Muscarinic receptor activation increases hERG channel expression through phosphorylation of

ubiquitin ligase Nedd4-2

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Muscarinic receptor modulation of hERG channels

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List of nonstandard abbreviations used in the paper:

BFA, brefeldin A; CCh, carbachol; hERG, human ether-a-go-go-related gene; I_{hERG}, hERG current; I_{Kr}, the rapidly activating delayed rectifier K^+ current; LQTS, long QT syndrome; Nedd4-2, neural precursor cell expressed developmentally down-regulated protein 4 subtype 2; Ub, ubiquitin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; 4-DAMP, 1,1-Dimethyl-4-diphenylacetoxypiperidinium iodide; AF-DX 116, 11[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b] [1,4]benzodiazepine-6-one.

ABSTRACT

The human ether-a-go-go–related gene (hERG) encodes the pore-forming subunit of the rapidly activating delayed rectifier potassium channel (I_{Kr}), which is important for cardiac repolarization. Reduction of hERG current due to genetic mutations or drug interferences causes long QT syndrome (LQTS), 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 EAG or Kv1.5 channels stably expressed in HEK cells. The carbachol-mediated increase in hERG expression was abolished by the selective M3 antagonist 4-DAMP but not by the M2 antagonist AF-DX 116. 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 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-a-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 (LQT2). 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.

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 Y *et al.*, 2013). M3 muscarinic receptor overexpression significantly shortens the APD and facilitates membrane repolarization by increasing the inward rectifying K^+ current (Liu Y *et al.*, 2013). Since hERG-encoded I_{Kr} 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 *PfuUltra* Hotstart 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. Lipofectamine 2000 (Invitrogen) 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 (MEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1% non-essential amino acids and 1% sodium pyruvate. For co-immunoprecipitation (co-IP) analysis of Ub-hERG interactions, the proteasome inhibitor ALLN (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 immunocytochemical 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 (I_{hERG}), Kv1.5 current ($I_{Kv1.5}$), and EAG current (I_{EAG}). The pipette solution contained (in mM) 135 KCl, 5 EGTA, 1 $MgCl_2$ and 10 HEPES (pH 7.2 with KOH). The bath solution contained (in mM) 135 NaCl, 5 KCl, 10

HEPES, 10 glucose, 1 $MgCl_2$, and 2 $CaCl_2$ (pH 7.4 with NaOH). From the holding potential of

−80 mV, I_{hERG}, $I_{Kv1.5}$, and I_{EAG} from the respective stable cell lines were evoked by 10-mV

incremental depolarization steps to voltages between −70 and 70 mV (50 mV for Kv1.5). The

hERG tail currents were recorded upon a repolarizing step to −50 mV. For the amplitude

analysis, the peak tail currents were used for I_{hERG}, and the pulse currents at the end of the

depolarizing steps were used for $I_{Kv1.5}$ and I_{EAG}. For the recording of native I_{Kr} 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

$MgCl_2$, 10 glucose, 10 HEPES, and 10 μM nifedipine (pH 7.4 with CsOH). From a holding

potential of −80 mV, the Cs^+-mediated I_{Kr} 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 co-Immunoprecipitation (co-IP)

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; Massaeli et al., 2010; Guo et al., 2011b). Proteins were separated on 8 or 15% SDS-

polyacrylamide electrophoresis gels, transferred onto PVDF membranes, and blocked for 1 h

with 5% non-fat milk. The blots were incubated with respective primary antibodies for 1 h 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 ECL detection kit (GE Healthcare). 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) for 4 h at 4 °C. The beads were washed three times with ice-cold RIPA (radioimmunoprecipitation assay) lysis buffer, resuspended in 2× Laemmli sample buffer and boiled for 5 min. The samples were centrifuged at $20,000 \times g$ for 5 min, 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 min, and blocked with 5% BSA (bovine serum albumin) in phosphate buffered saline for 1 h. ERG (rat I_{Kr} 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) were stained with rabbit anti-Nedd4-2 or anti-p-Nedd4-2 primary antibodies and the Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody. Images were acquired using a Leica TCS SP2 Multi Photon confocal microscope (Leica, Germany).

RNA Extraction and RT-PCR

Total RNA was extracted from control and carbachol-treated hERG-HEK cells using a Total RNA Mini Kit (Geneaid Biotech Ltd.). cDNA was generated using an Omniscript RT Kit (QIAGEN) 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), 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-min 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 s, 60 °C for 20 s, and 72 °C for 20 s, followed by a 5-min holding at 72°C. Primers for hERG cDNA amplification were: forward, 5'TCGCCTTCTACCGGAAAGAT3', and reverse, 5'CTCCATCACCACCTCGAAATTG3'. β-actin (internal control) primers were: forward, 5'CATCCTGCGTCTGGACCT3', and reverse, 5'TAATGTCACGCACGATTTCC3'. The amplified DNA was run on a 1% agarose electrophoresis gel containing SYBR Safe DNA gel stain and visualized under an ultraviolet transilluminator.

