed on Western blots using either antibody to hERG or to the HA epitope fused to ubiquitin. Lysates with His₆-ubiquitin were used as negative control.

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Sucrose gradients

HEK/hERG WT cells (pentamidine treated or untreated controls) were lysed in Digitonin lysis buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1% digitonin and protease inhibitors (Complete, Roche Diagnostics). Soluble material (400-800 µg total protein) was layered onto 15-45% sucrose gradients (150mM NaCl, 10mM Tris, pH 7.4, 0.1% digitonin). Gradients were made using BIOCOMP Gradient Mate (BIOCOMP, Fredericton, NB, Canada) as previously described (Wang et al., 2009). After centrifugation, 275 µl fractions were collected manually from the top of each gradient. Aliquots of individual fractions were concentrated using PAGEprep Protein Clean-Up and Enrichment Kit (Pierce) prior to loading onto a SDS/PAGE gel for Western blotting.

Pulse-chase and hERG-chaperone-interaction studies

Pulse-chase and hERG-chaperone-interaction experiments were performed as described (Ficker et al., 2003). Briefly, HEK/hERG WT cells were starved for 30 min and pulse labeled for 60 min in 100-150 µCi/ml [³⁵S]-methionine/cysteine containing medium. Cells were harvested immediately after labeling or following different chase periods in label-free medium.

Pentamidine was added for 24 h prior to labeling. Cells were lysed in a 0.1% NP40 buffer in the presence of protease inhibitor. Lysates were immunoprecipitated with anti-hERG antibody (Alomone Labs) and collected with Protein G Dynabeads (Dyna, Lake Success, NY).

Immunoprecipitated radiolabeled proteins were separated by SDS-PAGE, and analyzed with a STORM PhosphoImager (GE Healthcare). In pulse chase experiments, image densities of fully-glycosylated (fg) and core-glycosylated (cg) hERG were normalized to the signal of freshly synthesized, cg-hERG protein isolated immediately after radiolabeling at t=0.

To study hERG-chaperone interactions, HEK/hERG WT cells were labeled and chased in the absence or presence of pentamidine as described above. Immunoprecipitations were

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performed either with anti-hERG, anti-Hsp/c70 or anti-Hsp90 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), separated by SDS-PAGE, and analyzed with a STORM PhosphoImager (Ficker et al., 2003). HERG-chaperone interactions were studied following treatment of labeled HEK/hERG cells with the chemical crosslinker dithiobis(succinimidyl propionate) (DSP, Pierce). Cross-linking was reversed by boiling in β -mercaptoethanol /SDS buffer. Image densities of cg-hERG were normalized to densities measured immediately after labeling to assess time-dependent changes in hERG-chaperone interactions as a function of time and drug exposure.

Immunocytochemistry

HEK-hERG cells were grown overnight on poly-lysine coated glass coverslips under control conditions or in the presence of 30 μ M pentamidine. Following incubation, cells were washed with PBS and fixed in ice cold 4 % formaldehyde/PBS for 30 min. After fixation, cells were permeabilized with 0.1 % Triton X-100 and blocked in 5 % goat serum/PBS for 30 - 60 min at room temperature. For double labeling, permeabilized cells were incubated overnight at 4°C with rabbit anti-hERG GST antibody and mouse anti-KDEL antibody (Ficker et al., 2003). The tetrapeptide KDEL, located at the carboxy-terminal sequences of luminal ER proteins, is a common motif expressed in the ER that is well-suited as a compartment marker. For staining, cells were incubated for 2 h at room temperature with secondary anti-rabbit FITC (Jackson Labs, Bar Harbor, ME, USA) and anti-mouse Rhodamine RedX antibody (Jackson Labs). Coverslips were mounted with Vectashield and imaged using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

Data analysis

Data are expressed as mean \pm S.E. of n experiments or cells studied. Differences between means were tested using either a two-tailed Student's *t*-test or single factor analysis of

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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.

Results

Pentamidine inhibits hERG forward trafficking. We and others have previously reported that pentamidine reduces cell surface expression of hERG within 24 h in a concentration-dependent manner (Cordes et al., 2005; Kuryshv et al., 2005). However, whether pentamidine decreases hERG stability directly at the cell surface or, alternatively, inhibits hERG forward trafficking from the ER remained unresolved. To determine hERG surface stability as a function of drug exposure we studied the time course of pentamidine effects by incubating HEK/hERG cells for 0.5-24 h with 30 μ M pentamidine. When analyzed on Western blots (Fig. 1A), we found that the fully-glycosylated 155-kDa cell surface form of hERG (fg-hERG) decayed in the presence of pentamidine with a half-life of 10.5 h, which compared well to a half-life of about 11 h determined for fg-hERG under control conditions in pulse chase experiments (Ficker et al., 2003). In marked contrast, fg-hERG decayed much faster with a half-life of 2.4 h on incubation with 0 $[K^+]_{ex}$, which is known to increase endocytic uptake of hERG channels from the cell surface (Fig. 1B; Guo et al., 2009). The slow reduction of fg-hERG on Western blots was also reflected in electrophysiological recordings, where we measured hERG current densities of 67.1 ± 10.2 pA/pF (n=10) under control conditions, of 69.6 ± 20.8 pA/pF (n=7) following 6 h exposure to pentamidine, and of 4.9 ± 1.7 pA/pF following 24 h exposure to pentamidine (n=6; Fig. 1C, D). Furthermore, expression of the core-glycosylated, 135-kDa ER resident form of hERG (cg-hERG) was not altered upon short-term incubation, while it accumulated significantly on overnight exposure (16-24 h) to pentamidine (see Fig. 1A, B) which is thought to be indicative of impaired forward trafficking.

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To confirm independently that pentamidine inhibits hERG forward trafficking, we performed pulse chase experiments to examine hERG maturation in the absence and presence of pentamidine. In these experiments, the appearance of complex or fully glycosylated hERG was used to monitor ER exit and passage of the channel protein through the Golgi apparatus. We found that hERG maturation from the initially synthesized 135-kDa ER resident form to the fully-glycosylated 155-kD form was blocked, while the ER resident cg-form of hERG accumulated in the presence of 30 μ M pentamidine (Fig. 2A, B). To test whether inhibition of ER exit was also reflected in an altered subcellular localization of hERG, we co-stained HEK/hERG cells in immunocytochemical experiments with anti-hERG and anti-KDEL (a well-established ER marker) antibodies either under control conditions or following overnight exposure to pentamidine (Fig. 2C). While hERG staining could be detected both at the cell surface and in the ER under control conditions, hERG was restricted to the ER on incubation with pentamidine, as expected, when ER export is blocked.

