

β-Arrestin-mediated Regulation of the Human Ether-a-go-go-Related Gene (hERG)

Potassium Channel

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Abbreviations: CCh, carbachol; CNO, clozapine-N-oxide; co-IP, co-immunoprecipitation; DREADDs, designer receptors exclusively activated by designer drugs; EAG, ether-a-go-go; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEK, human embryonic kidney; hERG, human ether-a-go-go-related gene; I_{EAG} , EAG current; I_{hERG} , hERG current; I_{Kr} , rapidly activating delayed rectifier K^+ current; $I_{Kv1.5}$, Kv1.5 current; LQTS, long QT syndrome; M3D-arr, arrestin-biased M3 muscarinic receptor-based DREADD; PCR, polymerase chain reaction; PKC, protein kinase C; SGK, serum- and glucocorticoid-inducible kinases.

ABSTRACT

The rapidly activating delayed rectifier K⁺ channel (I_{Kr}) is encoded by the *human ether-a-go-go-related gene* (*hERG*), which is important for the repolarization of the cardiac action potential. Mutations in hERG or drugs can impair the function or decrease the expression level of hERG channels, leading to long QT syndrome (LQTS). Thus, it is important to understand hERG channel trafficking and its regulation. For this purpose, G protein-coupled receptors (GPCRs), which regulate a vast array of cellular processes, represent a useful route. The development of designer GPCRs known as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) has made it possible to dissect specific GPCR signaling pathways in various cellular systems. In the present study, by expressing an arrestin-biased M3 muscarinic receptor-based DREADD (M3D-arr) in stable hERG-expressing HEK (hERG-HEK) cells, we demonstrate that β -arrestin signaling plays a role in hERG regulation. By exclusively activating M3D-arr using the otherwise inert compound, clozapine-N-oxide (CNO), we found that M3D-arr activation increased mature hERG expression and current. Within this paradigm, M3D-arr recruited β -arrestin-1 to the plasma membrane, and promoted PI3K-dependent activation of Akt. The activated Akt acted through phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) and Rab11 to facilitate hERG recycling to the plasma membrane. Potential β -arrestin signaling mediated increases in hERG and I_{Kr} were also observed in hERG-HEK cells as well as in neonatal rat ventricular myocytes treated with the muscarinic agonist carbachol. These findings provide novel insight into hERG trafficking and regulation.

INTRODUCTION

The *human ether-a-go-go-related gene* (*hERG* or *KCNH2*) encodes the rapidly activating delayed rectifier K⁺ channel (I_{Kr}) (Sanguinetti *et al.*, 1995; Trudeau *et al.*, 1995). A decrease in I_{Kr} due to loss-of-function mutations or drug interactions can cause inherited or acquired long QT syndrome (LQTS), a cardiac electrical disorder characterized by a prolongation of QT intervals on an ECG (Curran *et al.*, 1995; Keating and Sanguinetti, 2001; Kuryshv *et al.*, 2005; Guo *et al.*, 2007; Guo *et al.*, 2009). Individuals with LQTS are predisposed to the ventricular arrhythmias or even sudden death (Keating and Sanguinetti, 2001).

Mutations in hERG can decrease I_{Kr} through various mechanisms (Zhou *et al.*, 1998) with trafficking deficiency being one of the most frequent reasons (Anderson *et al.*, 2006). Thus, rescue of mutant hERG trafficking is of clinical relevance. Due to the critical role of hERG in cardiac repolarization and the development of LQTS, it is important to understand the regulation of hERG protein trafficking and expression. The versatile signaling of G protein-coupled receptors (GPCRs) represents a useful route to study hERG regulation (Lee *et al.*, 2001; Takeda *et al.*, 2002). We previously showed that activation of M3 muscarinic receptor increases hERG channel expression through G_q protein-mediated activation of PKC (Wang *et al.*, 2014). However, in addition to G proteins, GPCRs recruit β-arrestins upon ligand binding (Reiter and Lefkowitz, 2006). β-arrestins not only play crucial role in desensitizing and internalizing GPCRs but also act as multi-protein scaffolds to promote G protein-independent signaling (Luttrell *et al.*, 2001; Ahn *et al.*, 2004; Shenoy *et al.*, 2006; Noma *et al.*, 2007). Traditionally, complex signaling and heterologous expression of GPCRs makes it difficult to analyze distinct β-arrestin signaling pathways (Hermans, 2003). However, the development of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) has allowed for the selective activation of GPCR

subtypes by an otherwise pharmacologically inert ligand (Wess *et al.*, 2013; Thiel *et al.*, 2013). In this regard, the β -arrestin-biased M3 muscarinic receptor-based DREADD (M3D-arr) represents a powerful tool to examine β -arrestin signaling. M3D-arr contains two point mutations (Y148C and A238G) that prevent binding of endogenous acetylcholine and allow for activation by the otherwise pharmacologically inert compound, clozapine-N-oxide (CNO) (Armbruster *et al.*, 2007; Alvarez-Curto *et al.*, 2011). A third point mutation in the conserved “DRY” (Asp-Arg-Tyr) motif, R165L, uncouples G_q proteins to produce exclusive β -arrestin signaling upon CNO-induced M3D-arr activation (Nakajima and Wess, 2012).

In the present study, utilizing the M3D-arr, we investigated the role of β -arrestin in the regulation of hERG channels. We found that CNO-mediated activation of M3D-arr enhanced hERG protein expression and current. Mechanistically, β -arrestin signaling stimulated PI3K-mediated activation of Akt (protein kinase B, PKB), and consequently promoted recycling of hERG channels to the plasma membrane through the phosphatidylinositol 3-phosphate 5-kinase, PIKfyve, and the small GTPase Rab11.