Reagents and Antibodies

Rabbit anti-Kv11.1 (hERG) and anti-ubiquitin antibodies were purchased from Sigma-Aldrich. Rabbit anti-Nedd4-2 and anti-Phospho-Nedd4-2 (Ser448) antibodies were purchased from Cell Signaling Technology. PhosSTOP phosphatase inhibitor mixture was purchased from Roche Applied Science. Goat anti-hERG (C-20 and N-20), rabbit anti-Kv1.5 (H-120), goat anti-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-Kv10.1 (EAG) antibodies, carbamylcholine chloride (carbachol), G418, brefeldin A (BFA), oxotremorine-M (Oxo-M), 4-DAMP, AF-DX 116, acetylcholine (ACh) chloride, H7 and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. The Total RNA Mini Kit was purchased from Geneaid (Taiwan). 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 (USA). The polyN hexamers were purchased from Eurofins (Germany). The 2× PCR Master Mix was purchased from Thermo Scientific.

Data are expressed as the mean ± the standard error of the mean (S.E.M.). A one-way ANOVA 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 carbamylcholine chloride (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 h 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 h, and peaked at 8 h (Fig. 1B). Thus, we used 50 μM carbachol to treat cells for 8 h for mechanistic investigations in our study. As shown in Fig. 2A, 50 μM carbachol (CCh) selectively increased the 155-kDa, but not the 135-kDa form of hERG channels. Carbachol treatment also significantly increased I_{hERG} whose amplitude was measured using the peak tail current upon repolarization to −50 mV following a 4-s depolarizing step to 50 mV. Carbachol treatment (50 μM) increased I_{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_{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 mV and 6.4±0.7 mV in control cells and −6.6±0.9 mV 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 s depolarizing step to 50 mV. The cell membrane was then repolarized to −100 mV for 10 ms 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 for 6 h did not change the hERG inactivation rate; the time constant at 50 mV was 13.1±0.1.2 ms in control cells, and 11.9±0.9 ms 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 Kv1.5 or EAG (Guo *et al.*, 2012). To test the specificity of carbachol treatment on potassium channels, HEK cells stably expressing Kv1.5 or EAG channels were examined. Carbachol treatment did not affect the expression or function of either Kv1.5 (Fig. 2C and D) or EAG channels (Fig. 2E 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, acetylcholine chloride (ACh, 2 mM) and oxotremorine-M (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 µM) (Thomas and Ehlert, 1994; Sawyer and Ehlert, 1998; Sawyer and Ehlert, 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 h did not change the hERG mRNA expression level (Fig. 4A).

To study the effect of carbachol on hERG degradation, brefeldin A (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; Guo *et al.*, 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-h 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 ubiquitin (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 mono-ubiquitinated 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.

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Recently, we have demonstrated that hERG channels are regulated by an E3 ubiquitin ligase, neural precursor cell expressed developmentally down-regulated protein 4 subtype 2 (Nedd4-2) (Guo *et al.*, 2012; Lamothe and Zhang, 2013; Cui and Zhang, 2013). Nedd4-2 contains a C2 domain, four WW domains and a ubiquitin ligase 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; Lamothe and Zhang, 2013; Cui 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 h. 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; Lamothe and Zhang, 2013; Cui 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 h. 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 serum and glucocorticoid inducible kinases (SGK) 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 phosphorylated Nedd4-2 (p-Nedd4-2). Whole-cell protein was extracted from hERG-HEK cells cultured in the absence (control) or presence of carbachol for 8 h. A phosphatase inhibitor was added to the lysate to stabilize the phosphorylated state of proteins. Nedd4-2 extracted from hERG-HEK cells displayed two bands with molecular masses of 110- and 120-kDa. The 110-kDa band was used for quantification due to the fact that it directly interacts with hERG protein (Cui and Zhang, 2013), as revealed in Fig. 5B. As shown in Fig. 6, carbachol treatment significantly increased the phosphorylated Nedd4-2 (p-Nedd4-2), but not the total level of Nedd4-2 in both WT-hERG and Δ1073-hERG cells. The increased p-Nedd4-2 is accompanied by an increased expression of the mature hERG band in WT-hERG, but not in Δ1073-hERG cells.

