MUSCARINIC RECEPTOR ACTIVATION INCREASES hERG CHANNEL EXPRESSION THROUGH PHOSPHORYLATION OF Ubiquitin Ligase Nedd4-2

Tingzhong Wang, Andrew Hogan-Cann, Yudi Kang, Zhi Cui, Jun Guo, Tonghua Yang, Shawn M. Lamothe, Wentao Li, AiQuan Ma, John T. Fisher, and Shetuan Zhang

ABSTRACT

The human ether-à-go-go–related gene (hERG) encodes the pore-forming subunit of the rapidly activating delayed rectifier potassium channel, which is important for cardiac repolarization. Reduction of hERG current due to genetic mutations or drug interferences causes long QT syndrome, leading to cardiac arrhythmias and sudden death. To date, there is no effective therapeutic method to restore or enhance hERG channel function. Using cell biology and electrophysiological methods, we found that the muscarinic receptor agonist carbachol increased the expression and function of hERG, but not ether-à-go-go or Kv1.5 channels stably expressed in human embryonic kidney cells. The carbachol-mediated increase in hERG expression was abolished by the selective M3 antagonist 4-DAMP (1,1-dimethyl-4-diphenylacetoxypiperidinium iodide) but not by the M2 antagonist AF-DX 116 (11[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b] 1,4-benzodiazepine-6-one). Treatment of cells with carbachol reduced the hERG-ubiquitin interaction and slowed the rate of hERG degradation. We previously showed that the E3 ubiquitin ligase Nedd4-2 mediates degradation of hERG channels. Here, we found that disrupting the Nedd4-2 binding domain in hERG completely eliminated the effect of carbachol on hERG channels. Carbachol treatment enhanced the phosphorylation level, but not the total level, of Nedd4-2. Blockade of the protein kinase C (PKC) pathway abolished the carbachol-induced enhancement of hERG channels. Our data suggest that muscarinic activation increases hERG channel expression by phosphorylating Nedd4-2 via the PKC pathway.

Introduction

The human ether-à-go-go–related gene (hERG) encodes the rapidly activating delayed rectifier potassium channel (IKr), which plays an important role in cardiac repolarization (Sanguinetti et al., 1995). A reduction in hERG current can cause long QT syndrome (LQTS), predisposing affected individuals to a high risk of fatal cardiac arrhythmias and sudden death (Curran et al., 1995; Keating and Sanguinetti, 2001). Naturally occurring loss-of-function mutations of hERG reduce IKr and cause inherited type 2 long QT syndrome. More frequently, many medications can cause acquired LQTS by impairing hERG function (Keating and Sanguinetti, 2001). In addition, a reduction in extracellular K+ concentration, clinically known as hypokalemia, causes LQTS, and we have previously demonstrated that hypokalemia induces endocytic degradation of hERG channels from the plasma membrane (Guo et al., 2009). Since a reduction in hERG current causes LQTS, measures to restore or enhance hERG channel function have been recognized as a strategy to treat LQTS (Rajamani et al., 2002). However, clinically useful measures that restore or enhance hERG channel function are limited. We recently demonstrated that the E3 ubiquitin ligase Nedd4-2 mediates hERG degradation (Guo et al., 2012). We also found that the serum- and glucocorticoid-inducible kinases (SGK) enhance the expression level of hERG channels partly through the inhibition of Nedd4-2 (Lamothe and Zhang, 2013). Whether other physiological/pathophysiological factors related to cardiac autonomic control also regulate hERG channel expression in a similar manner is not known and is the focus of the present study.

ABBREVIATIONS: Δ1073, C-terminal truncation mutation at position 1073; ACh, acetylcholine; AF-DX 116, 11[(2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b] 1,4-benzodiazepine-6-one; ALLN, N-acetyl-Leu-Leu-Norleu-al; APD, action potential duration; BFA, brefeldin A; CCh, carbachol; co-IP, coimmunoprecipitation; DAG, diacylglycerol; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; EAG, ether-à-go-go; ECG, electrocardiogram; ER, endoplasmic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GF100203X., 2-[(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide, or bisindolylmaleimide I; HEK, human embryonic kidney; hERG, human ether-à-go-go–related gene; IEAD, EAG current; IhERG, hERG current; IKr, rapidly activating delayed rectifier K+ current; l severity, K1.5 current; IP3, inositol 1,4,5-trisphosphate; LQTS, long QT syndrome; Oxo-M, oxotremorine-M; PCR, polymerase chain reaction; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; p-Nedd4-2, phosphorylated Nedd4-2; RT, reverse transcription; SGK, serum- and glucocorticoid-inducible kinases; Ub, ubiquitin; WT, wild-type.
Cardiac function is controlled by the sympathetic and parasympathetic nervous systems via adrenoceptors and muscarinic receptors, respectively (Harvey, 2012). Among five muscarinic receptor subtypes (Brodde and Michel, 1999), M1, M2, and M3 are present in the heart (Brodde and Michel, 1999; Fisher et al., 2004; Wang et al., 2007), with the M2 receptor having a dominant chronotropic effect (Fisher et al., 2004). The endogenous muscarinic receptor agonist acetylcholine (ACh) has been reported to shorten the QT interval on an electrocardiogram (ECG) (Pate et al., 2010), and stimulation of muscarinic receptors with carbachol (CCh) reduces the ventricular action potential duration (APD) in papillary muscles (Arreola et al., 1994). Recently, it was reported that M3 muscarinic receptor overexpression reduces the incidence of arrhythmias and mortality associated with myocardial ischemia/reperfusion in mice (Liu et al., 2011). M3 muscarinic receptor overexpression significantly shortens the APD and facilitates membrane repolarization by increasing the inward rectifying K⁺ current (Liu et al., 2011). Since hERG-encoded IKr is the main component determining the human cardiac action potential duration (Sanguinetti et al., 1995), we investigated the effect of muscarinic receptor activation on the hERG channel. Our data suggest that activation of muscarinic receptors enhances hERG channel expression, and this effect is via M3 muscarinic receptor-mediated phosphorylation of Nedd4-2.

