Post-transcriptional control of HERG potassium channel
protein by \( \alpha \)-adrenergic receptor stimulation.

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α-Adrenergic regulation of HERG translation.

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Non-standard abbreviations:
HERG, human ether-a-go-go-related gene;
WT, wild-type;
HEK, human embryonic kidney 293 cell line;
PKA, protein kinase A;
PKC, protein kinase C;
PMA, Phorbol 12-myristate 13-acetate;
Pseudo-HERG, HERG with 17 of 18 possible PKC target sites mutated to alanine;
Pseudo-HERGPKA, HERG with all 4 possible PKA target sites mutated to alanine;
RNCM, Rat Neonatal Cardiac Myocytes
Abstract

Stimulation of α1-adrenoreceptors (α1-AR) acutely alters ion channel behavior via several signaling pathways (calcium and protein kinase C (PKC)). Little is known about sustained α1-adrenergic/PKC signaling and channel regulation, as may occur during cardiovascular disease states. Here we describe effects of prolonged α1A-AR and PKC activity on HERG K+ channels (Kv11.1) expressed in a heterologous expression system. Stimulation of α1A-AR with phenylephrine (PE), or direct activation of PKC with phorbol ester increased HERG channel protein abundance and K+ current density in a time- and dose-dependent manner. Channel augmentation reached a steady-state plateau within 24 hours with a 2-6-fold induction. Phorbol ester and moderate α1A-AR stimulation enhanced HERG abundance in a PKC-dependent fashion but with stronger α1A-adrenergic stimulation PKA-dependent activity also contributed. Comparable channel induction of other cardiac K+ channels was not seen in this system. Comparison of wild type HERG and channels with either mutated PKC phosphorylation sites (HERGΔPKC) or mutated PKA phosphorylation sites (HERGΔPKA) suggested that the mechanisms of augmentation of HERG by the two kinases were partially overlapping. The PKC-dependent effect was largely due to enhanced synthetic rates. Stimulation of α1-AR in cultured rat neonatal cardiac myocytes also enhanced the abundance of ERG channels. These findings show that α1A-AR stimulation is capable of influencing the balance of HERG channel synthesis and degradation via multiple signaling pathways, a process that may have relevance in cardiac diseases and treatment.
**Introduction**

The human ether-a-go-go–related gene (HERG) encodes the pore-forming subunit of the channel responsible for the rapidly activating delayed rectifier K⁺ current, Iᵦᵣ (Sanguinetti, Jiang et al. 1995). HERG is the gene subject to mutations in the hereditary long QT syndrome, locus LQT2 (Curran, Splawski et al. 1995). Acquired long QT physiology has also been associated with HERG, particularly in cases of inadvertent pro-arrhythmic drug effects (Kamiya, Niwa et al. 2006). In both hereditary and acquired LQTS involving HERG the ultimate result is due to a reduction on Iᵦᵣ current density, or “repolarization reserve” (Thomas, Kiehn et al. 2003; Roden 2004). Dynamic regulation of the HERG channel in common acquired cardiovascular conditions is not well understood. Furthermore, even less is certain about how HERG participates in sudden arrhythmic death and cardiac electrical remodeling in chronic heart disease.

Tachyarrhythmia leading to sudden cardiac death complicates a variety of common chronic heart diseases. Hyper-adrenergic tone with elevated circulating catecholamine hormones is a hallmark of a wide variety of cardiovascular conditions. Among the physiological or disease states characterized by chronically elevated circulating catecholamines are myocardial ischemia (Singh 2002) and chronic heart failure (Eisenhofer, Friberg et al. 1996). Initially the adrenergic stimulation is compensatory for disturbed supply-demand issues in cardiac output however, as disease progresses it may become maladaptive. In the case of α-adrenergic receptors, chronically altered signaling through protein kinase C (PKC) may contribute to the
pathogenesis of acquired heart disease and play a role in electrical remodeling (Tomaselli and Marban 1999; Nerbonne and Guo 2002; Murphy and Frishman 2005). Electrical remodeling is the process where specific protein expression profiles that occurs under a variety of environmental stimuli (hemodynamic demands) lead to altered ion channel abundance, spatial distribution, and regulation. These changes may affect action potential morphology, synchrony of excitation and propagation. Any electrophysiological changes such as these may lead to susceptibility to cardiac rhythm disturbances. Most studies to date have focused on altered ion channel expression (primarily at the RNA level). Alterations in channel protein translation, assembly, processing and degradation as mechanisms of electrical remodeling have been less studied.

The present study was designed to investigate the effects of prolonged $\alpha$-adrenergic receptor stimulation upon HERG channels. Sustained $\alpha 1A$-adrenergic stimulation caused a profound augmentation of HERG channel protein that was largely PKC-dependent, involved direct phosphorylation of the channel and was achieved by enhanced synthesis or translation rates. These findings newly link a signaling pathway that may play an important role in the maintenance of cardiac electrical stability.

**Materials and methods**

**Cell culture and transfection.** HEK293 cell lines were cultured in RPMI 1640, and supplemented with L-glutamine, 10% fetal calf serum (Hyclone), and
penicillin-streptomycin (GIBCO). Cultured cells were maintained in 5% CO₂-95% humidified air at 37°C. For initial studies utilizing transient expression 4μg of C-terminal myc-tagged HERG plasmid (in pCI-NEO (McDonald, Yu et al. 1997)) plus 4μg α1A-adrenergic receptor (in pCDNA3) were transiently co-transfected into HEK293 cells together with 2μg of GFP cDNA by electroporation. Cells were washed and resuspended in a buffer mimicking cytoplasm (120mM KCl, 0.15mM CaCl₂, 10mM K₂HPO₄/KH₂PO₄, 25mM HEPES, 2mM EGTA, 5mM MgCl₂, 2mM ATP and 5mM glutathione, pH 7.6) and were electroporated in a 2-mm gap cuvette using a Gene Pulser Xcell (BIO-RAD) with the following settings: voltage = 110V; pulse length = 15ms. After electroporation, the cells were plated sparsely and grown on sterile glass cover slips within 100-mm tissue culture dishes. Cells were used for electrophysiological studies 24-72 hours after electroporation.