Astemizole rescues pentamidine-induced trafficking inhibition. Astemizole is an anti-histamine known to directly block hERG channels with high affinity in electrophysiological experiments, where IC_{50} values of 6-13 nM have been reported at physiological temperatures (hERGAPDbase; Hishigaki and Kuhara, 2011). More importantly, however, astemizole has been used previously as a pharmacological chaperone to correct trafficking defects in several LQTS-hERG mutations located in the transmembrane region of the channel protein, most likely via direct interactions with the universal drug binding site in the conduction pathway (Ficker et al., 2002). Consequently, we employed astemizole as a conformation-sensitive probe to further dissect inhibition of hERG forward trafficking by pentamidine. Astemizole restored hERG trafficking in the presence of 10 μ M pentamidine half-maximally at a concentration of 335 ± 33 nM (n=3) on Western blots. In the presence of 30 μ M pentamidine, astemizole rescue was half-maximal at 962 ± 89 nM (n=3) with the rescue-response curve parallel shifted to the right (Fig. 3A, B). Furthermore, astemizole was able to restore ER export to control levels at both

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pentamidine concentrations tested, as if pentamidine and astemizole were to compete for overlapping binding sites. To test whether pharmacological rescue would produce functional channels at the cell surface, we also measured hERG currents (Fig 3C). Tail current density was 43.5 ± 4.4 pA/pF (n=7) under control conditions and 4.9 ± 1.5 pA/pF (n=6) following overnight incubation with 30 μ M pentamidine. On co-incubation with 30 μ M pentamidine and 5 μ M astemizole, tail current density was increased to 19.3 ± 4.3 pA/pF (n=6) after astemizole had been washed out for 1 hour prior to recording. Since wash-out of a high affinity blocker like astemizole is notoriously difficult and never complete, we additionally determined tail current densities in HEK/hERG cells incubated solely with 5 μ M astemizole. Under these conditions we measured a current density of 16.4 ± 2.8 pA/pF (n=6) following a 1 hr wash-out of astemizole which was not different from measurements that followed co-incubation with both pentamidine and astemizole. Taken together, our data suggest that pentamidine-induced trafficking inhibition can be almost completely reversed by the pharmacological chaperone astemizole as judged from Western blots, and that fully functional channels are rescued to the cell surface.

Mutational analysis of pentamidine-hERG interactions. Guided by our rescue experiments we surveyed a series of mutations in the transmembrane domain of hERG (S1-S6) that replaced negatively charged glutamate or aspartate residues with alanine, because we considered negatively charged amino acid residues putative interaction partners for positively charged pentamidine. Mutations in the S1-S4 voltage sensor domain included hERG 4EC, which removes not one but 4 glutamate residues in the extracellular S1-S2 linker (Fernandez et al., 2005), hERG D456A, D460A, D466A, D509A, E518A, E519A, D540A and E544A. In the inner pore region (S5-S6) we tested three additional negative charge replacements, hERG E575A, D580A and D591A. We found that none of these mutations affected pentamidine-induced hERG trafficking inhibition (data not shown).

In marked contrast, pentamidine-induced trafficking inhibition was substantially attenuated by hERG F627Y, a unique mutation in the signature sequence of the selectivity filter

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(Fig. 4; supplemental Fig. S1). HERG F627Y was selected for analysis, because it rendered channels less sensitive towards cardiac glycosides, another well characterized class of hERG trafficking inhibitors (Wang et al., 2009; Wang et al., 2007). Since F627Y promotes channel inactivation (Gang and Zhang, 2006), we extended our analysis to hERG S641A, a mutation in the S6 transmembrane helix with accelerated inactivation (Fig. 4B; supplemental Fig. S2) (Bian et al., 2004), and to hERG S631A, a mutation in the extracellular mouth of the conduction pathway with disrupted inactivation (Zou et al., 1998). In both instances we found wildtype behavior which suggested that pentamidine effects were not correlated with inactivation gating (Fig. 4B, C; supplemental Fig. S1).

Next, we focused on the possibility that astemizole and pentamidine may share overlapping binding sites. We approached this hypothesis in a first set of experiments utilizing bovine ether-a-gogo (bEAG) channels instead of engineered hERG mutants. This is for the following reason: both hERG and bEAG belong to the EAG potassium channel family and show a high degree of sequence homology in the inner pore region (S5-6) where the canonical drug binding site of hERG is located (Warmke and Ganetzky, 1994). However, bEAG channels are relatively insensitive to block by ‘classical’ hERG blockers (Ficker et al., 1998). Similarly, astemizole blocks hERG half-maximally at concentrations of 6-13 nM, while bEAG is about 20 to 30-times less sensitive with an IC_{50} value of about 200 nM (Garcia-Ferreiro et al., 2004). Thus we speculated that bEAG may be less sensitive to pentamidine-induced trafficking inhibition, if a hERG-like drug binding site were to form an indispensable mechanistic requirement. Indeed, bEAG trafficking was not affected by pentamidine concentrations up to 100 μ M as judged from analysis of fully-glycosylated cell surface channels on Western blots (Fig. 4A, B). Furthermore, bEAG currents were much less sensitive on long-term exposure to pentamidine than hERG WT channels (Fig. 4C).

At this point our structure-function studies directed us towards the universal drug binding site of hERG as a candidate region for pentamidine interactions. The two most important

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determinants of drug binding to hERG include an aromatic tyrosine (Y652) and phenylalanine (F656) in the S6 transmembrane helix which are complemented by additional residues located at the intracellular mouth of the selectivity filter (Thr623, Ser624, Val625) when high affinity blockers such as astemizole are bound (Mitcheson et al., 2000). We studied the effects of mutations in three positions, hERG S624, Y652 and F656, to evaluate the effects of perturbations of drug binding on pentamidine-induced trafficking inhibition. When hERG S624A was transiently expressed in HEK cells and exposed overnight to either 10 or 30 μM pentamidine, fg-hERG S624A levels were reduced on Western blots to $61 \pm 4.5\%$ and $36 \pm 5.2\%$ ($n=3$), respectively, which was not different from WT levels which were reduced to 61 ± 3.8 and $33 \pm 3.9\%$ ($n=5-6$; supplemental Fig. S3).

Our Western analysis of hERG Y652A revealed an unusual biphasic change in surface expression on overnight incubation with pentamidine, i.e. an initial increase in cell surface hERG Y652A on incubation with 1 μM pentamidine was followed by a decrease with 10 and 30 μM pentamidine. In marked contrast, expression of the immature (cg) form of hERG Y652A was augmented in a dose-dependent manner over the entire concentration range studied (supplemental Fig. S4). The biphasic behavior of fg-hERG Y652A on Western blots was also reflected in electrophysiological recordings, where tail current density increased from -31 ± 5.0 pA/pF ($n=14$) under control conditions to -102.7 ± 19.5 pA/pF ($n=4$) on overnight incubation with 1 μM pentamidine, followed by a decrease to -75.3 ± 13.8 ($n=11$) and -48 ± 6.6 pA/pF ($n=14$) on incubation with 10 and 30 μM pentamidine, respectively.