**Materials and Methods**

**Molecular Biology.** hERG cDNA was provided by Dr. Gail Robertson (University of Wisconsin-Madison). The human embryonic kidney (HEK) 293 cell line stably expressing hERG channels (hERG-HEK cells) was obtained from Dr. Craig January (University of Wisconsin-Madison). The hERG Y1078A point mutation and C-terminal truncation mutation at position 1073 (Δ1073) were generated using Phusion Hotstart Polymerase Chain Reaction (PCR) Master Mix (Agilent Technologies, Santa Clara, CA) and confirmed by DNA sequencing (Eurofins MWG Operon, Huntsville, AL). Myc-DDK–tagged human M3 muscarinic receptor plasmid (CHR3) was purchased from Origene (Rockville, MD). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfecting plasmids into HEK 293 cells. Stable cell lines were generated using G418 for selection (Geneaid Biotech Ltd., New Taipei City, Taiwan). cDNA was generated using an Omniscript Reverse Transcription (RT) Kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. In brief, 1 μl of cDNA was added to a PCR reaction tube containing 10 μl of forward/reverse primer mixture, and 8 μl of Taq polymerase contained (in mM) 153 CsCl, 5 MgATP, 10 EGTA, and 10 HEPES (pH 7.2 with CsOH). The bath solution contained (in mM) 135 Cl⁻, 1 MgCl₂, and 2 CaCl₂ (pH 7.4 with NaOH). From a holding potential of −80 mV, the Cs⁺-mediated Ikᵣ was evoked by depolarizing pulses to voltages between −70 and 70 mV for Kᵣ,1.5. The hERG tail currents were recorded upon a repolarizing step to −50 mV. For the amplitude analysis, the peak tail currents were used for Iₖᵣ,1.5, and the pulse currents at the end of the depolarizing steps were used for Iₖᵣ,1.5 and Iₑₕᵣ. For the recording of native Ikᵣ in cultured neonatal rat cardiomyocytes, the pipette solution contained (in mM) 135 CsCl, 5 MgATP, 10 EGTA, and 10 HEPES (pH 7.2 with CsOH). The bath solution contained (in mM) 135 CsCl, 1 MgCl₂, 10 glucose, 10 HEPES, and 10 μM nifedipine (pH 7.4 with CsOH). From a holding potential of −80 mV, the Ca⁺+-mediated Ikᵣ was evoked by depolarizing pulses to voltages between −70 and 70 mV in 10-mV increments. Tail currents upon repolarization to the holding potential of −80 mV after the depolarizing pulse to 50 mV were used for the current amplitudes in control and treated groups of ventricular myocytes (Zhang, 2006). Patch-clamp experiments were performed at room temperature (22 ± 1°C).

**Western Blot Analysis and Coimmunoprecipitation.** Whole-cell proteins isolated from hERG-HEK cells and neonatal rat ventricular myocytes were used. Western blot analysis was performed using the previously described procedure (Guo et al., 2009, 2011b; Massaelli et al., 2010). Proteins were separated on 8% or 15% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and blocked for 1 hour with 5% nonfat milk. The blots were incubated with respective primary antibodies for 1 hour at room temperature and then with the corresponding horseradish peroxidase–conjugated secondary antibodies. β-Actin was used as a loading control. The blots were visualized with Fuji X-ray films using an enhanced chemiluminescence detection kit (GE Healthcare, Piscataway, NJ). To quantify the Western blot data, the band intensities of proteins of interest on each gel were first normalized to their respective actin intensities; the normalized intensities from treated cells were then compared with those from control cells, and expressed as relative values.

For co-IP, 0.5 mg of the protein was incubated with the appropriate primary antibody overnight at 4°C and then precipitated with protein A/G PLUS agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 hours at 4°C. The beads were washed three times with ice-cold radioimmunoprecipitation assay lysis buffer, resuspended in 2× Laemmli sample buffer, and boiled for 5 minutes. The samples were centrifuged at 20,000 g for 5 minutes, and the supernatants were analyzed using Western blot analysis.

**Immunofluorescence Microscopy.** Isolated neonatal rat ventricular myocytes grown on coverslips were cultured without (control) or with 50 μM carbachol for 24 hours. Cells were washed and fixed with 4% ice-cold paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, and blocked with 5% bovine serum albumin in phosphate-buffered saline for 1 hour. hERG (rabbit primary) was stained with a goat anti-hERG primary antibody and Alexa Fluor 488–conjugated donkey anti-goat secondary antibody. Nedd4-2 or phosphorylated Nedd4-2 (p-Nedd4-2) was stained with a mouse anti-Nedd4-2 primary antibody and Alexa Fluor 594–conjugated donkey anti-rabbit secondary antibody. Images were acquired using a Leica TCS SP2 Multi Photon confocal microscope (Leica, Heidelberg, Germany).

