Muscarnic Receptor Activation Increases hERG Channel Expression through Phosphorylation of Ubiquitin Ligase Nedd4-2

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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 (I_{Kr}), 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 I_{Kr} and cause inherited type 2 long QT syndrome. More frequently, many medications can cause acquired LQTS by interfering with the rapid activating delayed rectifier potassium channel (IKr), which plays an important role in cardiac repolarization.

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 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.
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) (Patel 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 of the ether-à-go-go (EAG) current (IEAG), the pipette solution contained (in mM) 135 NaCl, 5 KCl, 10 HEPES, 10 glucose, and 2 CaCl2 (pH 7.4 with NaOH). From the holding potential of −80 mV, the Cs+-mediated ICa was evoked by depolarizing pulses to voltages between −70 and 70 mV (50 mV for K1.5). The hERG tail currents were recorded upon a repolarizing step to −50 mV. For the amplitude analysis, the peak tail currents were used for ICa, and the pulse currents at the end of the depolarizing steps were used for ICa, and IEG. For the recording of native ICa 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 Cs+-mediated ICa 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; Massaeli et al., 2010). Proteins were separated on 8% or 15% SDS-PAGE, transferred onto polyvinylidene fluoride 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 20,000g 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 (rat Y1078A protein) 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 rabbit anti–Nedd4-2 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 poly(N 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

**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 (CHRM3) 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 (1 mg/ml) and maintenance (0.4 mg/ml). HEK 293 cells were cultured in Minimum Essential Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1% nonessential amino acids, and 1% sodium pyruvate. For coimmunoprecipitation (co-IP) analysis of ubiquitin (Ub)–hERG interactions, the proteasome inhibitor ALLN (N-acetyl-Leu-Leu-Norleucine; 50 μM) was added to the culture medium to inhibit hERG degradation.

**Neonatal Rat Ventricular Myocyte Isolation.** Experimental protocols used for animal studies were approved by the Queen’s University Animal Care Committee. Single ventricular myocytes were isolated from 1- to 2-day-old Sprague-Dawley rats of either sex by enzymatic dissociation as described previously (Guo et al., 2007). Cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Invitrogen, Burlington, OH) with 10% fetal bovine serum. After treatments, the myocytes were cultured on coverslips for immunochemistry analysis and electrophysiological recordings, and in 100-mm dishes for Western blot analysis.

**Patch-Clamp Recording.** The whole-cell patch-clamp method was used to record hERG current (ICa), K1.5 current (IK1.5), and ether-α-go-go (EAG) current (IEAG). The pipette solution contained (in mM) 135 KCl, 5 EGTA, 1 MgCl2, and 10 HEPES (pH 7.2 with KOH). The bath solution contained (in mM) 135 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1 MgCl2, and 2 CaCl2 (pH 7.4 with NaOH). From the holding potential of −80 mV, ICa, IK1.5, and IEAG from the respective stable cell lines were evoked by 10-mV incremental depolarization steps to voltages between −70 and 70 mV (50 mV for K1.5). The hERG tail currents were recorded upon a repolarizing step to −50 mV. For the amplitude analysis, the peak tail currents were used for ICa, and the pulse currents at the end of the depolarizing steps were used for ICa, and IEG.
holding at 72°C. Primers for hERG cDNA amplification were as follows: forward, 5'-TCGCCTTTCATCCGAAAGAT-3', and reverse, 5'-CTCC ATACCCACCTGAGAAATTG-3'. β-Actin (internal control) primers were as follows: forward, 5'-CATCTGTGAATCTGCACT-3', and reverse, 5'-TAATGTCACGCAGGATTC-3'. The amplified DNA was run on a 1% agarose electrophoresis gel containing SYBR Safe DNA gel stain and visualized under a UV transilluminator.

**Reagents and Antibodies.** Rabbit anti-K.11.1 (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), rabbit anti-K.1.5 (H-120), goat anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), rabbit anti-GAPDH, mouse anti-GAPDH, goat anti-M2 (C-18) and anti-M3 (H-20) antibodies, donkey anti-goat IgG, goat anti-rabbit IgG, goat anti-mouse IgG, and Protein A/G PLUS-Agarose for immunoprecipitation assays were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-actin (AC-10), rabbit anti-K.10.1 (EAG) antibodies, carbamylcholine chloride (carbachol), G418, brefendin A (BFA), oxotremorine-M (Oxo-M), 4-DAMP (1,1-dimethyl-4-diphenylacetoxy piperidiniodide), AF-DX 116 (11-[2-(diethylamino)-13-acetate (PMA) were purchased from Sigma-Aldrich. The Total RNA Mini Kit was purchased from Qiagen. The Omniscript RT Kit was purchased from Promega. 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 Eurofins (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. A 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 155 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 IhERG, 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 IhERG 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 IhERG 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 50 mV was 13.1 ± 0.12 milliseconds in control cells and 11.9 ± 0.9 milliseconds in CCh-treated cells (n = 6–9 cells, P > 0.05).