Disruption of the PKC Pathway Abolishes the Effect of Carbachol on hERG Channels

M3 muscarinic receptors couple via $G_{q/11}$ to phospholipase C (PLC), which cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Eglen, 2012). While IP_3 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 (10 µM) for 8 h. Whole-cell lysates were isolated, hERG and phosphorylated Nedd4-2 were detected using their respective antibodies during Western blot analysis. Carbachol significantly increased the expression of mature hERG (155-kDa band) and phosphorylated Nedd4-2 in control cells, 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 activator PMA (10 nM) for 8 h. PMA incubation significantly increased the expression level of hERG as well as phosphorylated Nedd4-2 (Fig. 7B). Thus, M3 muscarinic receptor activation may activate PKC, which in turn phosphorylates Nedd4-2 to reduce the degradation of hERG channels.

Carbachol Increases the Expression and Function of I_{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_{Kr}, neonatal rat ventricular myocytes were isolated and cultured under control

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conditions or with carbachol for 24 h. 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 neonatal ventricular myocytes (Fig. 8A). The increased expression of ERG protein was confirmed by Western blot analysis (Fig. 8B). The whole-cell patch clamp recordings of I_{Kr} using symmetrical Cs^+ solutions (Zhang, 2006) showed that carbachol significantly increased I_{Kr} (Fig. 8C).

DISCUSSION

hERG/I_{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 (Zhou *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 (Zarzoso and Noujaim, 2013; Lu *et al.*, 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). As well, 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 QT 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 (I_{K-Ach}). 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 (Wang *et al.*, 1999; Shi *et al.*, 1999). The effect of muscarinic receptor activation on hERG channels remains unknown. Since hERG-encoded I_{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. As well, carbachol treatment increases the expression and function of I_{Kr} channels in neonatal rat ventricular myocytes (Fig. 8).

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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-terminal to mediate hERG degradation (Guo *et al.*, 2012; Lamothe and Zhang, 2013; Cui 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 (Lamothe and Zhang, 2013). We showed that SGK1 or SGK3 overexpression 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 acetylcholine chloride (ACh, 2 mM) and oxotremorine-M (Oxo-M, 10 µM) increased hERG

expression (Fig. 3B). As well, the M3 selective antagonist 4-DAMP (Wess *et al.*, 1990; Ehlert, 1996), but not the M2 antagonist AF-DX 116 (Thomas and Ehlert, 1994; Sawyer and Ehlert, 1998; Sawyer and Ehlert, 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 $G_{q/11}$ proteins to activate PLC (Brodde and Michel, 1999; Eglen, 2012). This enzyme acts on membrane PIP_2, generating IP_3 that triggers Ca^{2+} 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 (TRH) decreased hERG current through a PKC-dependent acceleration of deactivation, and deceleration of activation. Cockerill *et al.* (2007) also reported that activation of $G_{q/11}$-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 or chronic down-regulation of PKC isoforms by 24 h pretreatment of cells with PMA. Furthermore, Ramstrom *et al.* (2010) reported that treatment of hERG-expressing HEK cells with 10 µM DAG for 1 h caused a significant reduction of mature hERG channels in the plasma membrane and this effect was

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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 h 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 by more than 2 folds (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 *vs.* 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_{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 $G_{q/11}$-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 non-selective) 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. While we studied the effect of muscarinic receptor activation on various K^+ channels (e.g. hERG, Kv1.5 and EAG) 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, Jr., 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:

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Wrote or contributed to the writing of the manuscript: Wang, Hogan-Cann, Kang, Cui, Guo,

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Legends for figures

Fig. 1. Carbachol increases the expression level of mature hERG channels in concentration- and time-dependent manners. A, Effects of carbachol (CCh) at 5, 20 and 50 μM on the expression of hERG channels. hERG-HEK cells were treated with carbachol for 12 h. Whole-cell lysates were extracted and hERG expression was analyzed using Western blot analysis. B, Effects of treatment of cells with 50 μM carbachol (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 h, 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 carbachol (CCh)-treated cells were normalized to those from control (Ctl) cells and expressed as relative values in the bar graphs beneath the Western blots (n=5, for A; n=4 for B). *$P<0.05$, **$P<0.01$ *vs.* Ctl.

Fig. 2. Carbachol selectively increases mature hERG expression levels and I_{hERG}. A, C, and E, Effects of carbachol (CCh) on the expressions of hERG, Kv1.5, and EAG channels. hERG-HEK cells were treated with carbachol (50 μM) for 8 h. Whole-cell lysates were obtained and analyzed using Western blot analysis. The band intensities of the respective channel proteins from carbachol (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 control (Ctl) or carbachol (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 (Ctl), and solid circles denote carbachol (CCh) treatments. **$P<0.01$ *vs.* Ctl.