**RNA Extraction and Reverse-Transcription Polymerase Chain Reaction.** Total RNA was extracted from control and carbachol-treated hERG-HEK cells using a Total RNA Mini Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan). cDNA was generated using an Omniscript Reverse Transcription (RT) Kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. In brief, 1 μg of RNA, 2 μl of polyN hexamers (100 μM), nuclease-free water, and an Omniscript RT Kit mixture were mixed to make a total volume of 20 μl. To amplify hERG cDNA, 1 μl of cDNA was added to a PCR reaction tube containing 10 μl of 2× PCR Master Mix (Thermo Scientific, Waltham, MA), 1 μl of the forward/reverse primer mixture, and 8 μl of nuclease-free water to reach a final volume of 20 μl. A 4-minute incubation at 94°C served to activate the Taq polymerase contained in the 2× PCR Master Mix. The protocol for PCR was 30 cycles of 94°C for 20 seconds, 60°C for 20 seconds, and 72°C for 20 seconds, followed by a 5-minute
holding at 72°C. Primers for hERG cDNA amplification were as follows: forward, 5′-TCGCTTTTACCGGAAGAT-3′, and reverse, 5′-CTCCCATACCCACCTGGAATTG-3′. β-Actin (internal control) primers were as follows: forward, 5′-CATTCTGGCCTGTGGACCT-3′, and reverse, 5′-TAATGTCACGCGAGTTC-3′. The amplified DNA was run on a 1% agarose gel containing SYBR Safe DNA gel stain and visualized under a UV transilluminator.

Reagents and Antibodies. Rabbit anti-K1.11 (hERG) and anti-ubiquitin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-Nedd4-2 and anti-phospho-Nedd4-2 (Ser448) antibodies were purchased from Cell Signaling Technology (Danvers, MA). PhosSTOP phosphatase inhibitor mixture was purchased from Roche Applied Science (Indianapolis, IN). Goat anti-hERG (C-20 and N-20), Kv10.1 (EAG) antibodies, carbamylcholine chloride (carbachol), 4-DAMP (1,1-dimethyl-4-diphenylacetoxy piperidinum iodide), AF-DX 116 (11-[2-[(diethylamino) benzodiazepine-6-one], ACh chloride, H7, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. The Total RNA Mini Kit was purchased from Geneaid. The Omniscript RT Kit was purchased from Qiagen. The 50 bp DNA ladder and SYBR Safe DNA gel stain were purchased from Invitrogen. The gel loading dye (6×) was obtained from New England Biolabs (Ipswich, MA). The polyN hexamers were purchased from Eurofin (Hamburg, Germany). The 2× PCR Master Mix was purchased from Thermo Scientific.

Data are expressed as the mean ± S.E.M. A one-way analysis of variance or two-tailed Student’s t test was used to determine statistical significance between the control and test groups. * P value of 0.05 or less was considered significant.

Results

Carbachol Selectively Increases hERG Channel Expression and Current. To investigate the effects of muscarinic receptor agonists on the hERG channel, we treated hERG-HEK cells with carbachol (CCh) at different concentrations for various periods. The hERG expression levels in CCh-treated and control cells were compared using Western blot analysis. Wild-type (WT) hERG channel protein is initially synthesized in the endoplasmic reticulum (ER) as the immature core-glycosylated form with a molecular mass of 135 kDa. It is then transported to the Golgi apparatus and fully glycosylated to become the mature form with a molecular mass of 155 kDa, which is destined to the plasma membrane as a functional channel (Zhou et al., 1998). Treatment of hERG-HEK cells with 5, 20, and 50 μM carbachol for 12 hours selectively increased the 155-kDa, but not the 135-kDa hERG band in a concentration-dependent manner (Fig. 1A). We then cultured hERG-HEK cells with 50 μM carbachol for various periods, and our data show that the carbachol-induced increase in hERG expression became obvious at 2 hours, and peaked at 8 hours (Fig. 1B). Thus, we used 50 μM carbachol to treat cells for 8 hours for mechanistic investigations in our study. As shown in Fig. 2A, 50 μM CCh selectively increased the 155-kDa, but not the 135-kDa form of hERG channels. Carbachol treatment also significantly increased I$_{\text{hERG}}$, whose amplitude was measured using the peak tail current upon repolarization to −50 mV following a 4-second depolarizing step to 50 mV. Carbachol treatment (50 μM) increased I$_{\text{hERG}}$ from 1.21 ± 0.14 nA (n = 17) to 1.75 ± 0.14 nA (n = 23, P < 0.05). To investigate whether carbachol treatment affects the biophysical properties of hERG channels, tail current-voltage (g-V) relationships were constructed in control and carbachol-treated cells (n = 5, respectively). Carbachol increased I$_{\text{hERG}}$ without affecting the voltage dependence of hERG activation (Fig. 2B). The half-activation voltage and the slope factor of the g-V curve were −2.8 ± 0.8 and 6.4 ± 0.7 mV in control cells and −6.6 ± 0.9 and 6.8 ± 0.6 mV in CCh-treated cells (n = 5–7 cells, P > 0.05). The CCh treatment did not change the inactivation properties of the hERG channel either. We examined the inactivation rates of hERG channels in control and CCh-treated cells using a three-pulse protocol (Spector et al., 1996). The hERG channels were inactivated by a 1-second depolarizing step to 50 mV. The cell membrane was then repolarized to −100 mV for 10 milliseconds to allow inactivated channels to recover to the open state. The cell membrane was then clamped to various voltages to determine the inactivation rates at each voltage. CCh treatment of 6 hours did not change the hERG inactivation rate; the time constant at ASPET Journals on June 25, 2017 molpharm.aspetjournals.org Downloaded from