MATERIALS AND METHODS

Molecular Biology

The human embryonic kidney (HEK) 293 cell lines that stably express hERG (hERG-HEK cells), Kv1.5 (Kv1.5-HEK cells) or EAG channels (EAG-HEK cells) were used (Guo *et al.*, 2009; Guo *et al.*, 2012; Wang *et al.*, 2014; Lamothe and Zhang, 2013; Lamothe *et al.*, 2016). hERG cDNA was acquired from Dr. Gail Robertson (University of Wisconsin-Madison). Kv1.5 cDNA was acquired from Dr. Michael Tamkun (Colorado State University, Fort Collins, Colorado). hEAG cDNA acquired from Dr. Luis Pardo (Max-Planck Institute of Experimental

Medicine, Göttingen, Germany). The HA-tagged rat arrestin-biased M3 Designer Receptor Exclusively Activated by Designer Drugs (DREADD) [Rq(R165L)] (M3D-arr) plasmid was obtained from Dr. Jürgen Wess (Nakajima and Wess, 2012) (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health). The FLAG-tagged human PTEN (phosphatase and tensin homolog deleted on chromosome 10) plasmid was obtained from Dr. Xiaolong Yang (Queen's University). The Rab11 dominant-negative (DN) mutant (Rab11 S25N) plasmid was obtained from Addgene (Cambridge, Massachusetts). hERG-HEK cells were maintained in normal culture medium which contained Minimum Essential Medium (MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1 × non-essential amino acids and 1 mM sodium pyruvate, and 0.4 mg/ml G418 (Invitrogen). Lipofectamine 2000 (Invitrogen) was used to transfect 2 or 8 µg of the intended plasmid into HEK cells cultured in 35 mm or 100 mm dishes, respectively. Empty pcDNA3 plasmid was used as a control. For electrophysiological studies, co-transfection of a GFP plasmid (pIRES2-EGFP, Clontech) with plasmids of interest at a ratio of 1:3 was used to identify transfected cells. Following transfections, cells were cultured for 24 h before experiments were performed.

Western Blot Analysis

Whole-cell proteins were isolated from HEK cells that express intended proteins after various treatments. Cells were washed twice and collected in ice-cold phosphate buffered saline (PBS). Cells were then suspended in radioimmunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF, 1 mM, Sigma) and a protease inhibitor cocktail (1×, Sigma). For detecting phosphorylated Akt, a phosphatase inhibitor PhosSTOP (1×, Roche) was added to the buffer solution to stabilize phosphorylated proteins. Whole-cell protein lysates were

generated via sonication and centrifugation at 10,000 \times g for 10 min. Protein concentration measurements were performed using the Bio-Rad DC Protein Assay Kit (Bio-Rad). 15 μ g of protein in 50 μ l of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) sample buffer was separated using 8% SDS-polyacrylamide gels. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes and blocked for 1 h with 5% non-fat milk. Membranes were treated with a primary antibody followed by a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody, each for 1 h. Incubations were followed by triple 10-min washes in Tris-Buffered Saline and Tween 20 (TBS-T, 0.1% Tween-20). An enhanced chemiluminescent kit (ECL, GE Healthcare) was used to visualize proteins on Fuji X-ray films. The expression of actin was detected for controlling potential differences. In each gel, the band intensities of intended proteins were normalized to their corresponding actin intensities. The normalized band intensities of treatment groups were compared to those of controls and expressed as relative values.

Co-immunoprecipitation (Co-IP)

For each sample, 0.5 mg of whole-cell protein was incubated with β -arrestin-1 or β -arrestin-2 antibodies in 0.5 ml lysis buffer overnight at 4° C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used as a negative control. Protein A/G PLUS agarose beads (Santa Cruz Biotechnology) was mixed with the protein-antibody complexes for 4 h at 4° C. The beads were washed 4 times with 0.5 ml of ice-cold RIPA lysis buffer and centrifuged at 10,000 \times g for 2 min. After being resuspended in 50 μ l of 2 \times Laemmli sample buffer and boiled for 5 min, samples were centrifuged at 10,000 \times g for 10 min. The supernatant was used for Western blot analyses to detect precipitated proteins.

RNA extraction and quantitative reverse transcription PCR

Twenty-four hours after transfection of empty pcDNA3 or HA-tagged M3D-arr plasmid into hERG-HEK cells, cells were treated with or without CNO for 24 h. Total cellular RNA was then extracted from cells using a Total RNA Mini Kit (Cat No: RB050, Geneaid Biotech Ltd., Taiwan), and treated with DNase I (Cat No: M0303S, New England BioLabs). RNA concentration was assessed using a spectrophotometer (Molecular Devices, spectra MAX plus, CA, USA).

Reverse transcription of total RNA (1 µg) to cDNA was performed using the Omniscript RT kit (Cat No: 205111, Qiagen). Quantitative real time PCR (RT-qPCR) was performed with TaqMan Gene Expression Master Mix (Cat No: 4369016, Life Technologies) and a thermal cycler (Model 7500, Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control (housekeeping) gene. Oligonucleotide primers (hERG: Assay ID, Hs04234270_g1; GAPDH: Assay ID, Hs03929097_g1) were obtained from Life Technologies. The PCR protocol used was: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and expressed as fold induction of hERG normalized to GAPDH.

Immunofluorescence Microscopy

hERG-HEK cells were cultured on glass coverslips and transiently transfected with empty pcDNA3 or HA-tagged M3D-arr plasmid for 24 h. The cells were then treated without (Ctrl) or with CNO. 24 hours after treatment, cells were fixed with an ice-cold 4% paraformaldehyde-PBS solution for 15 min, permeabilized with 0.1% Triton X-100 for 10 min,

and then blocked with 5% bovine serum albumin (BSA) for 1 h. To detect HA-tagged M3D-arr, a rabbit anti-HA primary antibody and an Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody were used. To detect β -arrestin-1 or β -arrestin-2, a goat anti- β -arrestin-1 or mouse anti- β -arrestin-2 primary antibody and an Alexa Fluor 488-conjugated donkey anti-goat or goat anti-mouse secondary antibody were used.

Patch-clamp Recording

The whole-cell voltage-clamp method was used to record hERG (I_{hERG}), Kv1.5 ($I_{Kv1.5}$) or EAG current (I_{EAG}) in respective stable HEK cells. Cells were held at a holding potential of -80 mV and elicited by a series of depolarizing steps ranging from -70 to 70 mV in 10 mV increments. The depolarizing steps were followed by repolarizing steps to -50 mV. Current amplitudes for I_{hERG} were analyzed using peak tail currents after 50 mV depolarizing steps. Current amplitudes for $I_{Kv1.5}$ and I_{EAG} were analyzed using currents upon the end of 50 -mV depolarizing steps. The bath solution contained 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES (pH 7.4 with NaOH). The pipette solution contained 135 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES (pH 7.2 with KOH). Ventricular myocytes were isolated from 1-day-old Sprague-Dawley rats of either sex by enzymatic dissociation (Guo *et al.*, 2007). Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Invitrogen, Burlington, ON) supplemented with 10% FBS. Myocytes were cultured on coverslips overnight and then subjected to various treatments prior to patch clamp recordings. I_{Kr} in neonatal rat ventricular myocytes was isolated using symmetrical Cs^+ solutions as Cs^+ -mediated I_{Kr} (I_{Kr-Cs}). Cells were depolarized to voltages between -70 and $+70$ mV in 10 mV increments and repolarized to a holding potential of -80 mV. Peak tail

currents upon -80 mV following depolarizing steps to 50 mV were used to measure I_{Kr-Cs} amplitude (Zhang, 2006).