In subsequent experiments stably transfected cells were generated. HERG (wild type, PCK mutant-HERG or PKA mutant-HERG) plasmid DNA was linearized and transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen/Life Technologies). 24-hours after transfection cells were sparsely re-plated and grown under the selective pressure of 500μg/ml G418 added to the culture media. 10-14 days later G418-resistant colonies appeared and were isolated, suspended by trypsinization, and subjected to serial dilution into 96-well dishes to obtain clonal cell lines. G418-resistant clonal lines were confirmed by immunoblot and patch clamp analyses.

The α1AR cDNA in the pEF6/V5-His vector (Invitrogen) was used to stably express the receptor in the HEK-HERG cells. After transfection, as above, stable
clones were selected by Blasticidin (5μg/ml) containing medium and detected by Western blot analysis using anti-V5-HRP antibody (Invitrogen) to confirm the a1AR-V5-His stable expression (HEK-HERG/a1AR cell).

**Isolation of Rat Neonatal Cardiac Myocytes (RNCM).** RNCMs were prepared following the protocol of Sadoshima’s group (Sadoshima et al., 1992) with minor modifications. Briefly, after washing extracted neonatal rat hearts with ice-cold PBS, the ventricles were separated and minced. The tissue was then digested for 6x15 minutes with 0.12% collagenase, type II (Worthington Biochemical) in PBS at 37°C; the enzyme was then neutralized with 15% horse serum, and the cells were collected by centrifugation. The resulting pellet was resuspended in buffer (in g/liter: 6.80 NaCl, 4.80 HEPES, 0.14 NaH₂PO₄, 0.60 glucose, 0.40 KCl, 0.205 MgSO₄*7H₂O), layered over a 45%/64% Percoll step gradient, and centrifuged at 3000rpm for 30min using Sorvall TJ-25 centrifuge. The myocytes were collected from the gradient interface, washed 3x with ADS and plated in media (per liter of DMEM/F-12: 0.33g sodium pyruvate, 0.72g glucose, 17.6 ascorbic acid, 15mM HEPES pH 7.6, 2g BSA fraction V, 4mg transferring, 0.4μmol selenite, fatty acid supplement (Sigma), 2.44g NaHCO₃) supplemented with 5% horse serum and 100μM BrdU. After 24hrs, the cells received fresh serum/BrdU-free media.

**Western blot.** HEK293 or RNCMs were prepared for immunoblot analyses by washing adherent cells with ice-cold PBS and then adding ice cold lysis buffer NDET buffer (150mM NaCl, 25mM Tris-HCl pH 7.5, 5mM EDTA, 1% NP-40, 0.4% deoxycholic acid and EDTA-free protease inhibitor cocktail tablets (Roche
Pharmaceuticals)). Lysates were then cleared by centrifugation at 16,000 × g for 5 min at 4°C. The cleared supernatants were assayed for total protein content (Bio-Rad Protein Assay), and equal amounts (50-100 μg) of cell lysate protein subjected to SDS-PAGE analysis. Protein samples were combined with 4 × SDS-PAGE sample buffer (4% (w/v) SDS, 40% glycerol, 20% (v/v) β-mercaptoethanol, 0.004% (w/v) bromphenol blue, 125 mM Tris buffer, pH 6.8) incubated for 5 min at room temperature, separated on a 7.5% SDS-PAGE, and electrophoretically transferred onto 0.2 µm nitrocellulose membrane (Bio-Rad Laboratories. Membranes were blocked in 10% non-fat dry milk and 0.05% Tween 20 in TBS for 2 hours at room temperature, and incubated with appropriate primary antibodies at 1:250-1:1000 dilution in 5% dry milk and 0.05% Tween 20 in TBS for 4 h at room temperature. Secondary antibodies conjugated to either horseradish peroxidase or infrared-fluorescence IRDye® (Rockland Immunochemicals Inc.) were incubated with the blots at a concentration of 1:10,000-1:50,000 in 0.05% Tween 20 TBS at room temperature for 1-hour and then washed. Antibody detection was performed by either chemiluminescence (Pierce, SuperSignal West Pico Chemiluminescent Substrate) with multiple exposures to ensure linearity of signal intensity; or with the Odyssey infrared imaging system (Li-Cor biotechnology). Identical results were obtained with either system. All gels in figures are representative of 3-6 independent experiments.

Antibodies: Anti-Kv11.1 (ERG)-extracellular was from Alomone or Santa Cruz Biotechnology. Anti-myc mouse monoclonal 9E10, anti-myc rabbit polyclonal A-14, anti-Hsc70 goat polyclonal, anti-Hsp70 mouse monoclonal, and anti-Hsp90 mouse
monoclonal antibodies were from Santa Cruz Biotechnology. Anti-tubulin rabbit monoclonal antibody was from Sigma. Anti-HERG antibodies were either from rabbit serum immunized with purified HERG N-terminus as previously reported (Kagan, Melman et al. 2002) or from mouse immunized with a mix of purified recombinant HERG fragments from the C-terminus (each fragment 100 amino acids in length).

**Immunofluorescent microscopy analysis.** Immunofluorescence staining of HERG, calnexin and GM130 were performed after fixation of cells in 4% paraformaldehyde and permeabilization with 0.3% Triton X-100. Images were acquired using an Olympus IX70 microscope with an X60 PlanApo objective and a Photometrics Censys cooled charge-coupled device camera. Images were deconvoluted to reduce fluorescence interference from beyond the focal plane with Powerhazebuster (Vaytek), and all images were displayed with Adobe Photoshop. HERG co-localization with sub-cellular markers as well as HERG intensity levels were measured using the Image Correlation Analysis plug-in for Image-J software (Wright Cell Imaging Facility, www.uhnresearch.ca). Pearson’s correlation quotient (ranging from 0 to 1) and intensity correlation quotient (ICQ, ranging from -0.5 to 0.5) were obtained as a quantification of HERG co-localization.