![Fig. 1. Carbachol increases the expression level of hERG channels in concentration- and time-dependent manners. (A) Effects of CCh at 5, 20, and 50 μM on the expression of hERG channels. hERG-HEK cells were treated with carbachol for 12 hours. Whole-cell lysates were extracted and hERG expression was analyzed using Western blot analysis. (B) Effects of treatment of cells with 50 μM CCh for various periods on the expression of hERG channels. After hERG-HEK cells were treated with 50 μM carbachol for 2, 4, or 8 hours, whole-cell lysates were extracted and hERG expression was analyzed using Western blot analysis. In both A and B, the band intensities of the 155-kDa and 135-kDa proteins from CCh-treated cells were normalized to those from control (Ctl) cells and expressed as relative values (Rel) in the bar graphs beneath the Western blots (n = 5 for A; n = 4 for B). * P < 0.05; ** P < 0.01 versus Ctl.](https://doi.org/10.1093/molhr/jax136)
HEK 293 cells, both M2 and M3 muscarinic receptors have been reported to be endogenously expressed (Kurian et al., 2009), and our data also confirmed their presence (Fig. 3A, upper panel). Carbachol treatment did not affect the expression of either M2 or M3 receptors expressed in HEK cells (Fig. 3A, upper panel). To confirm the presence of endogenously expressed M3, we overexpressed M3 for comparison. The overexpressed M3 displayed a size consistent with the endogenous M3 (Fig. 3A, lower panel).

To test whether carbachol treatment increases hERG channel expression through muscarinic receptor activation, we treated hERG-HEK cells with two other muscarinic receptor agonists, ACh chloride (2 mM) and Oxo-M (10 μM). As shown in Fig. 3B, both acetylcholine chloride and oxotremorine-M significantly increased the expression of the mature form, but not the immature form of hERG channels. Muscarinic receptor activation exerts its effects on hERG via M3 receptors since the preferential M3 selective antagonist 4-DAMP (1 μM) (Wess...
et al., 1990; Ehlert, 1996) completely abolished the effect of carbachol on hERG channels, whereas the selective M2 antagonist AF-DX 116 (1 \( \mu \)M) (Thomas and Ehlert, 1994; Sawyer and Ehlert, 1998, 1999) did not affect the carbachol-induced increase of hERG expression (Fig. 3C).

**Carbachol Slows the Degradation of hERG Channels.** WT hERG protein is synthesized at ribosomes, core-glycosylated at the ER, fully glycosylated at the Golgi apparatus, and then traffics to the plasma membrane (Zhou et al., 1998; Guo et al., 2007). As mentioned, the 135-kDa band protein represents the core-glycosylated, immature form in the ER, and the 155-kDa band protein represents the fully glycosylated, mature form in the plasma membrane (Zhou et al., 1998; Guo et al., 2007). Carbachol treatment may enhance hERG protein expression by promoting the biosynthesis or obstructing the degradation of hERG channels. To study the effects of carbachol on these processes, the hERG mRNA level and channel degradation rate were examined in the absence (control) and presence of carbachol. RT-PCR analysis showed that the treatment of hERG-HEK cells with 50 \( \mu \)M carbachol for 8 hours did not change the hERG mRNA expression level (Fig. 4A).

To study the effect of carbachol on hERG degradation, BFA (10 \( \mu \)M) was used to block the conversion of hERG from the immature 135-kDa form to the mature 155-kDa form (Guo et al., 2009, 2011a). The degradation rate of hERG was monitored by detecting the 155-kDa band expression level at different time points after BFA treatments in control and carbachol-treated cells. As shown in Fig. 4B, BFA incubation resulted in a time-dependent reduction of the 155-kDa hERG band, reflecting the degradation of mature hERG channels. Carbachol treatment significantly slowed the degradation rate of mature hERG channels. As shown in Fig. 4B, following an 8-hour BFA incubation, the 155-kDa hERG band intensity decreased by 75% \( \pm \) 8% in control cells, whereas it only decreased by 30% \( \pm \) 14% in carbachol-treated cells (\( n = 5, P < 0.01 \)).

![Fig. 4. Carbachol slows hERG channel degradation. (A) CCh treatment does not change hERG mRNA levels. hERG-HEK cells were treated with CCh (50 \( \mu \)M) for 8 hours, and the total RNA was extracted for RT-PCR analysis. The band intensities from carbachol-treated cells were normalized to the values from control (Ctl) cells and summarized (\( n = 4 \)). (B) Carbachol treatment slows hERG degradation. hERG-HEK cells were treated without (Ctl) or with carbachol (50 \( \mu \)M) in the presence of BFA (10 \( \mu \)M), which blocks protein transport from the ER to the Golgi. Cells were collected at the indicated time points for Western blot analysis. The intensities of mature hERG bands (155 kDa) at various time points were normalized to their initial value (time 0 hour) and summarized for Ctl or CCh-treated cells. **P < 0.01 versus Ctl. Rel, relative values.](https://molpharm.aspetjournals.org/content/aspetjournals/111/3/881/F4.large.jpg)