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 carbachol (CCh, n=3). Lower panel, the presence of

endogenous M3 was confirmed by overexpression of exogenous human muscarinic M3 receptor.

B, Muscarinic receptor agonists acetylcholine (ACh) and oxotremorine-M (Oxo-M) increase the

expression of mature hERG channels. Cells were treated with 2 mM ACh or 10 μM Oxo-M for

12 h 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 carbachol

(CCh) for 8 h 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 (Ctl) cells (n=3). *$P<0.05$, **$P<0.01$ *vs.* Ctl.

Fig. 4. Carbachol slows hERG channel degradation. A, Carbachol (CCh) treatment does not

change hERG mRNA levels. hERG-HEK cells were treated with carbachol (CCh, 50 μM) for 8 h

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 (control,

Ctl) or with carbachol (50 μM) in the presence of BFA (10 μM), 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 its initial value (Time 0 h), and summarized for control (Ctl) or carbachol (CCh)-treated cells. **$P<0.01$ *vs.* Ctl.

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) or with 50 µM carbachol (CCh) for 8 h were immunoprecipitated with an anti-Ub antibody and immunoblotted 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 carbachol (CCh) for 8 h. 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 carbachol (CCh). Whole-cell lysates from cells treated without (control, Ctl) or with 50 µM carbachol (CCh) for 8 h 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). **$P<0.01$ *vs.* Ctl.

Fig. 6. Carbachol treatment concomitantly increases hERG and phosphorylated Nedd4-2 expression levels. A and B, Effect of carbachol (CCh) on the expression of WT and Δ1073 mutant hERG, Nedd4-2, and phosphorylated Nedd4-2 (p-Nedd4-2) in cells cultured without

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(control, Ctl) or with 50 µM carbachol (CCh) for 8 h. 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). $*P<0.05$, $**P<0.01$ vs. Ctl.

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 carbachol (CCh) for 8 h 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 phorbol 12-myristate 13-acetate (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. $**P<0.01$ vs. Ctl.

Fig. 8. Carbachol increases ERG expression and I_{Kr} in cultured neonatal rat cardiomyocytes. A, Confocal images showing that carbachol (CCh, 50 µM, 24 h) 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-

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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 carbachol (CCh) treatment (50 µM,

24 h) 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 I_{Kr}. Cardiomyocytes were treated with 50 µM carbachol (CCh) for 24 h.