Next, we analyzed a series of point mutations in position phenylalanine(F)656 which is crucial to drug binding in hERG, including substitutions with tryptophan (W), methionine (M), threonine (T), cysteine (C), alanine (A) and valine (V) (Fernandez et al., 2004; Guo et al., 2006). In general, it is thought that high-affinity drug binding is correlated with measures of hydrophobicity. Specifically, it has been reported that IC_{50} values for several high affinity blockers were increased when F656 was mutated to T, A or V. We transiently expressed all six

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point mutations in HEK293 cells, exposed them overnight to either 10 or 30 μM pentamidine and analyzed the amount of mature fg-hERG on Western blots (Fig. 5A). We found that substitutions in 656 with T, C, A or V produced channels apparently less sensitive to pentamidine than WT or channels with substitutions of W or M (Fig. 5B). To correlate changes in surface expression with changes in current expression we also recorded maximal tail current amplitudes at -120 mV under control conditions or on incubation with either 10 or 30 μM pentamidine (Fig. 5C; supplemental Fig. S5). In line with our Western analysis, we found that three substitutions, threonine, cysteine and valine, were significantly less sensitive to pentamidine than WT or 656W and 656M mutant channels.

How does pentamidine interfere with ER export of hERG? To gain first insight of how pentamidine binding may be translated into inhibition of ER export, we tested, whether pentamidine destabilizes tetrameric channel assembly thereby producing incompletely assembled proteins that are retained in the ER. To assess the oligomerization state of hERG, we separated digitonin lysates of HEK/hERG cells incubated in the absence and presence of 30 μM pentamidine on sucrose gradients. Fig 6 shows a Western analysis of brefeldin A-treated hERG gradient fractions illustrating the distribution of hERG in two prominent protein peaks, P1 and P2, with the majority of hERG protein present in P2. Using Blue Native-PAGE we have previously determined that hERG is present as monomer or dimer in peak P1, while it forms channel tetramers in P2 (Wang et al., 2009). Importantly, we could not detect any quantitative differences in the distribution of hERG on sucrose gradients as a consequence of pentamidine exposure. To evaluate the sensitivity of our assay, we also studied a trafficking impaired LQTS2 mutation, hERG A561V (Ficker et al., 2000). When compared to WT, hERG A561V showed an increase in peak P1 with a concomitant reduction in P2 indicating that assembly of this trafficking-deficient LQTS2 mutant was destabilized (supplemental Fig. S6).

Other possible explanations for ER retention include (1) inhibition of chaperone association in the hERG export pathway as described for geldanamycin or (2) drug-induced

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channel misfolding leading to prolonged hERG-chaperone interactions and ER retention (Ficker et al., 2003). Consequently, we studied interactions of hERG WT with two major cytosolic chaperones, Hsp/c70 and Hsp90, immediately after synthesis, or following a chase period of 6 h in HEK/hERG cells cultured under control conditions or following overnight incubation with pentamidine (Fig 7A). Quite surprisingly, we did not find any difference in the formation and stability of either hERG/Hsp/c70 or hERG/Hsp90 complexes in the presence of pentamidine (Figs. 7B). Thus, pentamidine-induced inhibition of hERG forward trafficking may not be mediated via cytosolic chaperones.

Finally, inhibition of ER export has often been linked to increased channel ubiquitination (Ficker et al., 2003; Gong et al., 2005). Consequently, we measured hERG ubiquitination under control conditions or following overnight exposure to 30 μ M pentamidine. To this end, we expressed hERG WT together with HA- or His₆- tagged ubiquitin. Incubation with the Hsp90 inhibitor geldanamycin known to increase hERG ubiquitination was used as positive control. Following immunoprecipitation with anti-hERG antibody immunoprecipitates were blotted with either anti-hERG or anti-HA antibody to detect multi-ubiquitinated hERG proteins. We found that on Western blots, hERG ubiquitination was increased in the presence of geldanamycin, but not pentamidine (Fig. 8) which actually decreased hERG ubiquitination slightly.

Analysis of pentamidine effects on native hERG/I_{Kr} currents in cardiomyocytes. In addition to our experiments in a heterologous expression system, we have also studied pentamidine effects on native ERG protein and I_{Kr} currents in murine HL-1 cardiac myocytes (Claycomb et al. 1998) as well as in neonatal rat ventricular myocytes (NRVMs). To assess for pentamidine-induced inhibition of channel surface expression, we incubated HL-1 cells overnight with either 1 or 10 μ M pentamidine. On Western blots we found that pentamidine suppressed expression of fully glycosylated full-length mERG1a protein as well as of an N-terminal splice variant, mERG1b, present in HL-1 myocytes in a concentration-dependent manner. In contrast, Kv1.5 expression was not altered by incubation with pentamidine (Fig. 9A). Reduction of cell

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surface mERG was associated with suppression of the corresponding native ion current, I_{Kr} , as expected. Following depolarizing steps from a holding potential of -80 mV slow tail currents were activated on return to -50 mV that specifically reflect ERG-mediated I_{Kr} (see arrows in Fig. 9B). Maximal I_{Kr} density was reduced from 3.9 ± 0.6 pA/pF (n=14) under control conditions to 0.6 ± 0.1 pA/pF (n=5) following overnight incubation with 10 μ M pentamidine (Fig. 9C). When I_{Kr} was blocked by application of 5 μ M E4031, residual I_K currents were analysed at the end of depolarizing test pulses to +60mV. In contrast to I_{Kr} , I_K current density was not significantly affected by 10 μ M pentamidine. We measured 8.8 ± 2.7 pA/pF (n=7) under control conditions and 11.8 ± 3.6 pA/pF (n = 6) on incubation with 10 μ M pentamidine.

Furthermore, we have studied pentamidine effects on native I_{Kr} /rERG channels in cultured NRVMs. Overnight incubation with pentamidine (30 μ M) suppressed the fully glycosylated (fg) cell surface form of rERG on Western blots. Importantly, pentamidine effects could be reversed on co-incubation with 3 μ M astemizole as expected from our experiments with HEK/hERG cells (Fig. 9D). To complement our Western analysis in NRVMs, we also monitored I_{Kr} current density as a function of pentamidine exposure. NRVM I_{Kr} currents were isolated in symmetrical Cs^+ -solutions and elicited using 350ms depolarizing test pulses from a holding potential of -80 mV (Fig. 9E). Maximal I_{Kr} tail current amplitudes measured on return to -80 mV were reduced from -16.5 ± 3.1 pA/pF (n=5) under control conditions to -6.8 ± 1.6 pA/pF (n=5) following overnight incubation with 30 μ M pentamidine (Fig. 9F).