**Carbachol Decreases hERG-Ubiquitin and hERG–Nedd4-2 Interactions.** We have previously reported that hERG is internalized and degraded through enhanced ubiquitination under hypokalemic conditions (Sun et al., 2011). The covalent binding of Ub to proteins, a process known as ubiquitination, is known to label proteins for degradation (Piper and Luzio, 2007). To test whether carbachol treatment delays hERG degradation by impairing hERG ubiquitination, we performed co-IP experiments to determine the effects of carbachol on the hERG-Ub interaction. Whole-cell lysates were immunoprecipitated with an anti-Ub antibody, and hERG protein expression levels in the precipitates were detected. As shown in Fig. 5A, in proteins immunoprecipitated with an anti-Ub antibody, a band close to 155 kDa was detected, which represents the monoubiquitinated mature hERG channels (Guo et al., 2012). Carbachol treatment significantly decreased the hERG-Ub interaction. Compared with control cells, carbachol-treated cells displayed a less-intense ubiquitinated hERG band. Thus, carbachol treatment delays hERG degradation by reducing the ubiquitination of hERG channels.

Recently, we demonstrated that hERG channels are regulated by an E3 ubiquitin ligase, Nedd4-2 (Guo et al., 2012; Cui and Zhang, 2013; Lamotho and Zhang, 2013). Nedd4-2 contains a C2 domain, four WW domains, and a catalytic HECT domain (Ingham et al., 2004). The WW domain of the Nedd4-2 protein recognizes substrates for ubiquitination by binding to the PPIX motif. Nedd4-2 then transfers Ub from the HECT domain to the lysine residues of hERG proteins, leading to the internalization of hERG channels from the cell membrane (Guo et al., 2012; Cui and Zhang, 2013; Lamotho and Zhang, 2013). To investigate the role of Nedd4-2 in the carbachol-induced augmentation of hERG channels, we studied the hERG–Nedd4-2 interaction using co-IP analysis. Whole-cell lysates were extracted from control hERG-HEK cells and from those treated with carbachol for 8 hours. An anti-hERG antibody was used to immunoprecipitate hERG and associated proteins. The precipitates were then immunoblotted to detect Nedd4-2. In control cells, a clear Nedd4-2 band was detected, indicating an interaction between hERG and Nedd4-2. Carbachol treatment significantly decreased the hERG–Nedd4-2 interaction, as a less-intense Nedd4-2 band was observed (Fig. 5B). To further test the involvement of Nedd4-2 in the carbachol-induced enhancement of hERG channels, we investigated the effect of carbachol on two hERG mutants, point mutation Y1078A and C-terminal truncation mutation \( \Delta 1073 \) stably expressed in HEK cells. These two mutants have a disrupted Nedd4-2 interaction, as a disrupted Nedd4-2 binding site (Guo et al., 2012; Cui and Zhang, 2013; Lamotho and Zhang, 2013). As shown in Fig. 5C, both mutations eliminated the carbachol-induced augmentation of hERG expression levels. These data indicate that carbachol treatment increases hERG channel expression by decreasing the Nedd4-2–induced hERG ubiquitination.

**Carbachol Increases hERG Expression through Enhanced Phosphorylation of Nedd4-2.** To determine whether the decreased hERG–Nedd4-2 interaction resulted from reduced Nedd4-2 expression, levels of endogenous Nedd4-2 expression were examined using Western blot analysis. Whole-cell protein was extracted from HEK cells stably expressing WT-hERG or \( \Delta 1073 \)-hERG channels in the absence (control) and presence of carbachol for 8 hours. Carbachol treatment did not affect the total level of endogenous Nedd4-2 in WT-hERG cells (Fig. 6A) or \( \Delta 1073 \)-hERG cells (Fig. 6B).
Phosphorylation of Nedd4-2 leads to the inactivation of its catalytic activity (Debonneville et al., 2001; Lamothe and Zhang, 2013). We recently reported that the SGKs increase hERG channel expression through enhanced Nedd4-2 phosphorylation (Lamothe and Zhang, 2013). To examine whether carbachol increases hERG expression by affecting the phosphorylation level of Nedd4-2, we conducted Western blotting with an antibody selectively targeting p-Nedd4-2. Whole-cell lysates from cells treated without (control; Ctl) or with 50 μM CCh for 8 hours were immunoprecipitated with an anti-hERG (C-20) antibody and immunoblotted with an anti-Nedd4-2 antibody (n = 3). (C) Effects of carbachol on WT and mutant hERG channels. Y1078A displays the 155- and 135-kDa bands like WT hERG, whereas Δ1073 displays the 145- and 125-kDa bands. Mutations (both Δ1073 and Y1078A) that disrupt the Nedd4-2 binding site in the hERG channel abolished the effects of CCh. Whole-cell lysates from cells treated without (Ctl) or with 50 μM CCh for 8 hours were used. The intensities of mature hERG bands from carbachol-treated cells were normalized to their respective controls and summarized in bar graphs shown beneath the Western blots (n = 3–5). **P < 0.01 versus Ctl. Rel, relative values.