Reagents and Antibodies

MEM, FBS, G418, non-essential amino acids, sodium pyruvate, Alexa Fluor 594-conjugated donkey anti-rabbit and Alexa Fluor 488-conjugated donkey anti-goat and goat anti-mouse secondary antibodies were purchased from Invitrogen. Rabbit anti-Kv11.1 (hERG), anti-Kv10.1 (EAG-1), and anti-HA (H6908), mouse anti-FLAG (F3165), anti-HA (H3663), and anti-actin (A4700) primary antibodies, electrolytes, EGTA, HEPES, glucose, BSA, Akt1/2 kinase inhibitor (A6730), SC79 (Akt activator, SML0749), and carbamylcholine chloride (carbachol, C4382) were purchased from Sigma-Aldrich. Goat anti-hERG (C-20) (sc-15968), anti- β -arrestin-1 (sc-9182), anti-GAPDH (sc-20357), mouse anti- β -arrestin-2 (sc-13140), anti-GAPDH (sc-51907) and rabbit anti-Kv1.5, anti-Akt (sc-1618-R) and anti-p-Akt (sc-135650) primary antibodies, as well as goat anti-mouse (sc-2005), mouse anti-goat (sc-2354), goat anti-rabbit (sc-2004) IgG-HRP secondary antibodies and protein A/G PLUS agarose beads (sc-2003) were purchased from Santa Cruz Biotechnology. YM201636 (PIKfyve inhibitor, 13576) was purchased from Cayman Chemical. H7 (PKC inhibitor, I885000) was obtained from Toronto Research Chemicals. PhosSTOP phosphatase inhibitor (04906845001) was purchased from Roche Applied Science.

Statistical Analysis

All data are presented as the mean \pm the standard error of the mean (S.E.M.). In all cases, n indicates the number of independent replicates. Two-tailed unpaired Student's *t* test (Fig. 3 and

7) or ordinary one-way ANOVA (Fig. 1, 2, 6, 8-10) were used to determine statistical significance between control and treatment groups. GraphPad Prism was used for analysis, and Newman-Keuls post hoc tests were used in ANOVA to determine the differences between control and treatment groups. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Activation of M3D-arr Increases Mature hERG Protein Expression and Current

To investigate the effect of M3D-arr activation on hERG, hERG-HEK cells were transfected with empty pcDNA3 (control) or M3D-arr plasmid. 24 hours following transfection, cells were treated with the selective M3D-arr activator, CNO, at different concentrations and for various time periods. hERG protein expression was analyzed using Western blot analysis.

On Western blots, hERG proteins display two bands with molecular masses of 135 kDa and 155 kDa. It has been established that under normal culture conditions, the 135-kDa band represents the immature, core-glycosylated channel proteins localized in the endoplasmic reticulum (ER), and the 155-kDa band represents the mature, fully-glycosylated channel proteins localized in the plasma membrane (Zhou *et al.*, 1998; Gong *et al.*, 2002). The distinct cellular localization of the immature (135-kDa) and mature (155-kDa) hERG proteins has been consistently demonstrated by a series of previous works. For example, biotinylation-isolated cell-surface proteins from hERG-HEK cells only display the 155-kDa hERG band (Gianulis and Trudeau, 2011; Kang *et al.*, 2015). As well, selective cleavage of cell-surface proteins using extracellularly applied proteinase K completely eliminates the 155-kDa hERG band but does not affect the 135-kDa hERG band (Zhou *et al.*, 1998; Rajamani *et al.*, 2006; Lamothe *et al.*, 2016).

Furthermore, inhibition of hERG maturation by blocking protein Golgi transit using brefeldin A completely eliminates the 155-kDa hERG band as well as the presence of hERG in the plasma membrane (Guo *et al.*, 2009). Thus, we take advantage of the two separate bands to investigate the effects of M3D-arr activation on the mature and immature hERG protein expression. Treatment with 0.1, 1, and 10 μ M CNO increased mature hERG expression in M3D-arr-, but not in pcDNA3-transfected cells, in a concentration-dependent manner (Fig. 1A). Furthermore, treatment of cells with 10 μ M CNO caused a time-dependent increase in mature hERG expression (Fig. 1B). CNO is inert in hERG-HEK cells without M3D-arr; treatment of pcDNA3-transfected cells with 10 μ M CNO for 24 h had no effect on hERG expression (Fig. 1).

M3D-arr expression alone did not affect hERG expression (Fig. 2A). CNO significantly enhanced the expression of the 155-kDa, but not 135-kDa hERG protein, in M3D-arr-transfected hERG-HEK cells (Fig. 2A). Quantitative reverse transcription PCR (RT-qPCR) on pcDNA3- and M3D-arr-transfected hERG-HEK cells cultured without (Ctrl) or with CNO was performed. CNO did not alter the relative hERG mRNA expression levels in pcDNA3- or M3D-arr-transfected hERG-HEK cells (Fig. 2B). To record hERG current (I_{hERG}) whole-cell patch clamp experiments were conducted. Families of I_{hERG} were recorded with the protocol shown inset of Fig. 2C. The activation-voltage relationship of hERG channels was obtained by fitting the tail current-depolarizing voltage relationships to the Hill equation. In pcDNA3-transfected hERG-HEK cells, the half activation voltage and the slope factor were -1.7 ± 2.5 mV and 7.9 ± 0.4 in control cells ($n=11$), and were -5.2 ± 2.5 mV and 8.1 ± 0.6 in CNO-treated cells ($n=7$, $P>0.05$). Thus, CNO was inert in the absence of M3D-arr and did not affect I_{hERG} . However, CNO significantly increased I_{hERG} in M3D-arr transfected cells without affecting the activation-voltage relationship of hERG channels (Fig. 2D). In M3D-arr-transfected hERG-HEK cells, the half

activation voltage and the slope factor were -4.0 ± 2.8 mV and 7.3 ± 0.3 in control cells ($n=10$), and they were -0.5 ± 2.7 mV and 7.5 ± 0.2 for CNO treated cells ($n=12$, $P>0.05$).