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Discussion

Pentamidine is unusual in that it targets the cardiac potassium channel hERG by reducing its surface expression slowly over time which delays cardiac repolarization and contributes to prolongation of the QT interval on the electrocardiogram (Cordes et al., 2005; Kuryshv et al., 2005). To contrast this unique mode of action with direct hERG blockade, pentamidine has been classified in the past as a hERG trafficking inhibitor which tacitly implied disruption of forward trafficking from the ER as mechanism. However, channel endocytosis in combination with accelerated degradation has emerged more recently as an alternative mechanism used by proarrhythmic compounds to control hERG surface expression. For example, it has been shown that probucol, a cholesterol lowering drug causing acLQTS, increased endocytic hERG uptake from the cell surface (Guo et al., 2011). A similar increase in endocytosis has been described upon exposure of hERG to low extracellular potassium concentrations (Guo et al., 2009). To clarify the cellular and molecular mechanisms that control hERG surface expression in the presence of pentamidine, we have investigated the time course of pentamidine effects on Western blots and in electrophysiological current recordings. In contrast to fast changes in surface expression seen with probucol or on incubation with 0 $[K^+]_{ex}$, short-term effects of pentamidine were minimal over several hours and consistent with a half life of about 10-11 h determined for WT under control conditions (Ficker et al., 2003). Importantly, lack of short-term effects cannot be attributed to slow access of dicationic pentamidine to the cell interior, because acute inward rectifier block was established within 5-10 min following extracellular application of pentamidine (Cordes et al., 2005; de Boer et al., 2010; Kuryshv et al., 2005). Furthermore, we have shown that newly synthesized hERG did not reach the Golgi apparatus using pulse chase experiments and that hERG immunostaining was restricted to the ER in the presence of pentamidine. Thus, we conclude that pentamidine disrupts exclusively hERG forward trafficking from the ER.

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To further dissect inhibition of forward trafficking by pentamidine at the mechanistic level, we have used the pharmacological chaperone astemizole as a conformation-sensitive probe. Astemizole has been used previously to correct conformational defects in mis-folded, trafficking deficient LQTS hERG mutants, while folding and trafficking of wildtype channels were not affected (Ficker et al., 2002). In addition, astemizole rescue was restricted to mutations in the transmembrane domain of the channel protein (Anderson et al., 2006), and relied on binding to phenylalanine656 in the universal drug binding site of hERG (Ficker et al., 2002). By analogy, we speculated that successful rescue experiments with astemizole may help us to uncover and localize pentamidine-induced conformational defects in hERG. In fact, astemizole restored hERG trafficking in the presence of pentamidine indicating first of all that a ‘general’ shut down of protein export from the ER was not the root cause for pentamidine-induced trafficking inhibition. This was not unexpected, because pentamidine reduced hERG surface expression specifically among several other major cardiac ion channels tested (Kuryshv et al., 2005). More importantly, however, successful astemizole rescue suggested an induced conformational defect in the transmembrane region of hERG and provided first hints that astemizole and pentamidine may interact in a competitive manner.

Several predictions from rescue experiments could be verified directly in our mutational analysis. First of all, pentamidine effects were attenuated by hERG F627Y, a mutation in the narrow selectivity filter. Because the amino acid residue in hERG627 does not project in the direction of the conduction pathway, it is neither accessible to dicationic pentamidine nor astemizole. However, a tyrosine residue in hERG627 may provide additional hydrogen bonds to a cuff of aromatic residues that stabilizes the selectivity filter and ultimately the entire channel protein (Doyle et al., 1998). This may also explain why hERG F627Y attenuates not only pentamidine effects, but also trafficking defects induced by cardiac glycosides or on exposure to low K^+ (Massaelli et al., 2010; Wang et al., 2009).

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Secondly, pentamidine effects were reduced by mutations in position hERG F656 that retained relatively normal biophysical properties but decreased sensitivity to direct hERG block (Fernandez et al., 2004). This suggested to us that a binding event similar to the binding of direct blockers in native channels may render hERG trafficking deficient. For example, mutation of F656 to Tyr, Trp or Met did not affect pentamidine-induced trafficking inhibition in line with only modest effects on direct hERG block. In contrast, mutation of F656 to Thr, Cys or Val all of which are relatively insensitive to drug block strongly reduced trafficking inhibition. We also noted differences in the requirements for trafficking inhibition and drug binding. For example, mutation of F656 to Ala seemed to attenuate trafficking inhibition on Western blots but not in current recordings, while it has been shown to decrease direct hERG block very strongly. Similarly, the aromatic side chain in position 652 which is required to maintain drug sensitivity for high-affinity blockers such as MK-499 or cisapride (Mitcheson et al., 2000), was less important for pentamidine induced trafficking inhibition, since hERG Y652A retained at least some sensitivity to pentamidine.

Differences between the mechanisms of direct channel blockade and pentamidine-induced trafficking inhibition were anticipated, since terminally folded hERG channels are not blocked by pentamidine with high affinity in acute experiments (Cordes et al., 2005). In fact, the affinity of pentamidine for direct hERG block at physiological temperature was approximately 300 μM , while hERG trafficking was inhibited half-maximally by only 8 μM pentamidine (Kuryshv et al., 2005). For this reason, we propose that pentamidine binds to an emerging ‘universal drug binding site’ in a folding intermediate of hERG with higher affinity than to the native conformation present in the terminally folded channel protein. We speculate that pentamidine binding arrests conformational maturation of hERG at a level, where channels are not yet exportable from the ER. The pharmacological chaperone astemizole on the other hand rescues pentamidine-induced trafficking inhibition by competing with pentamidine for an at least partially overlapping binding site. However, while astemizole facilitates channel maturation,

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pentamidine binding abrogates it, which is conceptually similar to the binding of an inverse agonist to its receptor target.

This leaves us with the important question of how ER export of hERG is curtailed by cellular quality control mechanisms in the presence of pentamidine. Obviously, the proposed interactions of pentamidine with residues in transmembrane helix S6 could have destabilized channel assembly producing incompletely assembled proteins that are retained in the ER. However, we showed that hERG assembly into tetramers was not disturbed on long-term exposure to pentamidine. Alternatively, pentamidine could have arrested hERG in a slightly aberrant tetrameric conformation that is recognized by cellular chaperones. However, to our surprise association with two crucial cytosolic chaperones, Hsp/c70 and Hsp90, was not altered in the presence of pentamidine. While it is still possible that interactions with another chaperone system may be targeted by pentamidine, we consider this possibility unlikely, because altered chaperone interactions are most often accompanied by an increase in channel ubiquitination and degradation. Again, this could not be shown. In marked contrast, channel ubiquitination was decreased, and immature, ER resident hERG accumulated in the presence of pentamidine (see Fig. 2B). Based on these observations, we suggest that long-term exposure to pentamidine may uncover a chaperone-independent quality control step for hERG. We believe that in the presence of pentamidine hERG matures to a point where chaperone systems disengage and where channels are subjected to a last quality check that does not focus on conformational integrity. Instead, channel proteins may be surveyed for exposed ER retention or export signals similar to what has been described for various inward rectifier channels.