**Patch Clamp recording.** Cells on cover slips were taken directly from the cell culture incubator and placed in an acrylic/polystyrene perfusion chamber (Warner Instruments) for electrophysiological measurements. Patch pipettes were pulled and polished to obtain a tip resistance of 2-3Mohms in the patch clamp solutions. All experiments were carried out at room temperature (20-22°C). Cells were studied on
an inverted microscope equipped with electronic patch-pipette micromanipulators and epifluorescence optics for GFP (transfected cells). Axopatch-200B patch clamp amplifiers (Axon Instruments) were used for voltage clamp measurements. The series resistance were about 9-10 Mohms. Voltage clamp protocols were controlled via PC using pClamp8 acquisition and analysis software. To elicit HERG K+ currents depolarizing voltage pulses were applied to various levels from a holding potential of –70 mV for 4.5 s followed by stepwise repolarization to –40 mV and then to –120 mV to measure outward tail currents. Signals were analog-filtered at 2,000 Hz and sampled at 5-10,000 Hz. Voltage-dependent activation data were fitted to Boltzmann relation $I = I_e/(1+\exp((V_h-V)/k))$, where $I$ is the relative tail current amplitude, $V$ is the applied membrane voltage, $V_h$ is the voltage at half-maximal activation, and $k$ is the slope factor. To compare the effects before and after the administration of reagents, current amplitude was normalized to the control group before application of drugs.

For whole-cell voltage clamp the pipette solution consisted of (in mM) KCl 126, MgSO4 2, CaCl2 0.5, EGTA 5, Mg-ATP 4, and HEPES 25 (pH 7.2, osmolality, 280±10 mΩ). External bath solution consisted of (in mM) NaCl 150, CaCl2 1.8, KCl 4, MgCl2 1, glucose 5, and HEPES 10 (pH 7.4; osmolality: 320±10 mΩ).

**Analysis of mRNA.** Total RNA from HEK-HERG cells and rat neonatal cardiac myocytes was extracted by TRIzol® Reagent (Invitrogen) and digested with DNAase. cDNA synthesis was performed with Superscript (Invitrogen). We then used the Mx3000P Real-Time PCR System with the Brilliant SYBR Green qPCR kit (Stratagene) to quantify HERG mRNA (GAPDH was used for normalization).
Negative controls consisted of TRIzol- and DNase I-digested cell samples omitting the reverse transcriptase. HERG Forward primer: 5’ TCA ACC TGC GAG ATA CCA ACA TG 3’ and HERG Reverse primer: 5’ CTG GCT GCT CCG TGT CCT T 3’. The PCR program is 95°C 10 min, then 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s, 40-cycles.

**Pulse-chase and metabolic labeling.** Cell proteins were metabolically labeled with $^{35}$S-cysteine/methionine (Trans-label, ICN) for pulse chase studies as previously described (Chen, Sroubek et al. 2009). To estimate the rate of HERG synthesis cells were incubated in cysteine/methionine-free media for 1 hour followed by the fresh RPMI 1640 medium containing 1600$\mu$Ci/ml $^{35}$S-cysteine/methionine for various intervals. Labeling was stopped by addition of ice-cold detergent lysis buffer. Cell lysates were pre-cleared with Ultra-Link Protein G-agarose (Pierce) for 30 min at 4°C. Supernatant was collected by centrifugation (8000rpm 1 min) and incubated with 8 $\mu$g anti-

*myc* (A14G, Santa Cruz Biotech) for 1 h. As control, non specific rabbit IgG was used. Antibody-antigen complexes were precipitated by 15$\mu$l protein G-agarose for 3 h at 4°C. After thorough washing (3-times) with PBS, proteins were eluted from the resin with 4x Laemmli sample buffer, and subjected to SDS-PAGE. Specific labeling of HERG was measured by detect of $^{35}$S signal from immunoprecipitated channels and compared to both total cellular protein uptake of $^{35}$S and labeling of the housekeeping protein tubulin. For total protein $^{35}$S uptake, samples taken from cell lysates after HERG precipitation were run on SDS-PAGE, dissolved in scintillation fluid for $\beta$-emission counting. For tubulin normalization, anti-tubulin antibodies
were used in the immunoprecipitation and densitometry of autoradiography gels was measured.

**Reagents.** Phenylephrine, CPT-cAMP were purchased from Sigma. Phorbol 12-myristate 13-acetate (PMA), 5-methyl urapidil (5-MU), chelerythrine (Chele), Bisindolylmaleimide I, Gö6976, and Ro-32-0432 were purchased from Calbiochem. Collagenase type II was from Worthington and Protease inhibitor cocktail from Roche. PMA, CPT-cAMP, Chelerythrine, Bisindolylmaleimide I, Gö6976, and Ro-32-0432 were dissolved in DMSO as stock solutions and used at the desired final concentrations in such that the final DMSO concentration was <0.5%.

**Statistics** Values presented are means ± SE. ANOVA was employed for statistical analysis of the data and $P$-values of < 0.05 were considered to be significant.
Results

*Sustained α₁-adrenergic stimulation increases HERG protein abundance in HEK293 cells.* To examine the effect of α₁A-adrenergic stimulation on HERG channel abundance, cDNAs of α₁A-adrenergic receptor and HERG channels were stably co-expressed in HEK293 cells. As shown in Figure 1A, treatment with PE (1µM), an α₁-adrenergic receptor agonist, for 24 h markedly increased HERG protein abundance in both immature (~130KD) and mature forms of HERG (~150KD). These effects were abolished by 5-Mu (1 µM), a selective α1A-adrenergic receptor antagonist (Figure 1B), showing the specificity of the α1A-adrenergic receptor-mediated effect.