Families of Cs^+-mediated I_{Kr} along with the summarized tail current amplitudes (n=20 in Ctl,
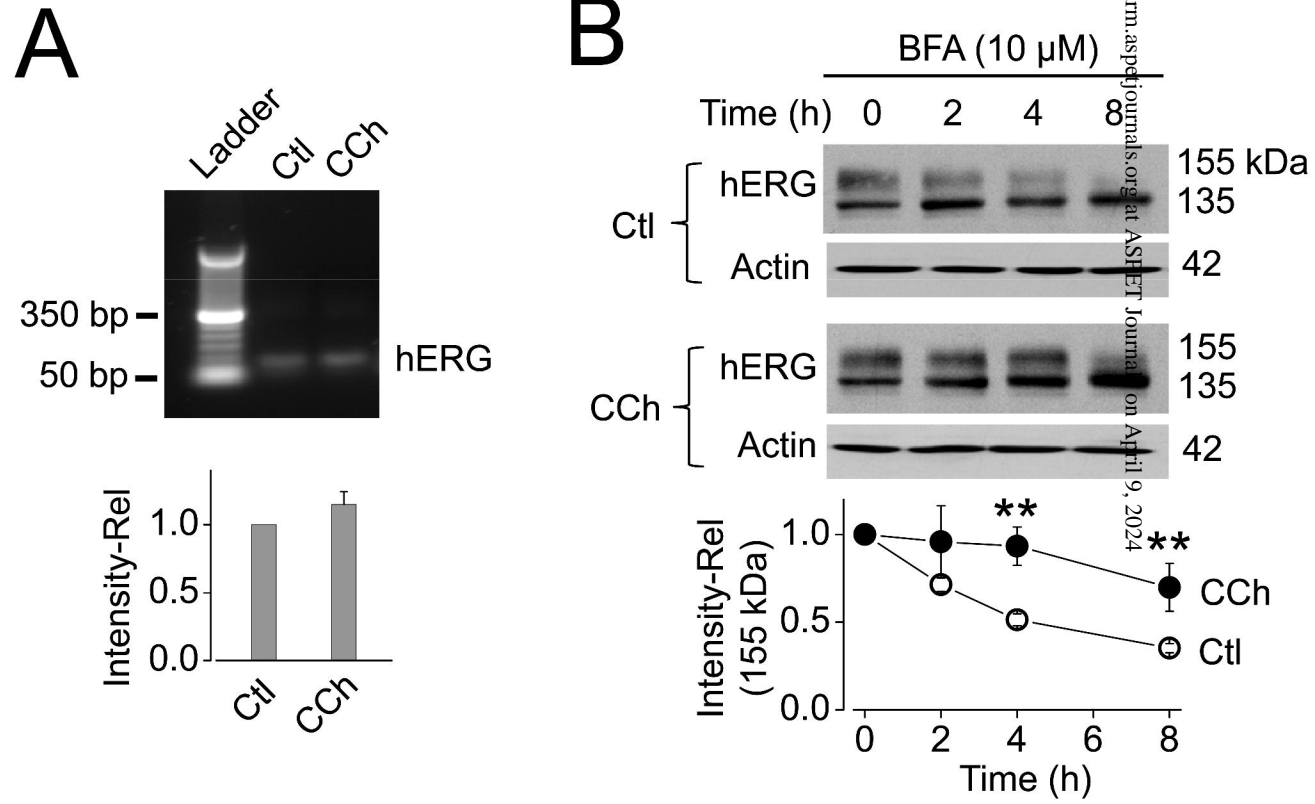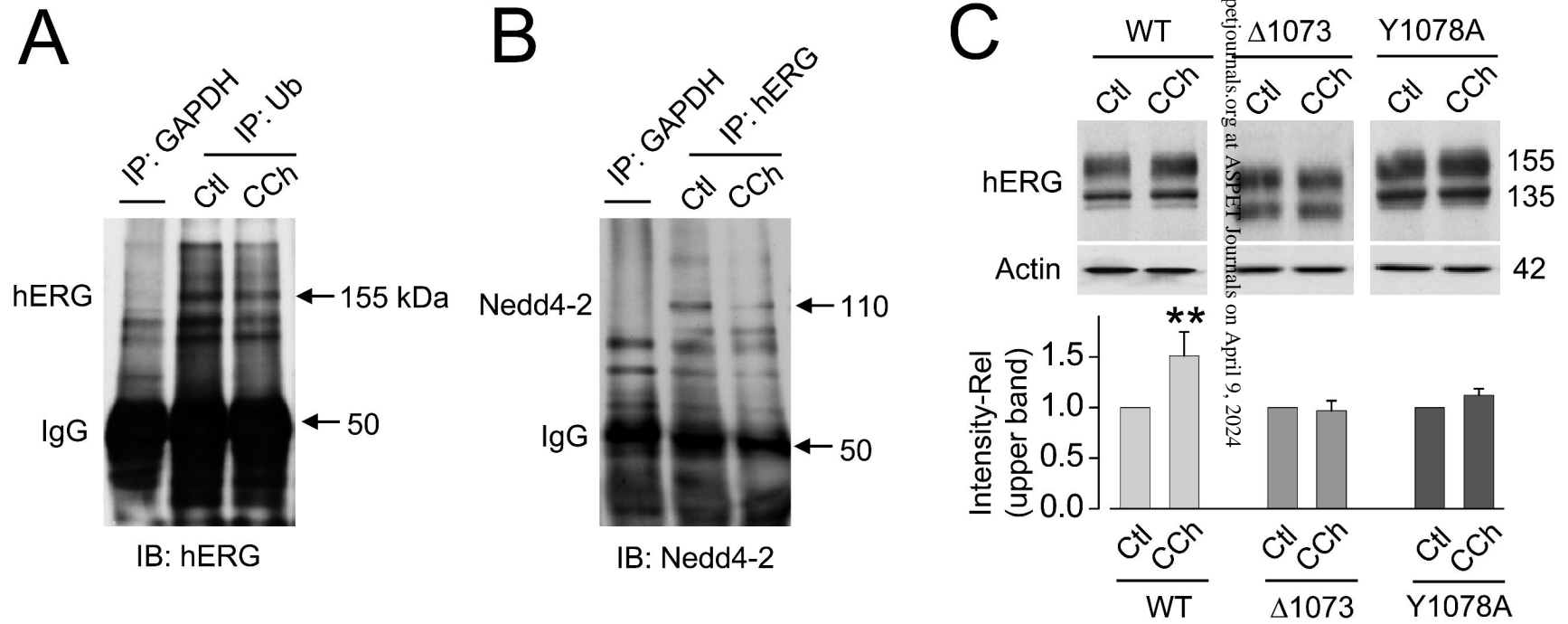
n=27 in CCh-treated cells) are shown. *$P<0.05$ *vs.* Ctl.