The hERG channel displays various unique properties. With regards to protein trafficking, 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). As well, we have shown that activation of the serum- and glucocorticoid-inducible kinase (SGK) enhances hERG, but neither Kv1.5 nor EAG, expression via Nedd4-2 and GTPase Rab11-mediated recycling (Lamothe and Zhang, 2013). To test the specificity of M3D-arr activation on potassium channels, HEK cells stably expressing Kv1.5 or EAG channels were transfected with M3D-arr plasmid. 24 h after transfection, cells were treated without (control) or with CNO (10 μ M). CNO treatment did not affect the expression or function of either Kv1.5 or EAG channels (Fig. 3).

CNO Enhances M3D-arr- β -arrestin Interaction and Co-localization at the Plasma Membrane

Among four arrestin isoforms, β -arrestin-1 (arrestin 2) and β -arrestin-2 (arrestin 3) exhibit 78% amino acid sequence homology and are ubiquitously expressed in various tissues (Sterne-Marr *et al.*, 1993; Attramadal *et al.*, 1992). Following agonist binding, β -arrestins associate with GPCRs to facilitate receptor desensitization and internalization, as well as G protein-independent signaling (Shenoy *et al.*, 2006; Goodman, Jr. *et al.*, 1996). β -arrestin recruitment is common to many GPCRs, including the β_2 -adrenergic receptor (β_2 -AR), angiotensin II type 1 receptor (AT₁R), muscarinic M2 receptor, and muscarinic M3 receptor (Novi *et al.*, 2005; Pals-Rylaarsdam *et al.*, 1997; Shenoy *et al.*, 2006; Ahn *et al.*, 2004). M3D-arr is designed to recruit β -arrestins upon CNO binding (Wess *et al.*, 2013; Wess, 2016; Nakajima and Wess, 2012). Since

β -arrestin isoforms display varying affinity for different GPCRs (Oakley *et al.*, 2000), we investigated whether β -arrestin-1, β -arrestin-2, or both interact with M3D-arr upon CNO treatment. To this end, co-immunoprecipitation (co-IP) experiments were performed on hERG-HEK cells transfected with M3D-arr and cultured without (control) or with CNO for 24 h. Specific anti- β -arrestin antibodies were used to immunoprecipitate either β -arrestin-1 or β -arrestin-2 and its associated proteins from whole-cell lysates. Precipitates were then immunoblotted with an anti-HA antibody to detect for M3D-arr. As shown in Fig. 4, CNO treatment significantly increased the interaction between M3D-arr and β -arrestin-1, but not β -arrestin-2.

To visualize the M3D-arr- β -arrestin interaction, immunocytochemical analysis was performed on hERG-HEK cells transfected with M3D-arr and cultured without (control) or with CNO for 24 h. After permeabilization, cells were probed with anti- β -arrestin-1 or anti- β -arrestin-2 and anti-HA (to detect M3D-arr) primary antibodies. Corresponding Alexa Fluor 594- and Alexa Fluor 488-conjugated secondary antibodies were then used to visualize M3D-arr and β -arrestin-1 or β -arrestin-2, respectively. As shown in confocal images in Fig. 5, there was increased co-localization between β -arrestin-1 and M3D-arr at the plasma membrane following CNO treatment (Fig. 5A). However, there was no increase in co-localization between β -arrestin-2 and M3D-arr at the plasma membrane following CNO treatment (Fig. 5B). These data are consistent with the co-IP results, and indicate that M3D-arr activation via CNO enhances the interaction between M3D-arr and β -arrestin-1 at the plasma membrane.

Activation of M3D-arr Increases hERG Expression through an Akt-dependent Pathway

Muscarinic M3 receptors couple with G_q proteins to activate phospholipase C, which

cleaves phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) to produce inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (Eglen, 2012). IP₃ stimulates the release of Ca²⁺ from the ER whereas DAG activates protein kinase C (PKC). We recently reported that activation of M3 receptor via carbachol (CCh) increases hERG expression through a PKC-dependent pathway (Wang *et al.*, 2014). However, M3D-arr contains a point mutation (R165L) that disrupts G_q protein-coupled PKC pathway (Nakajima and Wess, 2012). To verify that activation of M3D-arr increases mature hERG expression through a PKC-independent pathway, M3D-arr-transfected hERG-HEK cells were cultured without (control) or with CNO in the absence or presence of the PKC inhibitor, H7 (50 μM). After PKC inhibition, increased mature hERG expression induced by CNO-mediated M3D-arr activation was still present (Fig. 6A), indicating the involvement of a G_q protein independent pathway.

In addition to G_q proteins, stimulation of muscarinic M3 receptors promotes the activation of phosphoinositide 3-kinase (PI3K) and Akt (Guizzetti and Costa, 2001). β-arrestin-1 has been shown to mediate PI3K/Akt signaling (Povsic *et al.*, 2003). It has also been reported that PI3K-dependent activation of Akt increases hERG channel function in hERG-expressing HEK293 cells (Zhang *et al.*, 2003). We hypothesized that M3D-arr activation and subsequent recruitment of β-arrestin-1 enhances Akt activation, leading to increased mature hERG expression. We performed three sets of experiments to assess our hypothesis. First, we examined the effects of Akt inhibition on the increase mediated by M3D-arr activation. We cultured M3D-arr-transfected hERG-HEK cells without (control) or with CNO in the absence or presence of an Akt kinase inhibitor (Akt-I, 2.5 μM). As shown in Fig. 6B, inhibition of Akt abolished the CNO-induced increase in mature hERG expression. Second, we assessed total (non-phosphorylated and phosphorylated) Akt, and active (phosphorylated) Akt expression in M3D-arr-transfected hERG-

HEK cells upon CNO treatment. As shown in Fig. 7A, CNO treatment significantly increased the expression level of phosphorylated Akt, (but not total Akt) in conjunction with increased mature hERG expression. Third, we cultured hERG-HEK cells with the Akt activator, SC79 (12 μ M) to address the causative role of Akt activation in the increased mature hERG expression. SC79 significantly increased the expression level of phosphorylated Akt and mature hERG expression in hERG-HEK cells (Fig. 7B). SC79 treatment also significantly increased I_{hERG} (Fig. 7C).