The α₁A-adrenergic effect on HERG protein was concentration-dependent (Figure 1C) with a saturation above 1µM. The time course of the effect of PE was shown in Figure 1D. PE at 1 µM increased HERG protein abundance in a time-dependent manner with detectable effect observed within 4 hours. The absolute degree of HERG protein induction by α₁A-adrenergic was somewhat variable over periods of months but always showed a minimum of 2-fold increase after 24-hours of treatment. Accordingly, subsequent results show only experiments performed on cells under identical conditions and within a short time-frame (days-to-weeks).

*K⁺ current density through HERG channels is enhanced by prolonged α₁-adrenergic stimulation in HEK293 cells.* To investigate if α₁-adrenergic activation produced a concomitant increase in current through the HERG channels we
subjected cells to voltage clamp analysis via whole cell patch clamp technique. After 24-hours of treatment with 1μM PE the HERG current density increased significantly (Figure 2). The maximal current density as measured in I-V relationship increased from 13.4±3.5 pA/pF to 27.5±5.4 pA/pF (p< 0.05, n=6). PE also increased the density of the tail currents (Control: 34.4±3.8 pA/pF at +40mV, n=8; PE: 73.9±6.7 pA/pF; n=7, p<0.001). There was a trend towards a depolarizing shift in the voltage-dependence activation with sustained α1A-AR stimulation which did not reach significance (Control Vh: -33.6±2.6 mV, PE Vh: -26.9±2.6 mV).

*α1-adrenergic receptor-dependent augmentation of HERG protein involves activation of PKC.* Activation of α1A-adrenergic receptor is classically coupled to Gαq/Gα11 and as such, activates phospholipase C, Ca2+, and PKC signaling pathways. To determine whether α1A-AR-dependent augmentation of HERG channel protein is mediated by PKC, Bisindolylmaleimide I (Bis-I) and Ro-32-0432, pharmacological inhibitors of PKC were employed. 24-hour treatment of HEK cells stably expressing HERG and α1A-AR with 0.1μM PE increased HERG protein abundance and the effect was attenuated by Ro-32-0432 at 3μM and abolished by 10μM of Bis-I when the inhibitors were added one hour prior to treatment with PE and kept in the media (Figure 3A). When the concentration of PE was increased to 1μM (a saturating concentration, in terms of HERG augmentation, Figure 1C) both inhibitors were only partially capable of preventing the increase in HERG protein. A third PKC inhibitor,
Gö6976, yielded similar results suggesting that PKC was not the sole pathway for α1A-AR-dependent effects on HERG but that additional signaling may be involved (Figure 3B).

To directly examine PKC-dependent effects on HERG channel abundance we treated HEK cells stably expressing HERG with the phorbol ester PMA (Figure 4). Similar to the results with α1A-AR stimulation, prolonged treatment with PMA increased HERG protein abundance in a time- and dose-dependent fashion (Figure 4A,B). PMA-dependent increases in HERG were detected as early as 2-4 hours (when 10nM was used) and with concentrations between 1-10nM at the 24-hour point. Both Ro-32-0432 and Bis-I were capable of completely preventing PMA-dependent effects on HERG (Figure 4C). These results further support the interpretation that α1A-AR stimulation is, in part, mediated via PKC stimulation though additional signaling mechanisms may also contribute.

To further explore possible signaling mechanisms for α1A-AR-mediated regulation of HERG protein we compared the degree of channel augmentation in cells treated with either PMA or PE (Figure 4D). We consistently observed that α1A-AR stimulation produced a greater augmentation of channel abundance than did phorbol ester. This result further suggested that α1A-AR signaling enhanced HERG channel abundance by additional pathways from PKC. Since α1A-AR couples to Gαq which then activates phospholipase C additional signaling changes occur—elevation of cytoplasmic Ca^{2+} and transient consumption of PIP2. These two processes did not appear to be involved in the receptor-mediated effect on HERG channel protein.
abundance since pre-incubation of cells with either the Ca\textsuperscript{2+} chelator BAPTA-AM ester or PI3-kinase inhibitors (Wortmannin 100nM or LY 294002 10μM) that would prevent repletion of PIP2 failed to inhibit the α1A-AR effect (data not shown).

**PKC- and PKA-mediated phosphorylation of the HERG channel is required for α1A-AR-mediated increase in HERG protein abundance.** In view of previous work on acute regulation of HERG gating by α1A-AR that suggested cross-signaling between the receptor and PKC plus PKA (Kiehn, Karle et al. 1998) we sought to investigate a possible role for PKA in the present findings. When cells stably expressing HERG and α1A-AR were treated with a high concentration of PE (1μM) the effect on HERG protein was partially blocked by Bis-1 and to a lesser extent by the PKA inhibitor H89. The combination of Bis-1 plus H89, however, completely inhibited α1A-AR stimulation-dependent augmentation of HERG protein (Figure 5A).

To distinguish between kinase-mediated regulation of channel abundance by direct phosphorylation from phosphorylation of an intermediate regulator we employed a HERG mutant in which 17 of the 18 PROSITE-predicted PKC acceptor serines/threonines were changed to alanine (HERG\textsubscript{ΔPKC}) (Figure 5B)(Thomas 2003). HEK cells stably expressing HERG\textsubscript{ΔPKC} were subjected to PMA treatment for 24-hours and the increase in channel protein abundance was greatly decreased compared to wild type HERG (Figure 5C,D). We have previously shown a PKA-dependent augmentation of HERG abundance with enhanced protein synthesis as the mechanism (Chen, Sroubek et al. 2009). Two of the predicted PKC sites
(S890 and T895) are also known PKA phosphorylation sites on HERG (Cui, Melman et al. 2000). To investigate whether the effects of PKC were identical to or overlapped with those of PKA-mediated increase in HERG channels we employed a combination of pharmacological and mutation analyses. When cells stably expressing wild type HERG were treated with the membrane-permeable cAMP analog, CPT-cAMP the augmentation was not as great as that with PMA treatment. Moreover, when both reagents were used in combination, the effect was partially additive. The HERGΔPKC mutant was augmented by CPT-cAMP but the additivity with PMA was no longer seen. A mutant HERG in which the four acceptor serines/threonine were changed to alanine (HERGΔPKA, (Cui, Melman et al. 2000)) showed an elimination of CPT-cAMP-mediated increase and a reduced but present enhancement with PMA. Again, combination of PMA and CPT-cAMP failed to show additivity in augmenting the HERGΔPKA mutant.