Disruption of the Protein Kinase C Pathway Abolishes the Effect of Carbachol on hERG Channels. M3 muscarinic receptors couple via Gq/11 to phospholipase C, which cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate into IP3 (inositol 1,4,5-trisphosphate) and diacylglycerol (DAG) (Eglen, 2012). While IP3 binds to its receptor and mediates calcium release from the endoplasmic reticulum, DAG activates protein kinase C (PKC) (Brodde and Michel, 1999; Eglen, 2012). To examine whether M3 receptor activation increases hERG channel expression through the PKC pathway, hERG-HEK cells were cultured with the PKC activator PMA (10 nM) for 8 hours. PMA incubation significantly increased the expression level of hERG, but failed to do so in H7-treated cells (Fig. 7A). To further demonstrate the effect of PKC activation on hERG channel expression, hERG-HEK cells were cultured with the PKC inhibitor H7 (100 μM) for 8 hours. Whole-cell lysates were isolated, and hERG and phosphorylated Nedd4-2 were detected using their respective antibodies during Western blot analysis. Carbachol significantly increased the expression of mature hERG channel in WT-hERG, but not in Δ1073-hERG cells.

Fig. 5. Carbachol treatment decreases hERG-ubiquitin and hERG–Nedd4-2 interactions. (A) Carbachol treatment decreases the hERG-Ub interaction. Whole-cell lysates from cells treated without (control; Ctl) or with 50 μM CCh for 8 hours were immunoprecipitated with an anti-hERG antibody (n = 3). Anti-GAPDH was used as a negative control. (B) Carbachol treatment decreases the hERG–Nedd4-2 interaction. hERG-HEK cells were treated without or with 50 μM CCh for 8 hours. Whole-cell lysates were immunoprecipitated using an anti-hERG (C-20) antibody and immunoblotted with an anti-Nedd4-2 antibody (n = 3). (C) Effects of carbachol on WT and mutant hERG channels. Y1078A displays the 155- and 135-kDa bands like WT hERG, whereas Δ1073 displays the 145- and 125-kDa bands. Mutations (both Δ1073 and Y1078A) that disrupt the Nedd4-2 binding site in the hERG channel abolished the effects of CCh. Whole-cell lysates from cells treated without (Ctl) or with 50 μM CCh for 8 hours were used. The intensities of mature hERG bands from carbachol-treated cells were normalized to their respective controls and summarized in bar graphs shown beneath the Western blots (n = 3–5). **P < 0.01 versus Ctl. Rel, relative values.

Fig. 6. Carbachol treatment concomitantly increases hERG and phosphorylated Nedd4-2 expression levels. (A and B) Effect of CCh on the expression of WT and Δ1073 mutant hERG, Nedd4-2, and p-Nedd4-2 in cells cultured without (control; Ctl) or with 50 μM CCh for 8 hours. Band intensities of mature hERG (155 kDa for WT hERG; 145 kDa for Δ1073 hERG), Nedd4-2 (110 kDa), and p-Nedd4-2 (110 kDa) from carbachol-treated cells were normalized to their respective controls and summarized in bar graphs shown beneath the Western blots (n = 8 for hERG, n = 5 for Nedd4-2 and n = 5 for p-Nedd4-2, respectively). *P < 0.05; **P < 0.01 versus Ctl. Rel, relative values.
PKC, which in turn phosphorylates Nedd4-2 to reduce the degradation of hERG channels.

**Carbachol Increases the Expression and Function of \( I_{\text{Kr}} \) in Neonatal Rat Ventricular Myocytes.** M3 receptors are expressed in neonatal rat ventricular myocytes (data not shown). To determine whether the M3 muscarinic receptor agonist carbachol increases the expression and function of native cardiac \( I_{\text{Kr}} \), neonatal rat ventricular myocytes were isolated and cultured under control conditions or with carbachol for 24 hours. Our immunofluorescence microscopy findings paralleled the results obtained in HEK cells: carbachol increased the expression of hERG and phosphorylated Nedd4-2, but did not affect the basal Nedd4-2 expression level. hERG was detected in the same cells as Nedd4-2 or p-Nedd4-2. The Alexa Fluor 594-labeled hERG (C-20) primary antibody and Alexa Fluor 488–conjugated (green) donkey anti-goat secondary antibody. Nedd4-2 and p-Nedd4-2 were labeled with their respective rabbit primary antibodies and Alexa Fluor 594–conjugated (red) donkey anti-rabbit secondary antibody. ERG was detected in the same cells as Nedd4-2 or p-Nedd4-2. The Alexa Fluor 594–conjugated secondary antibody. ERG was detected in the same cells as Nedd4-2 or p-Nedd4-2. The Alexa Fluor 594–conjugated secondary antibody. ERG was detected in the same cells as Nedd4-2 or p-Nedd4-2. The Alexa Fluor 594–conjugated secondary antibody. ERG was detected in the same cells as Nedd4-2 or p-Nedd4-2. The Alexa Fluor 594–conjugated secondary antibody. ERG was detected in the same cells as Nedd4-2 or p-Nedd4-2. The Alexa Fluor 594–conjugated secondary antibody.