As a phospholipid kinase, PI3K phosphorylates PtdIns(4,5) P_2 to produce PtdIns(3,4,5) P_3 at the plasma membrane. Generation of PtdIns(3,4,5) P_3 promotes membrane localization and activation of downstream effectors containing a pleckstrin homology (PH) domain such as phosphoinositide-dependent kinase-1 (PDK1), leading to the activation of Akt (Milburn *et al.*, 2003; Alessi *et al.*, 1997; Stephens *et al.*, 1998). It has been established that PI3K-dependent activation of Akt is antagonized by the phosphatidylinositol 3'-phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) (Oudit and Penninger, 2009; Oudit *et al.*, 2004). We overexpressed PTEN in M3D-arr-transfected hERG-HEK cells and examined the effect of CNO treatment on the expression of Akt, as well as hERG expression and I_{hERG} . Western blot analyses showed that PTEN overexpression abolished the CNO-induced increase in phosphorylated Akt and mature hERG expression (Fig. 8A). Electrophysiological data showed that PTEN overexpression also eliminated the CNO-induced increase in I_{hERG} (Fig. 8B).

Inhibition of PIKfyve or Rab11 Abolishes the Effect of M3D-arr Activation on I_{hERG}

A downstream target of Akt, phosphatidylinositol-3-phosphate-5-kinase (PIKfyve), promotes endosomal trafficking of transmembrane proteins such as hERG and KCNQ1 channels, as well as GLUT4 carriers to the cell surface (Berwick *et al.*, 2004; Seebohm *et al.*, 2007;

Pakladok *et al.*, 2013). Upon phosphorylation by Akt, PIKfyve phosphorylates PtdIns(3)P to produce PtdIns(3,5)P₂, a phospholipid implicated in the stability of intracellular vesicles (McEwen *et al.*, 1999; de Lartigue *et al.*, 2009). To evaluate the role of PIKfyve in the CNO-mediated increase in I_{hERG}, we studied the effects of the PIKfyve inhibitor, YM201636, on the CNO-mediated increase of I_{hERG} in M3D-arr-transfected hERG-HEK cells. Inhibition of PIKfyve abolished the CNO-induced increase in I_{hERG} (Fig. 9).

We previously demonstrated that the small GTPase Rab11 is involved in hERG channel recycling (Lamothe and Zhang, 2013; Chen *et al.*, 2015). It was also reported that stimulation of PIKfyve enhances Rab11-mediated transport of KCNQ1/KCNE1 channels to the membrane (Seeböhm *et al.*, 2007). To examine the role of Rab11 in the CNO-mediated increase in I_{hERG}, we interfered with Rab11 function by overexpressing a Rab11 dominant-negative (Rab11 DN) mutant (Rab11 S25N) in M3D-arr-transfected hERG-HEK cells and examined the effect of CNO on I_{hERG}. Overexpression of Rab11 DN reduced I_{hERG} in control cells, indicating that Rab11-contributed hERG homeostasis had been disrupted (Chen *et al.*, 2015). Moreover, overexpression of the Rab11 DN mutant abolished the CNO-induced increase in I_{hERG} (Fig. 9).

We used CNO to activate M3D-arr to generate β -arrestin signaling in the present study. Using wild type and mutant M3 receptors, Alvarez-Curto *et al.* conclude that there is no functional selectivity associated with CNO, and activation of M3-DREADDs by CNO produces responses very similar to activation of wild type M3 receptors by acetylcholine (Alvarez-Curto *et al.*, 2011). To demonstrate whether activation of native M3 receptors enhances hERG expression via β -arrestin signaling, we stimulated endogenous M3 using CCh (50 μ M) in hERG-HEK cells in the presence of H7 which blocks G_q protein-mediated PKC activation. Our data show that although H7 blocked the CCh-induced increase in mature hERG expression following an 8-h

treatment (Wang *et al.*, 2014), it could not block the CCh-induced increase in mature hERG expression and I_{hERG} following a 24-h treatment (Fig. 10 A and B). Furthermore, the 24-h CCh-mediated hERG increase was eliminated by the Akt inhibitor, Akt-I (Fig. 10 A and B).

To investigate whether β -arrestin-mediated hERG regulation occurs in cardiac myocytes we examined the effects of CCh on I_{Kr} in neonatal rat ventricular myocytes in the presence of H7 to block PKC activation. Similar to results obtained in hERG-HEK cells, a 24-h treatment with CCh increased I_{Kr} in ventricular myocytes in the presence of H7, and the effect was abolished by Akt-I (Fig. 10 C).

Discussion

hERG K^+ channels are critical for the repolarization of the cardiac action potential and dysregulation of hERG channel expression or function underlies prolonged QT intervals under various pathological conditions (Keating and Sanguinetti, 2001). Blockade of hERG channel activity by the "off-target" actions of many therapeutic agents has been a major area of investigation and rationale for screening compounds during drug development to exclude long QT risks (Roden, 2004; Shah, 2005). Studies from our lab and others have indicated that aberrant hERG trafficking to the plasma membrane can also contribute to LQTS effects of some drugs (Dennis *et al.*, 2007; Guo *et al.*, 2007). Thus, understanding the basic cell physiology of hERG channel trafficking and its regulation by various receptor-mediated signaling pathways is important. We previously demonstrated that activation of M3 muscarinic receptors increases hERG channel expression by inhibiting the ubiquitin ligase Nedd4-2 through G_q protein-dependent activation of PKC (Wang *et al.*, 2014). Upon activation, M3 receptors also recruit β -arrestins to scaffold G_q protein-independent signaling (Novi *et al.*, 2005). However, the role of β -

arrestin signaling in hERG regulation has not been addressed.

The arrestin-biased M3 designer-receptor exclusively activated by CNO (M3D-arr) provides a powerful means for the investigation of β -arrestin signaling in the absence of G_q protein activity (Nakajima and Wess, 2012). It has been demonstrated that CNO activation of the M3D-arr is unable to stimulate a G-protein(q) coupled response but can stimulate β -arrestin signaling (Nakajima and Wess, 2012). It is noted that although CNO preferentially targets a mutant M3 receptor, M3D-arr, it does not produce functional selectivity regarding the signaling pathways associated with M3 receptor activation (Alvarez-Curto *et al.*, 2011). The β -arrestin-biased signaling of M3D-arr results from a mutation within the receptor which selectively uncouples G_q proteins but preserves β -arrestin signaling (Nakajima and Wess, 2012). Indeed, the β -arrestin signaling-mediated increase in hERG/ I_{Kr} was also observed in hERG-HEK cells and in neonatal ventricular myocytes upon activation of M3 receptors by CCh when the G-protein coupled response was blocked by H7 (Fig. 10).