One caveat for the HERGΔPKC results is that the baseline expression levels are relatively low. This mutant harbors many changes that may have unexpected effects in channel protein folding, assembly and trafficking—any of which can alter its abundance. Accordingly, we are cautious in relying completely on this mutant for our interpretations. Nevertheless, the combined data suggest that there is partial overlap in phosphorylation sites within HERG responsible of PKC- and PKA-mediated enhancement in channel protein. There is however, a clear quantitative and qualitative difference between the PKC- and PKA-mediated effects. Moreover, enhancement of HERG protein by α1A-AR stimulation appears to involve
cross-talk of PKC and PKA pathways. There remains a diminished but present enhancement of HERGΔPKC with PMA that suggests either indirect effects of the kinase on channel, additional unrecognized PKC sites, or that the 18th PROSITE-predicted PKC site (T74) may play a role in the effect. The T74A mutation was not used due to the fact that previous studies showed that this abolished all channel activity (Thomas 2003).

**Mechanism of α1A-AR and PKC augmentation of HERG channel protein.**

There are several potential ways that receptor stimulation and PKC could enhance the abundance of HERG protein: altered transcription; accelerated translation; or reduced degradation. To assess altered transcription we isolated RNA from HEK cells stably expressing HERG and α1A-AR under control conditions or after treatment with 1μM PE or 10nM PMA for 24-hours. Real Time-PCR analysis showed that there were no significant increases in HERG mRNA with either stimulus (PMA-treated cells expressed 60% as much HERG mRNA compared to controls and PE-treated cells expressed 109% compared to controls, n = 4, NS). Since the HERG cDNA is under control of the CMV promoter we investigated whether there were comparable changes in two other cardiac tetrameric voltage-gate K+ channels: KCNQ1 and Kv1.5—whose cDNAs were under control of the same promoter, epitope-tagged with myc, and were stably transfected into HEK cells. When these cells were treated with 10nM PMA for 24-hours they failed to show comparable increase in channel abundance as did HERG-expressing cells (Figure 6). Thus, regulation of transcription did not appear to be the mechanisms responsible for increased HERG. Moreover, the effect of PKC
on channel abundance was specific for HERG compared to KCNQ1 and Kv1.5.

To address regulation of protein synthesis or degradation we performed 35S-cysteine/methionine labeling. To measure HERG degradation we determined the stability or half-life using metabolic labeling and pulse-chase experiments followed by autoradiography of immunoprecipitated 35S-labeled HERG protein. Cells stably expressing HERG were pretreated with PMA (10nM) for 1-hour prior to pulse-chase. The half-life of HERG in these experiments was 17-18 hours, values similar to those published in other cultured cell types (McDonald, Yu et al. 1997; Ficker, Dennis et al. 2003, Chen, Sroubek et al. 2009). PMA treatment did not change the half life despite an apparent initial stabilization in PMA-treated cells in the first 12 hours (Figure 7A).

To examine the rate of channel synthesis we measured 35S-cysteine/methionine incorporation into HERG (relative to that of total cellular proteins and tubulin whose abundance was not altered by PMA). To normalize HERG synthesis values the densitometry of the autoradiography of HERG was divided by the rate of total cellular protein and specific tubulin 35S incorporation. Both methods resulted in comparable rates. The rate of 35S incorporation into HERG over a period of 60 minutes was nearly doubled in cells that had been pretreated with 10nM PMA (Figure 7B). Thus, the PKC-dependent augmentation of HERG protein abundance is due, primarily to an accelerated synthesis or translation of new channels although an enhanced stability of channels in the first few hours after they are formed may contribute.

We further examined whether PKC activation had any effect on HERG channel

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distribution within the cell by performing immunofluorescence confocal microscopy with double labeling of subcellular compartments. Antibody against GM130 was used to define the Golgi and antibody against calnexin was used for endoplasmic reticulum (ER). PMA treatment clearly enhanced the HERG signal (31-40%, n = 4) but had no detectable effect on calnexin or GM130 (Figure 8A,B). When merging of the HERG and subcellular marker signals was done it was seen that the enhanced HERG signal was present in ER, Golgi and periphery without obvious preferential accumulation in any one compartment. The degree of co-localization of HERG with calnexin and GM130 was reflected by the general increase in total HERG signal (49% for calnexin, 30% for GM130, as measured by ICQ).

Hsp90 and Hsp70 interaction with newly forming HERG channels has been established as a requirement for proper synthesis and trafficking (Ficker, Dennis et al. 2003). These chaperone interactions have been shown to play an important role in both maturation of normal channels and degradation of misfolded mutant HERG. We sought to determine if PKC-mediated augmentation of HERG abundance was associated with changes in either of these chaperones by immunoblot analysis. When cells stably expressing HERG were subjected to treatment with increasing concentrations of PMA for 24 hours we observed no change in the abundance of Hsp70, Hsc70 or Hsp90 (data not shown). Thus, changes in chaperone protein abundance did not appear to be responsible for PKC-mediated regulation of HERG protein.