Taken together, we have shown that in the presence of pentamidine hERG is no longer exported from the ER. Thus, inhibitors of hERG surface expression can be grouped in at least two functional classes: those that reduce hERG surface expression by increasing channel endocytosis, and those like pentamidine or arsenic trioxide that prevent channel export from the ER and are best characterized as hERG trafficking inhibitors. Furthermore, we provide the first

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example of a therapeutic compound that arrests hERG maturation in a non-exportable form via binding to residues in the canonical drug binding site of hERG. This is very different from what has been proposed for proarrhythmic compounds like probucol or progesterone (Guo et al., 2011; Wu et al., 2011), which are thought to mediate changes in hERG surface expression via effects on the composition of lipid rafts surrounding the channel protein. The reliance of pentamidine on hERG specific amino acid residues may also provide an answer to its specificity and may explain why pentamidine effects are restricted to ER export, because the native conformation of hERG which is present in post-ER compartments binds pentamidine with much lower affinity only.

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Authorship Contributions

Participated in research design: Deschenes and Ficker

Conducted experiments: Dennis, Wang, Wan, Nassal and Ficker

Performed data analysis: Dennis, Wang, Wan and Ficker

Wrote or contributed to the writing of the manuscript: Deschenes and Ficker

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Footnotes

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Legends for Figures

Fig. 1. Pentamidine does not reduce hERG surface expression on short-term exposure. **A**, Western blot showing time-dependent effects of incubation with 30 μ M pentamidine on hERG stably expressed in HEK293 cells. (fg) indicates fully-glycosylated, 150-kDa cell surface form of hERG; (cg) indicates core-glycosylated, 135-kDa ER-resident form of hERG. **B**, quantitative analysis of time-dependent changes in fg- and cg-hERG levels following exposure to 30 μ M pentamidine. Fast effects of $0K^+$ on fg-hERG (fg, 0K) are due to increased endocytic uptake. Image densities on Western blots were normalized to fg-hERG levels measured at t=0. Note that cg-hERG is increased at t=16-24 h (n=3-4). **C**, representative hERG current families recorded under control conditions, following a 6 h or a 24h (overnight) exposure to 30 μ M pentamidine. Currents were elicited using depolarizing voltage steps from -60 to +60 mV. Tail currents were recorded on return to -50 mV. Holding potential was -80 mV. **D**, time-dependent reduction of hERG tail current densities on exposure to 30 μ M pentamidine (n=6-10). Data are given as mean \pm S.E.

Fig. 2. Pentamidine inhibits hERG forward trafficking. **A**, pulse-chase analysis of hERG maturation in ^{35}S -labeled HEK/hERG cells under control conditions or following overnight incubation with 30 μ M pentamidine. Radiolabeled hERG was isolated by immunoprecipitation after chase periods indicated. Arrows indicate position of fully-glycosylated (fg), and core-glycosylated (cg) forms of hERG. **B**, quantitative analysis of time-dependent changes of fg- and cg-hERG densities measured under control conditions or following overnight incubation with 30 μ M pentamidine (n=3-4). **C**, subcellular localization of hERG protein under control conditions, and following overnight exposure to 30 μ M pentamidine. HEK/hERG cells were fixed, permeabilized and double labeled with anti-hERG and anti-KDEL antibody which was used as

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ER marker. In untreated controls hERG was localized to the cell surface. In cells treated with pentamidine hERG staining was no longer detectable at the cell surface. Instead, hERG staining was restricted to the ER. Scale bar: 20 μ m.

Fig. 3. Rescue of pentamidine-induced trafficking inhibition by incubation with the pharmacological chaperone astemizole. A, Western blot showing effects of overnight treatment with increasing concentrations of astemizole on HEK/hERG cells co-incubated with either 10 or 30 μ M pentamidine; (fg) indicates fully-glycosylated, 150-kDa cell surface form of hERG; (cg) indicates core-glycosylated, 135-kDa ER-resident form of hERG. B, quantitative analysis of concentration-dependent rescue of fg-hERG by astemizole following co-incubation with either 10 or 30 μ M pentamidine. Astemizole restored hERG trafficking in the presence of 10 μ M pentamidine half-maximally at a concentration of 335 ± 33 nM (n=3). In the presence of 30 μ M pentamidine, astemizole rescue was half-maximal at 962 ± 89 nM (n=3). C, maximal hERG tail current densities measured on return to -50 mV (holding potential was -80 mV; 2 sec depolarizing test pulses) under control conditions, on overnight incubation with 30 μ M pentamidine, 30 μ M pentamidine + 5 μ M astemizole (washout, 1 h) and 5 μ M astemizole to determine residual block after 1 h washout (n=6-7). Note, that co-incubation with 5 μ M astemizole significantly increased hERG tail currents following overnight incubation with 30 μ M pentamidine. Current densities measured on rescue were not significantly different from densities measured on incubation with astemizole alone.

Fig. 4. A mutation in the selectivity filter alters sensitivity of hERG to pentamidine induced trafficking inhibition. A, Western blots showing effects of overnight treatment with increasing concentrations of pentamidine on bEAG, hERG WT, hERG F627Y and hERG S631A transiently expressed in HEK293 cells. (fg) indicates the fully-glycosylated, mature form of channel. (cg) indicates core-glycosylated ER resident form of channel. B, quantitative analysis of fully-

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glycosylated form of bEAG (n=3), hERG WT (n=5-6), hERG F627Y (n=6), hERGS641A (n=4) and hERG S631A (n=3) after long term-exposure to 10 and 30 μ M pentamidine. Image densities of fully-glycosylated protein bands on Western blots were captured using a Kodak Imager, quantified and normalized to untreated controls. Black bars indicate a significant difference between fg-WT and bEAG or fg-hERG F627Y on treatment with either 10 or 30 μ M pentamidine (Dunnett's t test; $p < 0.05$). C, hERG WT (n=7-8), bEAG (n=6-7) and hERG S631A (7-9) currents were activated in the presence of 5 mM $[K^+]_{ex}$ from a holding potential of -80 mV with depolarizing pulses (2sec). Maximal hERG WT tail current amplitudes were recorded on return to -120 mV. BEAG and hERG S631A current amplitudes were recorded at the end of depolarizing pulses to +20mV. For hERG F627Y (n=9-7) maximal tail current amplitudes were recorded on return to -80 mV in the presence of 140 mM $[K^+]_{ex}$. Tail current amplitudes were converted into current densities and normalized to amplitudes measured in untreated controls. Black bars indicate a significant difference between WT and bEAG or hERG F627Y current densities on exposure to 30 μ M pentamidine (Dunnett's t test; $p < 0.05$).