Upon ligand-binding, G protein-coupled receptor kinases phosphorylate Ser-Thr residues in the cytoplasmic C-terminus of GPCRs to promote β -arrestin recruitment (Reiter and Lefkowitz, 2006; Oakley *et al.*, 2001). Many GPCRs have been identified to recruit and interact with β -arrestin at the plasma membrane (Shenoy *et al.*, 2006; Ahn *et al.*, 2004; Novi *et al.*, 2005). In the context of M3 receptors, stimulation by CCh promotes the recruitment and interaction with β -arrestin-1 (Novi *et al.*, 2005). Our data show that, following stimulation by CNO, M3D-arr selectively interacts with β -arrestin-1, but not β -arrestin-2 (Fig. 4 and 5). We further demonstrated that M3D-arr and β -arrestin-1 co-localize at or near the plasma membrane (Fig. 5), indicating that β -arrestin-1 is primarily recruited to stimulated M3D-arr receptors.

Activation of M3 receptors elicits G_q -protein-dependent (PKC) and independent (β -

arrestin) pathways (Nakajima and Wess, 2012). While our previous (Wang *et al.*, 2014) and present studies indicate that both pathways regulate hERG expression via distinct mechanisms, the proportional contribution of each pathway to the increased hERG expression is complex and remains to be elucidated in future studies. This is partially due to the observations that there is a difference in response time between G_q-protein and β -arrestin signaling-mediated hERG modulation. We previously demonstrated that CCh-mediated activation of M3 receptor increases mature hERG expression and I_{hERG} . The CCh-mediated increase occurs in 2 h and becomes obvious at 8 h (Wang *et al.*, 2014), and is blocked by the PKC inhibitor H7 (Wang *et al.*, 2014). On the other hand, in the present study, we found that the CNO-induced increase in mature hERG expression displayed a slow-onset and became obvious at 24 h (Fig. 1), and the effect could not be blocked by H7 (Fig. 6A). These data indicate that our previously observed 8-h CCh-mediated hERG increase is mainly due to activation of the G protein-dependent PKC pathway (Wang *et al.*, 2014), in which the G-protein-independent β -arrestin pathway had not sufficiently developed.

In the present study, our data show that the 24-h CNO-treatment induces similar increases in hERG expression in hERG-HEK cells without H7 or with H7 treatment (Fig. 6A). Moreover, the 24-h CCh-induced hERG increase was prevented by the Akt inhibitor, Akt-I (Fig. 10 A and B). We previously showed that a 24-h treatment of neonatal rat ventricular myocytes with CCh (50 μ M) in the absence of H7 increased I_{Kr} (Wang *et al.*, 2014). In the present study, we performed similar experiments in neonatal rat ventricular myocytes with inclusion of H7 to block the G-protein-dependent signaling pathway. Interestingly, CCh-treatment induced I_{Kr} increase in the presence of H7 (present study, Fig. 10C) is not different from that in the absence of H7 (previous study, (Wang *et al.*, 2014), Fig. 8C). Furthermore, the effect of the 24-h CCh-treatment

on I_{K_r} was abolished by Akt-I (Fig. 10C). These data suggest the 24-h CCh-mediated I_{K_r} increase is primarily mediated through β -arrestin signaling pathway. It has been shown that β -arrestin and G_q -protein signaling pathways are independent of each other (Shenoy *et al.*, 2006; Ahn *et al.*, 2004). For example, regarding ERK1/2 activation following AT_1R stimulation, β -arrestin-mediated ERK1/2 activation is slow, whereas G protein-mediated activation is rapid (Ahn *et al.*, 2004). Thus, it is likely that M3 receptor stimulation increases mature hERG expression through temporally distinct signaling mechanisms: an early G_q protein-dependent pathway and a late β -arrestin-dependent pathway. The existence of temporally distinct signaling mechanisms is interesting and provides novel insights into the sophisticated cellular regulatory systems that offer flexibility in hERG regulation.

Our data show that Akt plays a key role in the CNO-M3D-arr mediated β -arrestin signaling pathway. Inhibition of Akt abolished the CNO-induced increase in mature hERG expression (Fig. 6B). An increase in active (phosphorylated) Akt expression paralleled the increase in mature hERG expression following CNO treatment (Fig. 7A). Furthermore, activation of Akt by the small-molecule activator, SC79, increased the expression of phosphorylated Akt and mature hERG expression (Fig. 7B) and I_{hERG} (Fig. 7C).

Akt is modulated through a variety of upstream regulators. Of notable interest is the lipid kinase PI3K, a well-known activator of Akt that is constitutively expressed in the heart and vasculature (Oudit *et al.*, 2004; Crackower *et al.*, 2002). PI3K phosphorylates PtdIns(4,5) P_2 to produce PtdIns(3,4,5) P_3 , a phospholipid responsible for the selective recruitment of downstream effectors to the plasma membrane. By binding to a common PH domain, PtdIns(3,4,5) P_3 associates PDK1 with various kinase substrates such as Akt to facilitate their activation (Alessi *et al.*, 1997; Bellacosa *et al.*, 1998). The activity of PI3K is antagonized by the lipid phosphatase,

PTEN, which opposes production of PtdIns(3,4,5)P₃ (Maehama and Dixon, 1998). Our data showed that inhibition of Akt activation by PTEN overexpression abolished the CNO-induced increase in hERG expression and I_{hERG} in M3D-arr-transfected hERG-HEK cells (Fig. 8), suggesting that enhancement of Akt activity during this process occurs primarily through PI3K. The activity of PI3K is enhanced by tyrosine phosphorylation of its regulatory subunit, which relieves inhibition of its catalytic domain (Cuevas *et al.*, 2001). While this study does not evaluate the mechanism of PI3K activation, β-arrestin is known to scaffold non-receptor tyrosine kinases that may lead to its phosphorylation (Luttrell *et al.*, 1999).

Channel recycling contributes to the homeostasis of hERG expression level in the plasma membrane (Chen *et al.*, 2015). PIKfyve phosphorylates PtdIns(3)P to produce PtdIns(3,5)P₂, a phospholipid implicated in the stability of trafficking vesicles (Berwick *et al.*, 2004). Akt-mediated activation of PIKfyve increases PtdIns(3,5)P₂ production on the surface of intracellular vesicles, which subsequently modulates trafficking (Berwick *et al.*, 2004). Lang and colleagues showed that enhanced Akt and PIKfyve activity increases hERG current (Pakladok *et al.*, 2013). Our data are consistent with this notion, showing that PIKfyve inhibition attenuates the effect of CNO-mediated activation of M3D-arr on I_{hERG} (Fig. 9). Therefore, increases in hERG expression and current are likely a result of enhanced vesicle trafficking to the plasma membrane.