**α1-AR stimulation effects on ERG protein level in rat neonatal cardiac**
myocytes. To determine whether sustained stimulation of α1-adrenergic receptor could also increase native ERG protein abundance, cardiomyocytes we used the rat neonatal cardiac myocyte culture system in which the ERG channel has been shown to be expressed (Guo, Massaeli et al. 2007). Cells were used shortly after isolation in culture and were treated with PMA (10nM), PE (1μM) or vehicle (DMSO) for 24 h. As shown in Figure 9, treatment with PE for 24 h also measurably increased ERG protein abundance (Figure 9) with a statistically significant average increase in ERG of 29±0.12%, (n=6 when normalized to tubulin). PMA treatment resulted in an increase in ERG protein of 8±0.055% that did not meet statistical significance. This result suggests that native ERG channel protein may also be up-regulated by prolonged stimulation of α1-adrenergic receptor. Since the native ERG gene is under its own promoter rather than the CMV used in heterologous systems we quantified the amount of ERG mRNA from myocytes under control conditions and 24-hour treatment with PMA or PE by Real Time-PCR. Compared to control PMA-treated cells expressed 33±30% as much ERG Message (n = 4, not significantly different) and PE-treated cells expressed 82±79% as much (n = 4, not statistically different). These measurements indicate that enhanced transcription was not the mechanism for increased ERG protein in PE-treated cells. To the contrary, it appears that there is a trend towards reduced transcription of ERG, which taken with the protein abundance, further highlights the enhanced translation of channel protein.
Discussion

The results we report here describe the effects of sustained stimulation of \( \alpha_1 \)-adrenoceptors on the HERG channel protein. With prolonged receptor stimulation in a heterologous expression system the HERG channel protein abundance was markedly increased over 24 hours. The increase in protein was largely due to PKC activation and phosphorylation of the channel protein but at the highest stimulation of \( \alpha_1 \)-AR additional signaling pathways contributed, including PKA activation. The mechanism(s) of \( \alpha_1 \)-AR/PKC-mediated augmentation of channel protein involves post-transcriptional acceleration of channel synthesis or translation. Enhanced mRNA transcription or stability did not appear to be involved in the effect and the augmentation of channel abundance was specific to HERG compared to two other cardiac K\(^+\) channels.

Regulation of ion channels by means of GPCRs and downstream kinases is well established. Most studies of receptor- and kinase-mediated regulation have focused on short-term effects on channel gating. Acute PKA-mediated regulation of HERG channel behavior has been shown to involve both direct and indirect signaling (Kiehn, Karle et al. 1998; Thomas, Zhang et al. 1999; Cui, Melman et al. 2000; Cui, Kagan et al. 2001; Kagan, Melman et al. 2002). The situation for \( \alpha_1 \)-AR and PKC regulation of HERG is less clear. Acute stimulation of \( \alpha_1 \)-adrenergic receptors has been shown to decrease HERG channel activity via stimulation of PKC (Thomas 2003) and consumption of PIP\(_2\) (Bian, Kagan et al. 2004). Several studies showed
activation of PKC by phorbol esters leads to suppression of HERG current amplitude in heterologous expression systems (Kiehn, Karle et al. 1998; Thomas 2003). The phorbol ester-dependent suppression has been ascribed to accelerated deactivation, slowed activation, depolarizing shift in activation and reduction in current density. It has also been reported that the phorbol ester-dependent effect was due to PKA activation (Kiehn, Karle et al. 1998) and that removal of PKC consensus phosphorylation sites from HERG failed to abolish phorbol ester-mediated regulation, suggesting involvement atypical receptor-kinase crosstalk (Thomas 2003). Another report provided evidence that PKC phosphorylation of C-terminal portions of HERG were necessary for the acute PKC regulation of the channel (Cockerill, Tobin et al. 2007). The $I_{Kr}$ regulation in cardiac myocytes however, may be more complicated. In guinea pig cardiac myocytes, PKC activation enhanced $I_{Kr}$ (Heath and Terrar 2000). In rabbit cardiac myocytes $\alpha$-adrenergic stimulation acutely reduced HERG/$I_{Kr}$ primarily due to PIP2 consumption (Bian, Kagan et al. 2004). All of these studies were conducted within a time-frame of minutes, thus mimicking acute effects rather than sustained stimulation as may occur over hours to days.

We recently reported that prolonged stimulation of PKA (hours to days) enhanced HERG channel protein abundance (Chen, Sroubek et al. 2009). The PKA-dependent increase in HERG showed some similarities to those described in this report in that there was acceleration of synthesis and the effect occurred over a similar time frame. Notable differences however, occur with $\alpha1A$-AR/PKC-dependent changes in HERG. The most notable, for instance, is that the degree of channel protein increase is greater.
and K+ current increased occurred earlier with α1A-AR/PKC stimulation. Mutation of all PROSITE-predicted PKC sites (with the exception of T74) in HERG greatly reduced the phorbol-ester-induced augmentation of channel protein but did not completely abolish it; whereas mutation of the four PKA phosphorylation sites completely prevented PKA-dependent effects on the channel. There is some degree of overlap in the PKC- and PKA-dependent effects in that their ability to increase HERG was not entirely additive and several of the predicted phosphorylation sites within the C-terminus of HERG are common to the two kinases. Moreover, at high levels of α1A-AR stimulation both kinases appeared to play a role in increasing HERG. Taken together, these results suggest that the PKA-dependent regulation of HERG abundance is rather straightforward while that of α1-AR/PKC is more complex and possibly stronger.

Our results indicate that accelerated synthesis or translation of channel protein accounted for the α1A-AR/PKC effect rather than altered transcription rates. The exact mechanism(s) of how this signaling pathway changes HERG abundance remains an open question however. Phosphorylation of the nascent channels appears to play the largest role but additional, kinase effectors may also contribute since mutation of the PKC consensus sites did not completely suppress the effect. The biosynthesis of HERG appears to be a tenuous process—a concept underscored by the fact that most LQT2 mutation result mis-folded product that never reaches to cell surface due to either faulty production, early degradation or mis-trafficking (Anderson et al., 2006; Ficker et al., 2000; Gong et al., 2005; Kagan et al., 2000; Thomas et al.,
MOL #62216

2003). If newly forming proteins contain considerable stretches of hydrophobic segments they may be prone to aggregation if not properly shielded by chaperones prior to reaching their final conformation. In this event translation may be delayed or the newly synthesized proteins may be processed by quality control mechanisms of the cell leading to early degradation (Chirico, Waters et al. 1988; Meacham, Lu et al. 1999). This may occur with some non-mutant proteins under normal circumstances (Schubert, Anton et al. 2000; Qian, Princiotta et al. 2006). A possible interpretation of our data is that newly forming HERG protein is made more soluble or less likely to aggregate into non-productive conformation if the cytoplasmic segments are subject to phosphorylation by either PKA or PKC. Further work is needed to precisely distinguish between this and alternative mechanisms such as regulation of endosomal recycling (Delisle, Underkofler et al. 2009), lysosomal degradation and altered chaperone interactions with HERG (despite stable amounts of heat shock proteins) (Ficker, Dennis et al. 2003).