Fig. 5. Mutation of phenylalanine 656 in S6 transmembrane helix alters sensitivity to pentamidine-induced trafficking inhibition. A, Western blot showing effects of overnight treatment with increasing concentrations of pentamidine on hERG F656W and hERG F656V transiently expressed in HEK293 cells. (fg) indicates the fully-glycosylated, mature 160 kDa form of hERG (fg). (cg) indicates core-glycosylated ER resident 135 kDa form of hERG. B, quantitative analysis of fully-glycosylated (fg), 160 kDa form of hERG F656 (WT, n=5-6), hERG 656W (n=5), 656Y (n=4), 656M (n=3), 656T (n=4), 656C (n=3), 656A (n=3) and 656V (n=4) after overnight treatment with 10 or 30 μ M pentamidine. Image densities of fully-glycosylated protein bands on Western blots were captured using a Kodak Imager, then quantified and normalized to untreated controls. Black bars indicate a significant difference between fg-F656 (WT) and fg-656T and 656V on treatment with 30 μ M pentamidine (Dunnett's t test; $p < 0.05$).

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C, WT (n=7-8) or mutant currents (W, n=6-8; M, n=7-8; T, n=7-12; C, n=4-6; A, n=6-8; V, n=5-7) were activated in the presence of 5 mM $[K^+]_{ex}$ from a holding potential of -80 mV with a depolarizing pulse to +60 mV (2sec). Tail currents were recorded on return to -120 mV. Tail current amplitudes were converted into current densities and normalized to amplitudes measured under control conditions. Black bars indicate a significant difference between WT and F656T, F656C and F656V on treatment with 30 μ M pentamidine (Dunnett's t test; $p < 0.05$).

Fig. 6. Long-term exposure to pentamidine does not alter tetrameric structure of hERG. A, sedimentation analysis of heterologously expressed hERG WT channels incubated overnight either in the presence of 1 μ g/ml brefeldin A to inhibit complex glycosylation of the channel protein or in a combination of 1 μ g/ml brefeldin A and 30 μ M pentamidine. Digitonin lysates were separated on 15-45% sucrose gradients. Gradient fractions were analyzed by Western blotting using anti-hERG antibody. The direction of sedimentation is indicated by an arrow. Fraction numbers are indicated below the Western blot. Image densities of gradient fractions were analyzed using a Kodak Imager. B, quantification of steady-state sedimentation gradients as shown in (A). Signals from an entire gradient were summed up and set at 100% (total). Individual signals in each fraction were expressed as percent of total. P1 and P2 denote two major hERG peaks corresponding to channel monomers/dimers (P1) and channel tetramers (P2) (n=3).

Fig. 7. Pentamidine does not interfere with Hsp/c70 and Hsp90 function. A, shown is a pulse-chase experiment performed in the absence and presence of 30 μ M pentamidine. Radiolabeled hERG protein was isolated using immunoprecipitation with anti-hERG, anti-Hsp/c70 or anti-Hsp90 antibody after chase periods indicated. To the left of the panel the positions of fg/cg hERG, Hsp/c70 and Hsp90 on the autoradiogram are indicated. Rightmost lane represents a negative control with no primary antibody added. B, quantitative analysis of time-dependent

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formation of hERG-Hsp/c70 and hERG-Hsp90 complexes in the absence and presence of pentamidine (n=3-4). Note that chaperone association is not different between control and pentamidine treated cells.

Fig. 8. Pentamidine does not increase HERG ubiquitination. Western blot analysis of HEK/hERG cells transiently transfected with HA-tagged ubiquitin and treated overnight with either 30 μ M pentamidine or 10 μ M geldanamycin (GA) used as positive control. Transfection with His₆-ubiquitin was used as negative control. Whole cell lysates were immunoprecipitated with anti-hERG antibody, resolved by SDS-PAGE and immunoblotted using either anti-hERG antibody (hERG-IP) or an antibody recognizing the HA epitope fused to ubiquitin (HA-Ub). Immunoblotting with anti-HA antibody identifies high molecular weight forms of ubiquitinated hERG that are increased on treatment with geldanamycin (GA) but not pentamidine.

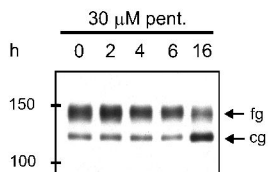
Fig. 9. Pentamidine suppresses endogenous ERG/I_{Kr} currents in HL-1 cardiomyocytes and in neonatal rat ventricular myocytes (NRVMs). A, Western blot showing mERG in HL-1 cardiomyocytes under control conditions and following overnight exposure to either 1 or 10 μ M pentamidine. Note, that both mERG1A and mERG1B were reduced by pentamidine, while mKv1.5 was not affected. Actin was used as loading control. B, mERG currents recorded in HL-1 cardiomyocytes under control conditions or following overnight exposure to 10 μ M pentamidine. Currents were elicited using 2 s depolarizing test pulses from -60 to +60 mV. Holding potential was -80 mV. Tail currents were recorded on return to -50 mV. C, quantitative analysis of maximal mERG tail current densities under control conditions or following overnight exposure to 10 μ M pentamidine (n=6-7). Data are represented in statistical box blots. Note that current densities were significantly different at p<0.05 level. D, Western blot showing rERG in NRVMs under control conditions and following overnight exposure to 30 μ M pentamidine, to 30 μ M pentamidine + 3 μ M astemizole, and to 3 μ M astemizole alone. Note that co-incubation with

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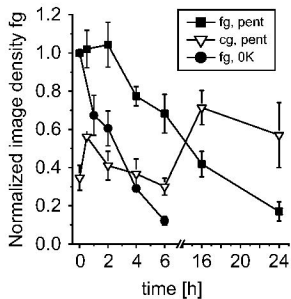
astemizole rescued fg-rERG in the presence of pentamidine. E, rERG currents in NRVMs were elicited in symmetric Cs⁺ solutions using depolarizing test pulses from -60 to + 60 mV. Holding potential was -80 mV. F, quantitative analysis of maximal rERG tail current densities recorded on return to -80mV under control conditions or following overnight exposure to 30 μM pentamidine (n=5). Data are given as mean ± S.E. Note that current densities were significantly different at p<0.05 level.

Figure 1

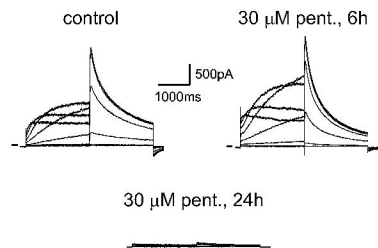
A



B



C



D

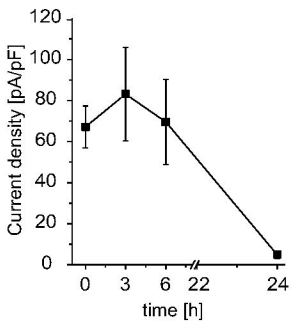
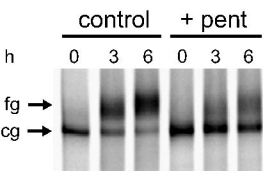
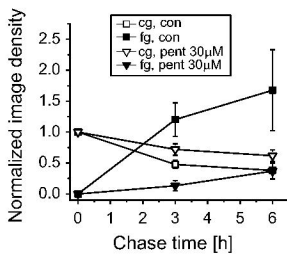