The hERG channel displays various unique properties. With regards to the degradation pathways of K+ channels, we have previously shown that the ubiquitin ligase Nedd4-2 targeting motif PPXY exists in hERG, but not in K1.5 or EAG (Guo et al., 2012). To test the specificity of carbachol treatment on potassium channels, HEK cells stably expressing K1.5 or EAG channels were examined. Carbachol treatment did not affect the expression or function of either K1.5 (Fig. 2, C and D) or EAG channels (Fig. 2, E and F).

**Carbachol Increases hERG Expression through Muscarinic Receptor Activation.** Carbachol is a cholinomimetic drug that binds and activates muscarinic receptors. In
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 Fig. 2. Carbachol selectively increases mature hERG expression levels and I_{hERG}. (A, C, and E) Effects of CCh on the expressions of hERG, Kv1.5, and EAG channels. hERG-HEK cells were treated with carbachol (50 μM) for 8 hours. Whole-cell lysates were obtained and analyzed using Western blot analysis. The band intensities of the respective channel proteins from CCh-treated cells were normalized to the values from control (Ctl) cells and summarized in the bar graphs beneath the Western blots (n = 7 for hERG, n = 5 for Kv1.5 and EAG, respectively). (B, D, and F) Effect of carbachol on I_{hERG}, I_{Kv1.5}, and I_{EAG}. Families of hERG, Kv1.5, and EAG currents recorded from Ctl or CCh-treated cells. The summarized current-voltage relationships of the hERG tail currents, Kv1.5, or EAG pulse currents (n = 5–8 cells, respectively) are shown beneath the current traces. Open circles denote controls, and solid circles denote CCh treatments. **P < 0.01 versus Ctl. Rel, relative values.

Fig. 3. Effects of muscarinic receptor activation on hERG expression. (A) Upper panel: Western blots showing that the endogenous M2 and M3 receptors are present in HEK 293 cells, and their expression levels are not affected by CCh (n = 3). Lower panel: the presence of endogenous M3 was confirmed by overexpression of exogenous human muscarinic M3 receptor. (B) Muscarinic receptor agonists ACh and Oxo-M increase the expression of mature hERG channels. Cells were treated with 2 mM ACh or 10 μM Oxo-M for 12 hours and analyzed using Western blot analysis. The 155-kDa band intensities from cells treated with agonists were normalized to the values from the respective control (Ctl) cells (n = 3). (C) M3 muscarinic receptor antagonist 4-DAMP, but not M2 receptor antagonist AF-DX 116, abolishes the effect of carbachol on hERG channel expression. Cells were treated with 50 μM CCh for 8 hours in the absence or presence of 1 μM AF-DX 116 or 1 μM 4-DAMP. The 155-kDa band intensities from CCh-treated cells were normalized to the values from their respective control cells (n = 3). *P < 0.05; **P < 0.01 versus Ctl. Endo, endogenous; Over-ex, over-expression; Rel, relative values.
et al., 1990; Ehlert, 1996) completely abolished the effect of carbachol on hERG channels, whereas the selective M2 antagonist AF-DX 116 (1 μM) (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 μM 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 μM) 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% ± 8% in control cells, whereas it only decreased by 30% ± 14% in carbachol-treated cells (n = 5, P < 0.01).

**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 by 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 PPxY 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 Δ1073 stably expressed in HEK cells. These two mutants have 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 Δ1073-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 Δ1073-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 treated with carbachol in the absence (control) or presence of 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 band in WT-hERG, but not in Δ1073-hERG cells.

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 = 3 for Nedd4-2 and p-Nedd4-2, respectively). *P < 0.05; **P < 0.01 versus Ctl. Rel, relative values.
Carbachol, which in turn phosphorylates Nedd4-2 to reduce the degradation of hERG channels.