We recently found that Rab11 maintains homeostatic levels of mature hERG expression by facilitating recycling of channels to the cell surface (Chen *et al.*, 2015). Previous studies have shown that activation of PIKfyve positively regulates Rab11-mediated transport of hERG and KCNQ1/KCNE1 channels to the membrane (Pakladok *et al.*, 2013; Seeböhm *et al.*, 2007). Therefore, Rab11 is likely involved in the M3D-arr activation-induced increase in hERG expression and function. Our results are consistent with this notion, showing that overexpression

of a Rab11 dominant-negative mutant (Rab11 DN) abolishes the effect of CNO-mediated activation of M3D-arr on I_{hERG} (Fig. 9). Rab11 DN also decreased I_{hERG} (Fig. 9), confirming that Rab11 contributes to the homeostasis of hERG expression in the plasma membrane (Chen *et al.*, 2015).

PI3K/Akt cascades can be activated by insulin- or insulin-like growth factor 1 (IGF-1) receptor signaling or other serum growth factors (Shepherd *et al.*, 1998; Povsic *et al.*, 2003). It has been reported that β -arrestin-1 mediates IGF-1 activation of PI3K and anti-apoptosis (Povsic *et al.*, 2003). In a previous study focusing on the role of SGK in hERG regulation, we demonstrated that activation of SGK by overexpressing the SGK1 or SGK3 plasmid in hERG-HEK cells or applying serum, dexamethasone or insulin to the culture medium, increases mature hERG expression partially through promoting Rab11-mediated hERG recycling (Lamothe and Zhang, 2013). SGK and Akt are closely related enzymes and share an overlapping set of substrates (Sommer *et al.*, 2013). While our previous study did not elucidate the specific roles of SGK and Akt, these data are consistent with our present finding that activation of PI3K/Akt cascades upregulate trafficking of the hERG channels.

In summary, we demonstrated a novel mechanism of β -arrestin-dependent regulation of hERG channels (Fig. 11). CNO-mediated activation of M3D-arr leads to the recruitment of β -arrestin-1, promoting PI3K-dependent activation of Akt. Enhancement of Akt activity stimulates PIKfyve, which promotes Rab11-mediated hERG recycling to the membrane (Fig. 11). These findings provide novel insight into hERG regulation, which is useful for understanding impaired hERG function and identifying therapeutic targets in the treatment of LQTS. In addition, the cellular signaling pathways identified using hERG regulation in the present study extend our understanding of molecular mechanisms underlying the regulation of other molecules involved

in various cellular functions.

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Author contributions:

Participated in research design: Sangoi, Lamothe, Fisher and Zhang.

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Performed data analysis: Sangoi, Lamothe, Guo, Yang, and Li.

Wrote or contributed to the writing of the manuscript: Sangoi, Lamothe, and Zhang.

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Footnotes

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FIGURES AND LEGENDS

Fig. 1. Activation of β -arrestin-biased M3 muscarinic receptor-based DREADD (M3D-arr) by CNO increases mature hERG expression levels in a concentration- and time-dependent manner. (A) Concentration-dependent effect of CNO on hERG expression (n=4). (B) Time-dependent effect of 10 μ M CNO on hERG expression (n=3). In both A and B, hERG-HEK cells were transfected with empty pcDNA3 (control) or HA-tagged M3D-arr plasmid. Twenty-four hours after transfection, cells were treated with CNO under various conditions. Whole-cell lysates were collected for Western blot analysis. In each gel, band intensities of the 155-kDa hERG proteins with CNO-treatments were normalized to the control and expressed as relative values. Summarized data are presented in the graphs beneath the Western blots. $**P<0.01$ vs. Ctrl (0 μ M and 0 h, respectively).

Fig. 2. CNO treatment increases mature hERG expression and I_{hERG} without affecting hERG mRNA level or the activation-voltage relationship of I_{hERG} in M3D-arr expressing cells. hERG-HEK cells were transfected with pcDNA3 or HA-tagged M3D-arr plasmid. Twenty-four hours after transfection, cells were cultured without (Ctrl) or with 10 μ M CNO for 24 h. (A) Effect of CNO treatment on the expression of hERG channels. Band intensities of the 155-kDa and 135-kDa hERG proteins from CNO-treated cells were normalized to respective controls and expressed as relative values in each gel and summarized beneath the Western blot images (pcDNA3, n=4; M3D-arr, n=7). $**P<0.01$ vs. Ctrl. (B) Effect of CNO on the relative hERG mRNA expression level. Quantitative reverse transcription PCR was conducted using RNA from cells transfected with pcDNA3 or M3D-arr and cultured without (Ctrl) or with CNO (pcDNA3,

n=3; M3D-arr, n=3). GAPDH was used as a control housekeeping gene. (C) Families of hERG currents elicited using the protocol shown inset in hERG-HEK cells transfected with pcDNA3 or M3D-arr after a 24-h CNO (10 μ M) treatment. (D) The effects of CNO treatment on the activation-voltage relationships of hERG channels in hERG-HEK cells transfected with pcDNA3 (n=7-11 cells) or M3D-arr plasmid (n=10-12 cells from 4 independent trials). ** P <0.01 vs. Ctrl for the maximal tail currents.

Fig. 3. CNO treatment affects neither Kv1.5 nor EAG channels in M3D-arr expressing cells. (A) Representative $I_{Kv1.5}$ or I_{EAG} in respective stable HEK cells transfected with M3D-arr with or without 24-h CNO (10 μ M) treatment. Summarized current amplitudes are shown below the traces. The numbers above the bar graphs indicate the number of cells examined from 4 independent experiments. (B). Effects of CNO on the expression of Kv1.5 and EAG channel proteins. The relative band intensities of channel proteins in cells treated with CNO are normalized to control and shown beneath the representative Western blot images (n=4 for Kv1.5, and n=4 for EAG).