Activation of α1A-receptor may lead to the activation of various PKC isoforms in cardiac myocytes (Puceat, Hilal-Dandan et al. 1994). The PKC involved isoforms in protein degradation include PKCδ, PKCε and PKCζ. Future studies will be necessary to determine which isoform(s) of PKC is/are involved in the effect of sustained stimulation of α1A-adrenergic receptor on HERG protein abundance. Although signaling through α1-AR is generally ascribed to Gαq/Gα11 and PKC, earlier studies have shown that cAMP generation and stimulation of PKA may also occur to varying degrees in several systems by either direct or indirect pathways (Cotecchia, Kobilka et
al. 1990). This appears to be the case in our experiments. Whether signaling cross-talk of α1AR and cAMP/PKA occurs in cardiac tissue in a similar manner is yet to be determined.

Heterologous expression systems (such as HEK293 cells) provide a malleable approach enabling precise manipulations to determine molecular mechanisms of protein interactions through biochemistry and electrophysiology. It may not however, accurately reflect the milieu of the same proteins in vivo. Therefore we investigated the α1A-adrenergic regulation of HERG/I_{Kr} in cardiac tissue and found that sustained α1A-receptor stimulation also significantly increased rat cardiomyocyte ERG channel protein expression, albeit to a more modest degree. Further investigation is merited to delineate the signaling events that mediate post-transcriptional regulation of I_{Kr} density and channel protein abundance in vivo.

Hyper-adrenergic stimulation frequently accompanies cardiovascular disease states and may result in internalization/down-regulation of β-adrenergic receptors; altered Gαs-mediated cAMP/PKA signaling; switching of β-AR-coupling to Gαi; and up-regulation of α1-AR/Gαq signaling, (Ungerer, Bohm et al. 1993; Rockman, Koch et al. 2002; Lohse, Engelhardt et al. 2003; Woodcock, Du et al. 2008). How α1-adreneric receptors impact these situations is less characterized but they also appear to have altered signaling during hyper-stimulation in disease states with differing consequences in terms of heart function (O'Connell, Swigart et al. 2006; Woodcock, Du et al. 2008). α1-AR, β-AR, PKC, and PKA have each been implicated in the pathogenesis of cardiovascular diseases that may be complicated by
ventricular arrhythmias (Lohse, Engelhardt et al. 2003). Medical therapies are presently targeted to, or under investigation for each of these proteins. The experience with adrenergic antagonists is the most obvious example however; there is continued research and interest in developing more specific reagents. How such therapies would specifically affect the long-term regulation of the HERG channel is important since membrane repolarization and risk for sudden arrhythmic death may be impacted. Our results suggest a novel regulation of HERG/I\textsubscript{Kr} potassium channels by α1A-adrenoceptor activity that may represent another potential link between stress and potentially life-threatening ventricular arrhythmias. Further investigation into molecular mechanisms of HERG channel regulation is warranted as it may significantly impact prevention and treatment of cardiac arrhythmias.
Footnotes

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References


Footnotes:

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Figure legends:

**Figure 1. α-1A-adrenergic augmentation of HERG protein.**

A) Immunoblots from clonal HEK cell lines stably expressing both HERG and the α-1A-adrenergic receptor with and without 24 hours phenylephrine treatment with tubulin loading control and immunoblots for the α-1A-adrenergic receptor below.  

B) The specific α-1A-adrenergic receptor antagonist 5-MU blocked the phenylephrine-dependent augmentation of HERG protein (Histogram provides summary data for different treatment groups with separate bars for the immature 135kDa and mature 155kDa forms).  

C) Clone 1 from panel A treated with differing concentrations of phenylephrine applied for 24 hours.  

D) Clone 1 from panel A treated with 1μM PE for varying durations.  

**Figure 2. α-adrenergic augmentation of HERG potassium current density.**  

A) Traces show representative current traces from HEK cells stably expressing HERG in response to a series of depolarizing voltage steps.  The left set of traces is shown from untreated cells and those on the right from a cell expressing the α-adrenergic receptor and treated with phenylephrine for 24 hours.  

B) The graph on the lower left shows the isochronal current-voltage relationship during the depolarizing steps (with current normalized to cell capacitance).  

Lower right graph shows the voltage-dependant activation curves as measured at the maximal tail current.
(normalized to cell capacitance) plotted against the preceding depolarizing step voltage.

**Figure 3.** PKC involvement in α1A-adrenergic augmentation of HERG channel protein.

A) Immunoblots from a clonal cell line stably expressing HERG and α1A-AR. PE treatment for 24-hours increases HERG abundance an effect that is reduced by Ro-32-0432 (Ro-32) and abolished by Bisindolylmaleimide I (Bis-I) when PE concentration is 0.1μM. When the PE concentration is increased to 1μM both PKC inhibitors only partially prevent the augmentation of HERG (histogram to the right shows densitometry results. * p < 0.02, ** p < 0.002, n = 3.). B) Similar conditions as in panel A showing inhibition of the receptor-mediated augmentation of HERG by another PKC inhibitor, Gö6976. A similar pattern of incomplete inhibition is seen when receptors are maximally stimulated.