Carbachol Increases the Expression and Function of Ikᵣ 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 Ikᵣ, 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 ERG and phosphorylated Nedd4-2, but did not affect the expression level of the total Nedd4-2 in both A and B, the intensities of the 155-kDa band of hERG and the 110-kDa p-Nedd4-2 in treated cells were normalized to the values from their respective control (Ctl) cells and summarized in the bar graphs beneath the Western blots. **P < 0.01 versus Ctl. Rel, relative values.

Discussion

hERG/Ikᵣ 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 (Zhou et al., 1999), sarcoplasmic/ER Ca²⁺-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⁺ solutions (Zhang, 2006) showed that carbachol significantly increased Ikᵣ (Fig. 8C).

Fig. 7. PKC activation is involved in the carbachol-induced hERG enhancement. (A) Inhibition of PKC abolishes the carbachol-induced hERG and p-Nedd4-2 enhancements. Cells were treated with CCh for 8 hours in the absence or presence of the PKC inhibitor H7 (100 µM). Carbachol increased the hERG and p-Nedd4-2 expression in control cells but not in H7-treated cells (n = 3). (B) PKC activator PMA significantly increases hERG and p-Nedd4-2 expression in hERG-HEK cells (n = 4). In both A and B, the intensities of the 155-kDa band of hERG and the 110-kDa p-Nedd4-2 in treated cells were normalized to the values from their respective control (Ctl) cells and summarized in the bar graphs beneath the Western blots. **P < 0.01 versus Ctl. Rel, relative values.

Fig. 8. Carbachol increases ERG expression and Ikᵣ in cultured neonatal rat cardiomyocytes. (A) Confocal images showing that CCh (50 µM, 24 hours) treatment increases the native ERG as well as p-Nedd4-2 expression, but does not affect the basal Nedd4-2 expression level. ERG was labeled with an anti-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 the 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 Nedd4-2 and p-Nedd4-2 were detected in separate cells. Images of control or carbachol-treated cells were taken with the same settings. Adjustments of brightness and contrast were uniformly applied to every pixel in each of the images. (B) Western blots showing that CCh treatment (50 µM, 24 hours) increases the expression of ERG. The intensity of the 150-kDa band from CCh-treated cells was normalized to that in control cells and expressed as relative values (n = 7). (C) Carbachol increases native Ikᵣ. Cardiomyocytes were treated with 50 µM CCh for 24 hours. Families of Cs⁺-mediated Ikᵣ along with the summarized tail current amplitudes (n = 20 in control (Ctl), n = 27 in CCh-treated cells) are shown. *P < 0.05 versus Ctl. Ikᵣ, Cs⁺-mediated Ikᵣ, Rel, relative values.
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 methoctramide, 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_K 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_K 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 Nedd4-2 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 Nedd4-2 and WT hERG channels (Fig. 5B). Furthermore, disruption of the Nedd4-2 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 Nedd4-2 pathway.

The activity of Nedd4-2 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 Nedd4-2 (Guo et al., 2009). We showed that SGK1 or SGK3 over-expression increases Nedd4-2 phosphorylation, which is known to inhibit Nedd4-2 activity. In the present study, we found that muscarinic receptor activation also enhances the expression level of phosphorylated Nedd4-2, but not the total Nedd4-2. Phosphorylation of Nedd4-2 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 μM) increased hERG expression (Fig. 3B). In addition, the M3 selective antagonist 4-DAMP (Wess et al., 1990; Ehler, 1996), but not the M2 antagonist AF-DX 116 (Thomas and Ehler, 1994; Sawyer and Ehler, 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_{q11} proteins to activate phospholipase C (Brodde and Michel, 1999; Eglen, 2012). This enzyme acts on membrane phosphatidylinositol 4,5-bisphosphate, generating IP₃ that triggers Ca²⁺ release from intracellular stores, and DAG that activates PKC. We hypothesize that activation of PKC may phosphorylate Nedd4-2, leading to Nedd4-2 inhibition and thus an increase in hERG expression by decreasing the hERG–Nedd4-2 interaction. This hypothesis is supported by our data that the PKC activator PMA significantly increased the expression levels of both hERG channels and phosphorylated Nedd4-2. 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. Cockerill et al. (2007) also reported that activation of G_{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 μM 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 α1A-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_K, 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_{q11}-coupled receptor, the M3 muscarinic receptor is known to generate the second messengers IP₃ and DAG, which mobilize calcium from the IP₃-sensitive stores and activate PKC. However, recent molecular and genetic studies have revealed that M3 muscarinic...
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BALIJEPALLI SY, ANDERSON CL, LIN EC, AND JANUARY CT (2010) Rescue of mutated known enzyme activation on various K+ 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.
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References


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