Figure 2

A



B



C

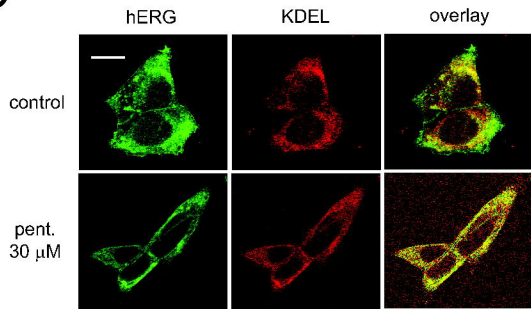
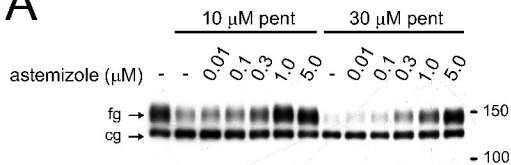
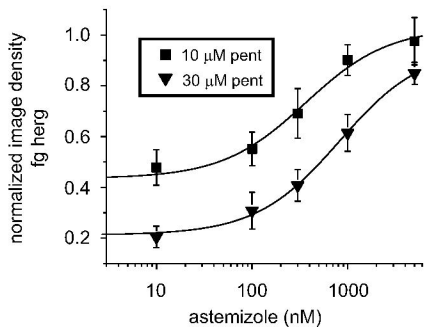


Figure 3

A



B



C

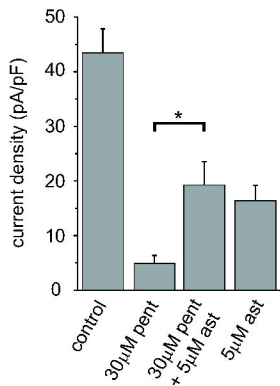


Figure 4

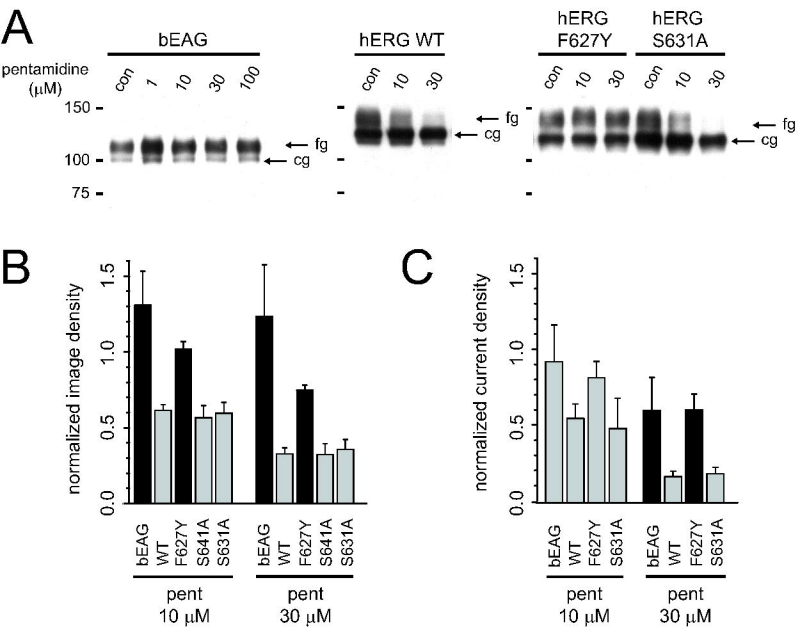
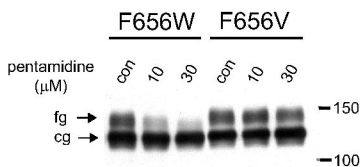
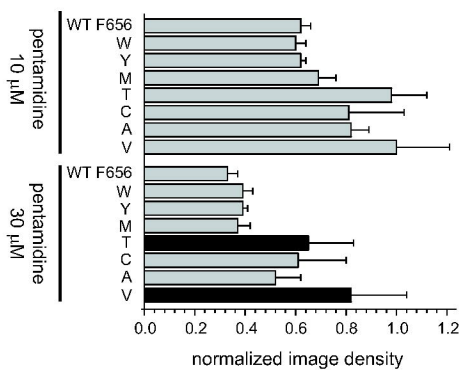


Figure 5

A



B



C

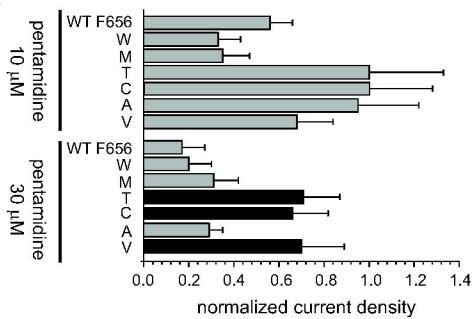
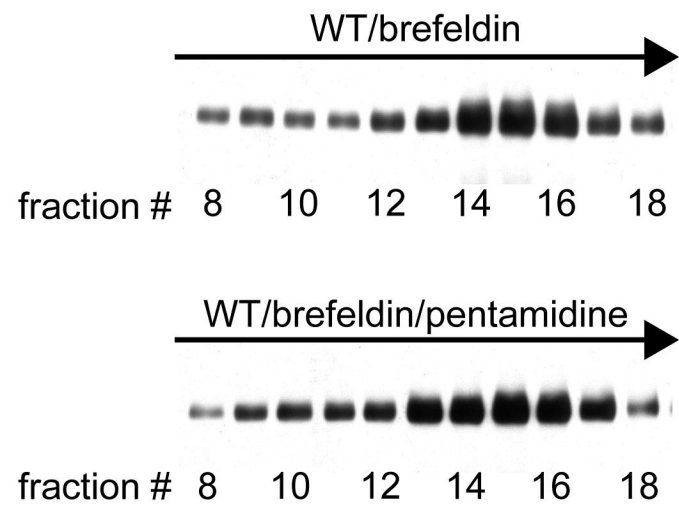