**Figure 4.** Direct PKC activation enhances HERG protein abundance.

Immunoblot analysis of HEK cells stably expressing HERG in response to PMA treatment. A) Shows changes in HERG with increasing duration of treatment with PMA at 10nM. B) Shows changes in HERG with increasing concentration of PMA treatment for 24 hours. C) Two PKC inhibitors, Bisindolylmaleimide I and R0-32-0432 block the PMA-dependent increase in HERG protein. D) A comparison of α1A-AR receptor stimulation (PE) is made with direct PKC activation (PMA).(n=3; * p<0.05; ** p<0.025; *** p<0.01).
Figure 5. Phosphorylation of HERG channels by PKC and PKA affect protein abundance.

A) Inhibition of PKC (with Bis-1) reduces the α1A-AR-mediated increase in HERG more than inhibition of PKA (with H-89) and inhibition of both kinases completely prevents the effect. (* p<0.05, n=5).  

B) Representative current traces from HEK cells expressing either HERGΔPKC (left) or HERGΔPKA (right).  

C) Immunoblot of HERG, HERGΔPKC and HERGΔPKA before and after treatment of cells with either PMA or CPT-cAMP.  

D) Summary densitometry data from HERG immunoblot analysis shown in C (* indicates significance compared to CPT-cAMP-treated cells p<0.05, n=2). The effects of PKA and PKC stimulation on HERG abundance are partially cumulative. Direct phosphorylation of the channel protein largely accounts for the kinase effects on HERG.

Figure 6. PKC-mediated effects on cardiac K channels Kv1.5 and KCNQ1.  

Anti-myc Immunoblot analysis of HEK cells stably expressing myc-epitope-tagged HERG, Kv1.5 or KCNQ1 after treatment with control vehicle or PMA 10nM for 24-hours. Histogram shows the relative PMA-dependent change in channel protein abundance. (n = 2, * p < 0.05).

Figure 7. PKC effects on synthesis and stability of HERG protein.  

A) Pulse chase of HERG labeled with 35S-cysteine/methionine with and without PMA 10nM treatment. Graph to the right shows time-dependent decrease in 35S-HERG normalized to initial incorporated amount at beginning of chase period (n = 4).  

B) Early incorporation of 35S-cysteine/methionine in HERG shown in the first 60 minutes.
of labeling with and without PMA treatment. Graph to right represents the densitometry data normalized to total cellular $^{35}$S-incorporation showing time-dependent new synthesis of HERG (n = 2).

**Figure 8. Subcellular localization of HERG after 24-hours of PKC activation.**

Confocal immunofluorescence assays with double staining for HERG-\textit{myc} (red channel) and either the Golgi marker GM130 (A) or the ER marker calnexin (B) (green channel). PMA treatment of 10nM for 24-hours results in globally increased HERG signal in both compartments as well as on the surface.

**Figure 9. $\alpha_1$-AR stimulation enhances ERG channel abundance in rat neonatal cardiac myocytes.**

Left panel, anti-HERG immunoblots from isolated rat neonatal cardiac myocytes under control conditions (CON) and after 24-hours of treatment with either PMA 10nM (PMA) or PE 1$\mu$M (PE). ERG channel bands are indicated by arrows. Lower gel shows tubulin immunoblot from same gel used to normalize for loading variances. Right panel, show summary data for 6 experiments with ERG normalized to tubulin densitometry (* indicates p < 0.05, n=6).
Figure 1

A

- α1AR cell lines
  - Clone 1
  - Clone 2
  - Clone 3

- PE
  - Control
  - +

- HERG-Myc
- Tubulin
- α1AR

B

- 5-MU
  - PE
  - -
  - +
- HERG-myc
- Tubulin

- 155 KD
- 135 KD

- Fold increase HERG protein

C

- PE
  - 0
  - 0.01
  - 0.1
  - 1
  - 10
  - 100 μM
- HERG-myc
- Tubulin

- Fold increase HERG protein

D

- HERG-myc
  - 0
  - 2
  - 4
  - 8
  - 18
  - 24 hours
- Tubulin

- Fold increase HERG protein

- Time (hours)
**Figure 3**

**Panel A**

<table>
<thead>
<tr>
<th>WT HERG +α1AR clone 5</th>
<th>Bis-I</th>
<th>Ro-32</th>
<th>PE</th>
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**Panel B**

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</table>

**Fold Increase HERG Protein**

- Control
- PE 0.1 μM
- PE 1.0 μM
- Gö6976 (μM)

**Fold Increase HERG Protein**

- Ro-32
- Bis-I
- *
Figure 4

**A**

![Western blot analysis over time (hr)](image)

- HERG myc
- Tubulin

**B**

![Western blot analysis at different concentrations (nM)](image)

- HERG myc
- Tubulin

**C**

<table>
<thead>
<tr>
<th></th>
<th>Bis-1</th>
<th>RO-32-0432</th>
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<tr>
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<td>PMA 10</td>
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**D**

![Western blot analysis with various treatments](image)

- HERG myc
- Tubulin

**Fold-increase HERG**

- control: 0.5
- CPT-cAMP: 1.2
- PMA: 3.0
- PMA & CPT-cAMP: 4.0
- PE (0.1 μM): 1.0
- PE (1 μM): 2.0

**Statistical Significance**

- ***: p < 0.001
- **: p < 0.01
- ns: not significant
Figure 5

A

H-89  0  0  0  10  10 μM
Bis-I  0  0  10  0  10 μM
PE     0  1  1  1  1 μM

HERG-myc
Tubulin

Fold-increase HERG
Control
PE 1μM

B

HERGΔPKC
HERGΔPKA

100 pA
0.5 s

2000 pA
0.5 s

C

<table>
<thead>
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<th>HERG-WT</th>
<th>HERGΔPKC</th>
<th>HERGΔPKA</th>
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<tr>
<td>CPT-cAMP (50μM)</td>
<td>-</td>
<td>+</td>
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HERG-myc
Tubulin

D

Fold-increase over control HERG protein

* ns

CPT-cAMP
PMA
CPT-cAMP & PMA