Figure 1
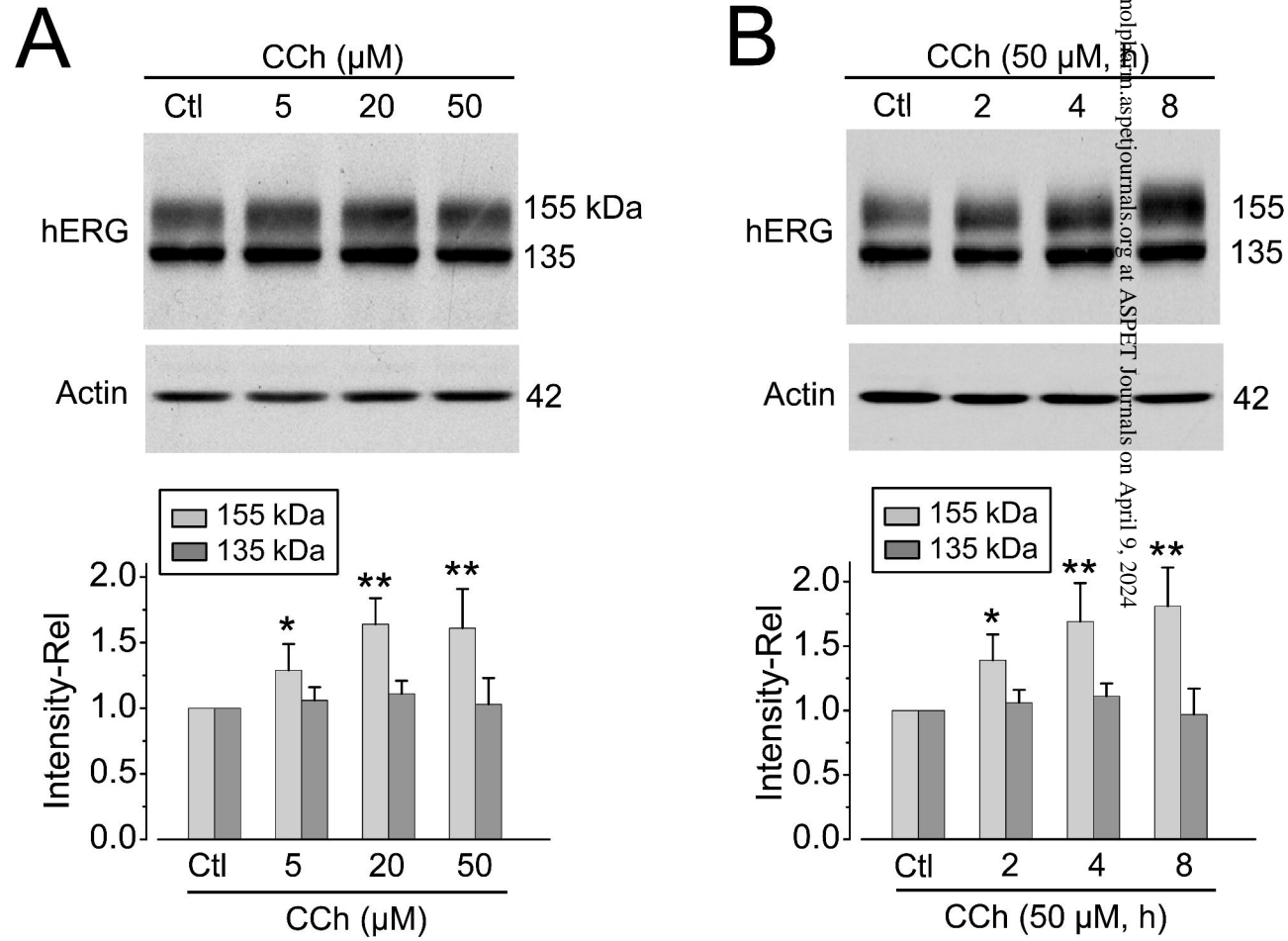
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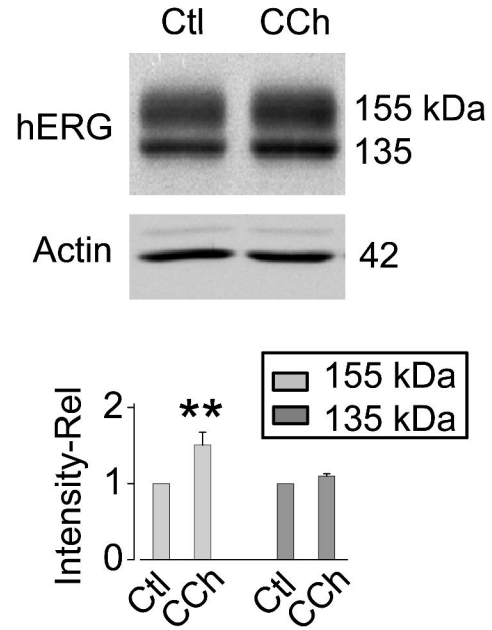