**Discussion**

hERG/\( I_{\text{Kr}} \) channels are crucial for the repolarization of cardiac action potentials. Genetic mutations or drug interactions are known to disrupt hERG channel function and consequently cause LQTS. Patients with LQTS have a high risk of sudden death due to cardiac arrhythmias. Thus, strategies to enhance or restore hERG channel function have the potential to lead to new treatments for LQTS. For this reason, a great deal of effort has been made for interventions that rescue or enhance the function of WT and especially mutant hERG channels. Approaches that have been found to increase hERG channel function include low-temperature incubation (Zhang et al., 1999), sarcoplasmic/ER Ca\(^{2+}\)-ATPase inhibition (Delisle et al., 2003), hERG channel blockers (Zhou et al., 1999; Ficker et al., 2002), glycerol (Zhou et al., 1999), small RNA interference targeting dominant negative mutant hERG channels (Lu et al., 2013; Zarzoso and Noujaim, 2013), and intragenic suppression (Delisle et al., 2005). Although these approaches are effective for the rescue of some mutant hERG channels in vitro, most of them are difficult to apply clinically (Balijepalli et al., 2010).

In addition, we have previously shown that extracellular K\(^{+}\) regulates hERG function and its stability in the plasma membrane (Guo et al., 2009; Massaeli et al., 2010). Increasing extracellular K\(^{+}\) has been shown to correct the prolonged QT1 intervals in humans (Compton et al., 1996; Choy et al., 1997). In addition, although safety and efficacy issues remain to be adequately addressed, hERG activators which increase hERG channel currents by attenuating the channel inactivation or enhancing the channel conductance are promising strategies for the treatment of LQTS (Perry et al., 2009). In the present study, we demonstrated that M3 muscarinic receptor activation enhances hERG channel expression via phosphorylation of the ubiquitin ligase Nedd4-2, which provides further information on manipulations that can increase hERG currents.
Muscarinic receptor agonists have been reported to reduce the cardiac APD and shorten the QT interval on ECGs, although the underlying mechanisms are not well understood. It was reported that muscarinic receptor agonists, such as choline (Shi et al., 1999) and pilocarpine (Wang et al., 1999), induce a delayed rectifier K\(^+\) current in dispersed cardiomyocytes from guinea pig and canine atria. This delayed K\(^+\) current is distinct from acetylcholine-activated K\(^+\) current. It is suppressed by the M3 selective antagonist 4-DAMP, but not by the M1 selective antagonist pirenzepine, the M2 selective antagonist methoctramine, or the M4 selective antagonist tropicamide (Shi et al., 1999; Wang et al., 1999). The effect of muscarinic receptor activation on hERG channels remains unknown. Since hERG-encoded I\(_{\text{Kr}}\) is the main component responsible for cardiac repolarization, its enhancement by muscarinic receptor activation would shorten the cardiac APD as well as the QT interval on ECGs. Indeed, our data support this possibility. We have demonstrated that the muscarinic receptor agonist carbachol increases mature hERG protein expression as well as hERG current. In addition, carbachol treatment increases the expression and function of I\(_{\text{Kr}}\) channels in neonatal rat ventricular myocytes (Fig. 8).

The expression of hERG channels on the plasma membrane is a balance between anterograde trafficking to and retrograde trafficking from the cell surface (Anderson et al., 2006; Guo et al., 2009). Our data showed that carbachol slowed the degradation rate of mature hERG channels (Fig. 4B). We have previously found that the ubiquitin ligase Ned4-2 targets the PPxY motif in hERG channels at the C terminus to mediate hERG degradation (Guo et al., 2012; Cui and Zhang, 2013; Lamotte and Zhang, 2013). In the present study, we found that carbachol treatment decreased the interaction between Ned4-2 and WT hERG channels (Fig. 5B). Furthermore, disruption of the Ned4-2 binding site in hERG by removing the PPxY motif completely abolished the effect of carbachol on hERG channels (Fig. 5C). Thus, muscarinic receptor activation increases hERG channel expression through the Ned4-2 pathway.

The activity of Ned4-2 is regulated by various factors. We recently demonstrated that the serum- and glucocorticoid-inducible kinases SGK1 and SGK3 regulate hERG channel expression partially via the ubiquitin ligase Ned4-2 (Lamothe and Zhang, 2013). We showed that SGK1 or SGK3 overexpression increases Ned4-2 phosphorylation, which is known to inhibit Ned4-2 activity. In the present study, we found that muscarinic receptor activation also enhances the expression level of phosphorylated Ned4-2, but not the total Ned4-2. Phosphorylation of Ned4-2 reduces its ability to recognize target proteins including hERG channels, leading to an increase in hERG channel expression in the plasma membrane.

Our data showed that, in addition to carbachol, other muscarinic receptor agonists including ACh chloride (2 mM) and Oxo-M (10 \(\mu\)M) increased hERG expression (Fig. 3B). In addition, the M3 selective antagonist 4-DAMP (Wess et al., 1990; Ehlerdt, 1996), but not the M2 antagonist AF-DX 116 (Thomas and Ehlerdt, 1994; Sawyer and Ehlerdt, 1998, 1999), abolished carbachol’s effect on hERG expression (Fig. 3C), implicating the involvement of M3 muscarinic receptor activation in this phenomenon. M3 receptors couple with \(G_{\text{q11}}\) proteins to activate phospholipase C (Brodde and Michel, 1999; Eglen, 2012). This enzyme acts on membrane phosphatidylinositol 4,5-bisphosphate, generating IP\(_3\) that triggers \(Ca^{2+}\) release from intracellular stores, and DAG that activates PKC. We hypothesize that activation of PKC may phosphorylate Ned4-2, leading to Ned4-2 inhibition and thus an increase in hERG expression by decreasing the hERG–Ned4-2 interaction. This hypothesis is supported by our data that the PKC activator PMA significantly increased the expression levels of both hERG channels and phosphorylated Ned4-2. Furthermore, incubation of cells with the PKC inhibitor H7 abolished the effects of carbachol on hERG channels (Fig. 7).