Figure 6

A



B

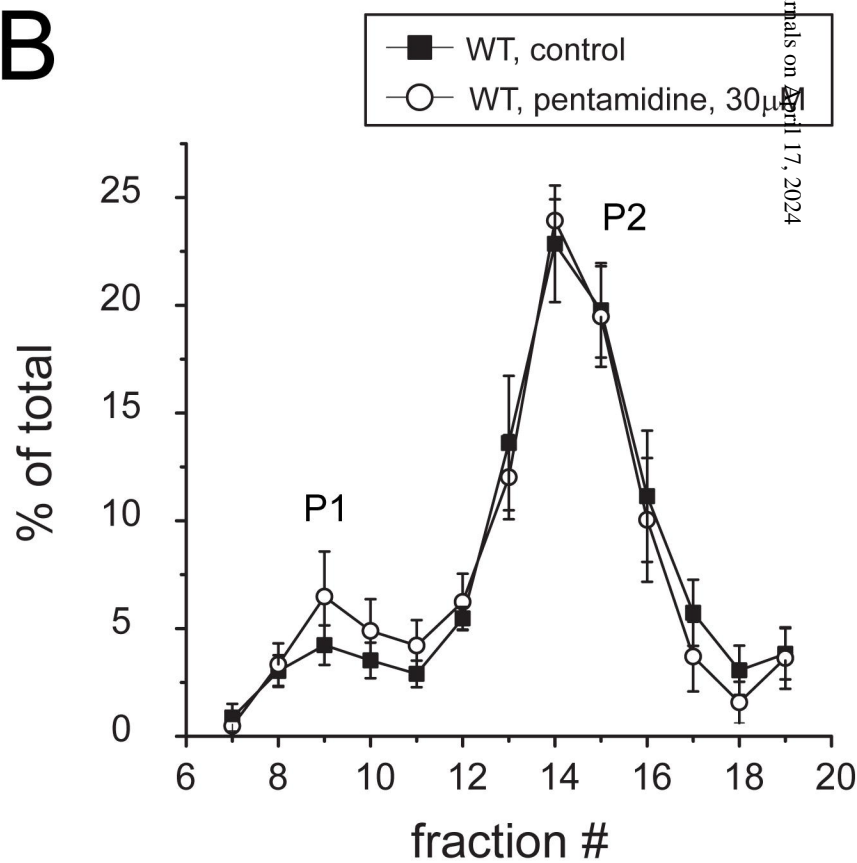
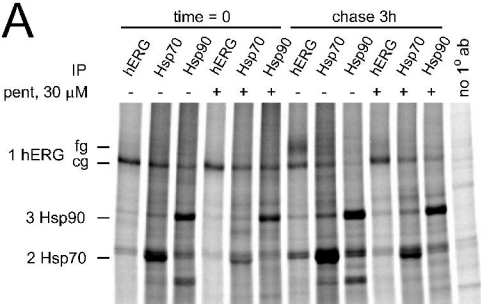


Figure 7

A



B

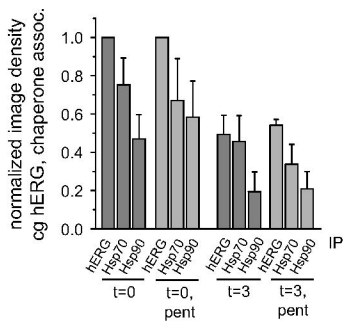


Figure 8

IB	hERG					HA				
His-Ub	-	-	-	+	+	-	-	-	+	+
HA-Ub	+	+	+	-	-	+	+	+	-	-
Pent	-	+	-	-	+	-	+	-	-	+
GA	-	-	+	-	-	-	-	+	-	-

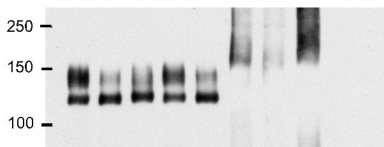
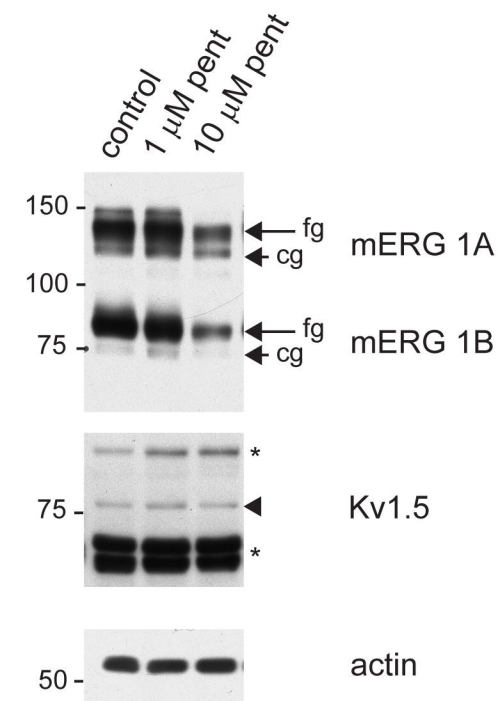
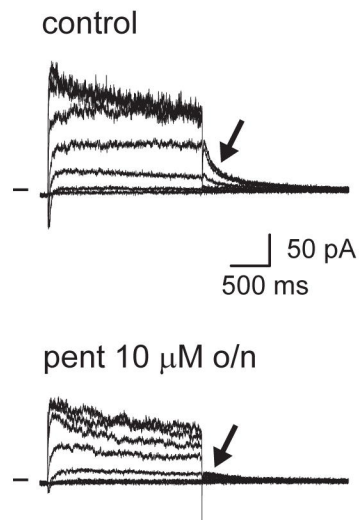


Figure 9

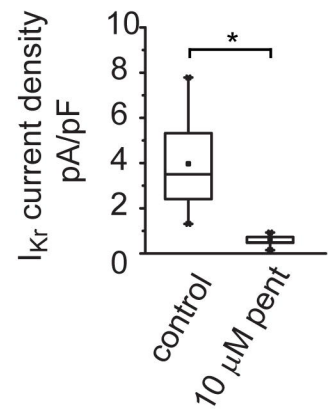
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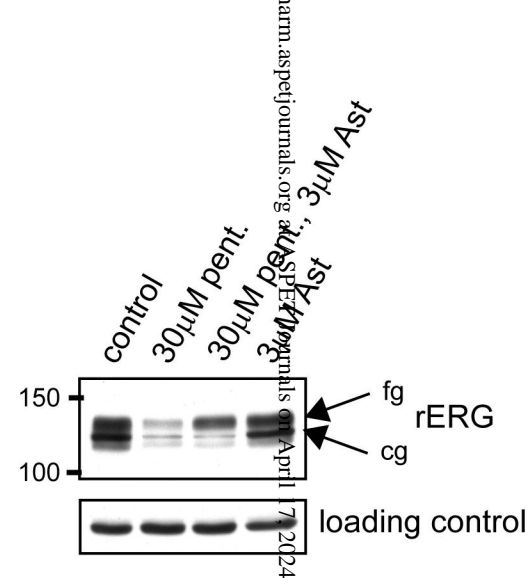
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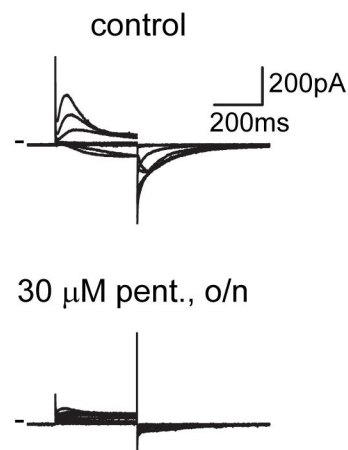
C



D



E



F