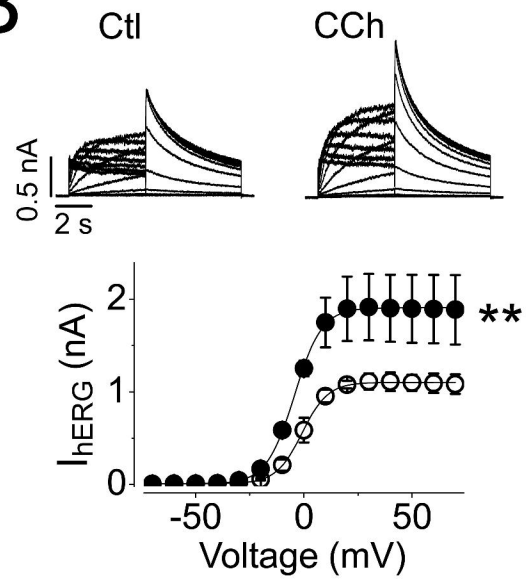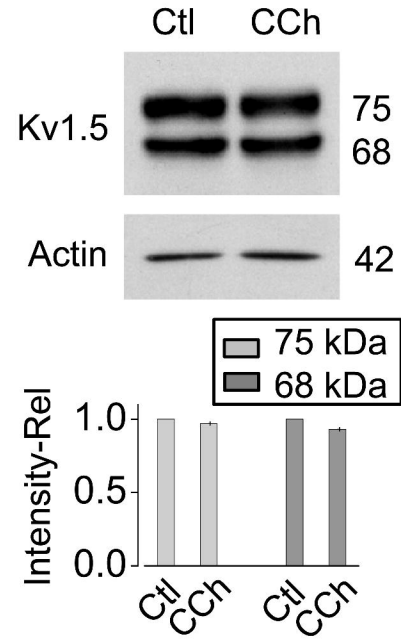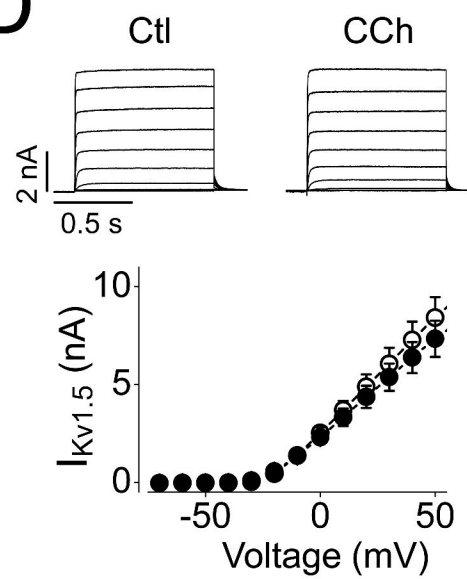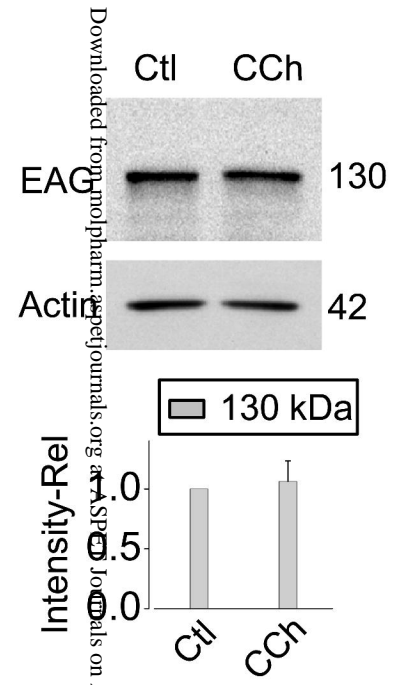