The effects of PKC activation on hERG channels appear to be complex. Barros et al. (1998) found that, in hERG-expressing Xenopus oocytes, thyrotropin-releasing hormone decreased hERG current through a PKC-dependent acceleration of deactivation, and deceleration of activation. Cockrell et al. (2007) also reported that activation of \(G_{\text{q11}}\)-coupled M3 muscarinic receptors with methacholine reduced hERG current amplitude with minor effects on the voltage dependence of activation and inactivation. The response to methacholine was attenuated by either acute inhibition of PKC with 300 nM GF109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide, or bisindolylmaleimide I) or chronic down-regulation of PKC isoforms by 24 hours pretreatment of cells with PMA. Furthermore, Ramstrom et al. (2010) reported that treatment of hERG-expressing HEK cells with 10 \(\mu\)M DAG for 1 hour caused a significant reduction of mature hERG channels in the plasma membrane, and this effect was believed to be related to PKC activation. In contrast, Chen et al. (2010) reported that stimulation of the \(\alpha1A\)-adrenergic receptor with phenylephrine or direct activation of PKC with phorbol ester (10 nM) increased hERG channel protein abundance and current density in a time- and dose-dependent fashion. Channel augmentation reached a steady-state plateau within 24 hours with a 2- to 6-fold induction (Chen et al., 2010; Krishnan et al., 2012). In principle, our observations are consistent with these latter reports (Chen et al., 2010; Krishnan et al., 2012). Nonetheless, although our data showed that PMA increased the expression levels of both the mature and immature forms of hERG more than 2-fold (Fig. 7B), a significant increase in hERG current was not detected (data not shown).

The reasons for the discrepant reports regarding PKC regulation of hERG channels are unknown, but are likely multifaceted. First, different expression systems (e.g., Xenopus oocytes versus HEK 293 cells) have been used, which would provide different cellular environments. There are different types of PKC isoforms. Their presence/abundance, along with chaperone molecules, in specific expression systems may be an important aspect that needs to be further elucidated. Second, various PKC inhibitors/activators have been used. In addition to PKC-mediated effects, these agents may exert direct effects on the channels. For example, it has been shown that the PKC inhibitor bisindolylmaleimide I directly blocks expressed hERG channels and native I\(_{\text{Kr}}\), and prolongs the action potential duration in freshly isolated guinea pig ventricular cardiomyocytes (Thomas et al., 2004). As direct effects of these agents may vary in potency, this would add another layer of interaction, which could contribute to this discrepancy. It should be noted that, as a prototypical \(G_{\text{q11}}\)-coupled receptor, the M3 muscarinic receptor is known to generate the second messengers IP\(_3\) and DAG, which mobilize calcium from the IP\(_3\)-sensitive stores and activate PKC. However, recent molecular and genetic studies have revealed that M3 muscarinic...
receptors can mediate additional cellular processes, such as β-arrestin–dependent activation of protein kinase D1 (Kong et al. 2010). In addition, stimulation of M3 muscarinic receptors has been shown to activate the sodium channel NALCN (sodium leak channel nonselective) via an SFK (Src family of tyrosine kinase)–dependent pathway without the involvement of G proteins (Swayne et al., 2009). There are other potential limitations in the present study. Although we studied the effect of muscarinic receptor activation on various K+ channels (e.g., hERG, Kc,1.5, and EAG1) stably expressed in HEK cells and on ERG channels in neonatal rat cardiomyocytes, we did not study the effect of muscarinic receptor activation on sodium and calcium channels, which also play important roles in the cardiac action potential (George, 2013). Thus, extrapolation of our observations to mammalian and human in vivo function must be made with caution.

In summary, our data demonstrate that M3 muscarinic receptor agonists increase hERG channel function by phosphorylating Nedd4-2. This finding provides new information on hERG channel regulation. Further investigation is warranted to elucidate whether and how the muscarinic receptor–mediated regulation of the hERG channel contributes to in vivo cardiac electrophysiology under physiological and pathophysiological conditions.

**Authorship Contributions**

**Participated in research design:** Wang, Hogan-Cann, Kang, Cui, Guo, Yang, Zhang.

**Conducted experiments:** Wang, Hogan-Cann, Kang, Cui, Guo, Yang, Lamothe, Li.

**Performed data analysis:** Wang, Hogan-Cann, Kang, Cui, Guo, Yang, Lamothe, Zhang.

**Wrote or contributed to the writing of the manuscript:** Wang, Kang, Cui, Guo, Yang, Lamothe, Li, Ma, Fisher, Zhang.

**References**


Arrieta J, Dirksen RT, Perez-Cornejo P, Piech KM, and Sheu SS (1994) Autonomic receptor agonists increase hERG channel function by phosphoinositide 3-kinase–mediated regulation of the hERG channel contributing to in vivo cardiac electrophysiology under physiological and pathophysiological conditions.


Address correspondence to: Dr. Shetuan Zhang, Department of Biomedical and Molecular Sciences, Queen’s University, Botterell Hall, Room 429, 18 Stuart Street, Kingston, ON, K7L 3N6 Canada. E-mail: shetuan.zhang@queensu.ca