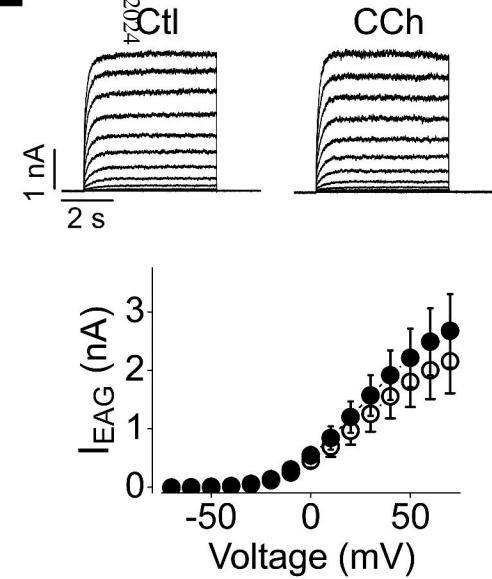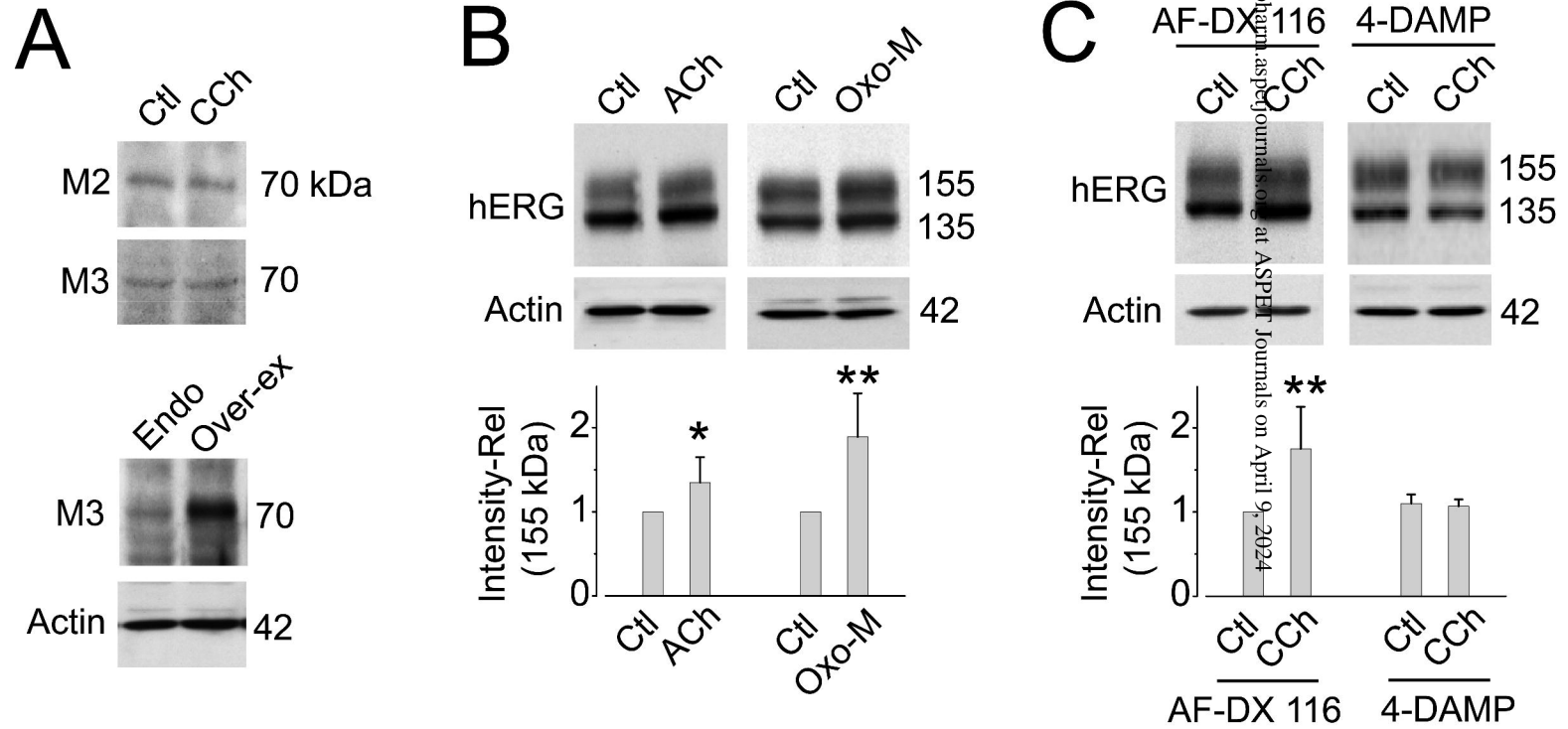
Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8