Fig. 4. CNO treatment enhances the interaction between M3D-arr and β -arrestin-1. Co-IP experiments illustrating that CNO treatment intensifies the interaction between M3D-arr and β -arrestin-1 (left) but not between M3D-arr and β -arrestin-2 (right). pcDNA3 or HA-tagged M3D-arr transfected hERG-HEK cells were treated with 10 μ M CNO for 24 h. Whole-cell lysates were immunoprecipitated with a goat anti- β -arrestin-1 or mouse anti- β -arrestin-2 antibody. HA-tagged M3D-arr was detected in the immunoprecipitates using an anti-HA primary and appropriate secondary antibodies. Anti-GAPDH was used as a negative control. The difference

in background noise between the two panels is due to the different species of antibodies used for immunoprecipitations. M: molecular weight marker. Similar data were obtained in 5 independent experiments.

Fig. 5. CNO enhances the β -arrestin-1-M3D-arr interaction. Confocal images showing that CNO-treatment intensifies the interaction between β -arrestin-1 and M3D-arr (A), but not the interaction between β -arrestin-2 and M3D-arr (B), at the plasma membrane. HA-tagged M3D-arr-transfected hERG-HEK cells grown on glass coverslips were cultured without (Ctrl) or with 10 μ M CNO for 24 h. Cells were fixed and permeabilized. M3D-arr was labeled with a rabbit anti-HA primary antibody and an Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody. β -Arrestin-1 or β -Arrestin-2 was labelled with a goat anti- β -Arrestin-1 or mouse anti- β -arrestin-2 primary antibodies and an Alexa Fluor 488-conjugated donkey anti-goat or goat anti-mouse secondary antibodies. Cells are shown in DIC images on the left. Intensities of the fluorescence signals of the lines across the representative cells in each condition are quantified by ImageJ and shown in the right panels. Similar data were obtained in 4 independent experiments.

Fig. 6. CNO treatment increases hERG expression through a PKC-independent but an Akt-dependent pathway in M3D-arr-transfected hERG-HEK cells. (A) PKC inhibition does not affect the CNO-induced increase in hERG expression. M3D-arr-transfected hERG-HEK cells were cultured without (Ctrl) or with 10 μ M CNO for 24 hours in the absence or presence of 50 μ M of H7, a PKC inhibitor (n=9). (B) Akt inhibition abolishes the CNO-induced increase in hERG expression. M3D-arr-transfected hERG-HEK cells were cultured without (Ctrl) or with 10

μ M CNO for 24 hours in the absence or presence of 2.5 μ M of Akt-I (n=6). Whole-cell lysates were extracted and assessed using Western Blot analysis. Band intensities of the 155-kDa hERG were normalized to the controls and expressed as relative values in each gel and summarized beneath the Western blot images. $**P<0.01$ vs. Ctrl.

Fig. 7. CNO-mediated M3D-arr activation increases the expression level of hERG as well as phosphorylated Akt (p-Akt). (A) Effect of CNO on hERG, total Akt and phosphorylated Akt (p-Akt) in M3D-arr-transfected hERG-HEK cells. Whole-cell lysates were collected 24 h after culture without (Ctrl) or with 10 μ M CNO (n=5-10). (B) Effect of Akt activator, SC79, on hERG, total Akt, and p-Akt expression in hERG-HEK cells. Whole-cell lysates were extracted from cells treated with DMSO (Ctrl) or 12 μ M SC79 (n=7). In A and B, band intensities of the 155-kDa hERG, Akt, and p-Akt in CNO- or SC79-treated cells were normalized to their respective controls and expressed as relative values in each gel and summarized beneath Western blot images. (C) Effect of SC79 on I_{hERG} . Summarized I_{hERG} amplitudes are shown beneath the representative families of I_{hERG} from cells treated with DMSO (Ctrl) or SC79. The numbers above the bar graphs indicate the number of cells examined from 4 independent experiments. $**P<0.01$ vs. Ctrl.

Fig. 8. CNO treatment increases mature hERG expression and I_{hERG} through PI3K-mediated activation of Akt in M3D-arr-transfected hERG-HEK cells. M3D-arr-transfected hERG-HEK cells were additionally transfected without or with the PI3K inhibitor, PTEN. Cells were then cultured without (Ctrl) or with 10 μ M CNO for 24 h. PTEN blocks the CNO-induced increase in mature hERG and p-Akt expression (A) and I_{hERG} (B). For Western blot analysis,

band intensities of the 155-kDa hERG and p-Akt from CNO-treated cells were normalized to their respective controls and expressed as relative values in each gel and summarized beneath the Western blot images (n=6). For whole-cell patch clamp, summarized I_{hERG} is shown beneath the representative families of I_{hERG} in each condition. The numbers above the bar graphs indicate the number of cells examined from 3 independent experiments. $**P < 0.01$ vs. Ctrl.

Fig. 9. The CNO-mediated increase in I_{hERG} is abolished by inhibition of PIKfyve or Rab11 in M3D-arr-transfected hERG-HEK cells. hERG-HEK cells were transfected with M3D-arr alone or together with Rab11 dominant negative (DN) mutant S25N. Twenty-four hours after transfection, cells were treated with CNO (10 μ M, 24 h) in the absence or presence of PIKfyve inhibitor, YM201636 (0.2 μ M, in M3D-arr-transfected cells). Cells were then collected for I_{hERG} recordings. The summarized I_{hERG} amplitudes are shown beneath the representative families of I_{hERG} in each condition. The numbers above the bar graphs indicate the number of cells examined from 3-5 independent experiments. $**P < 0.01$ vs. Ctrl.

Fig. 10. CCh increases hERG and native I_{Kr} in the presence of H7, and its effects are blocked by Akt-I. (A) Effects of CCh (50 μ M, 24 h) on hERG expression in H7-treated cells in the absence or presence of Akt-I (n=4). (B) Effects of CCh (50 μ M, 24 h) on I_{hERG} in 50 μ M H7-treated cells in the absence or presence of Akt-I (2.5 μ M). The numbers above the bar graphs indicate the number of cells examined from 4 independent experiments. (C) Effects of CCh (50 μ M, 24 h) on I_{Kr-Cs} in H7-treated neonatal rat ventricular myocytes in the absence or presence of Akt-I. The numbers above the bar graphs indicate the number of cells examined from 5 independent experiments. $**P < 0.01$ vs. Ctrl.

Fig. 11. Illustration of M3D-arr-mediated β -arrestin signaling in hERG regulation.

Activation of M3D-arr by CNO leads to the recruitment of β -arrestin-1, which promotes PI3K-dependent activation of Akt. Enhancement of Akt activity stimulates PIKfyve which promotes Rab11-mediated hERG recycling, leading to an increased mature hERG expression.

Figure 1

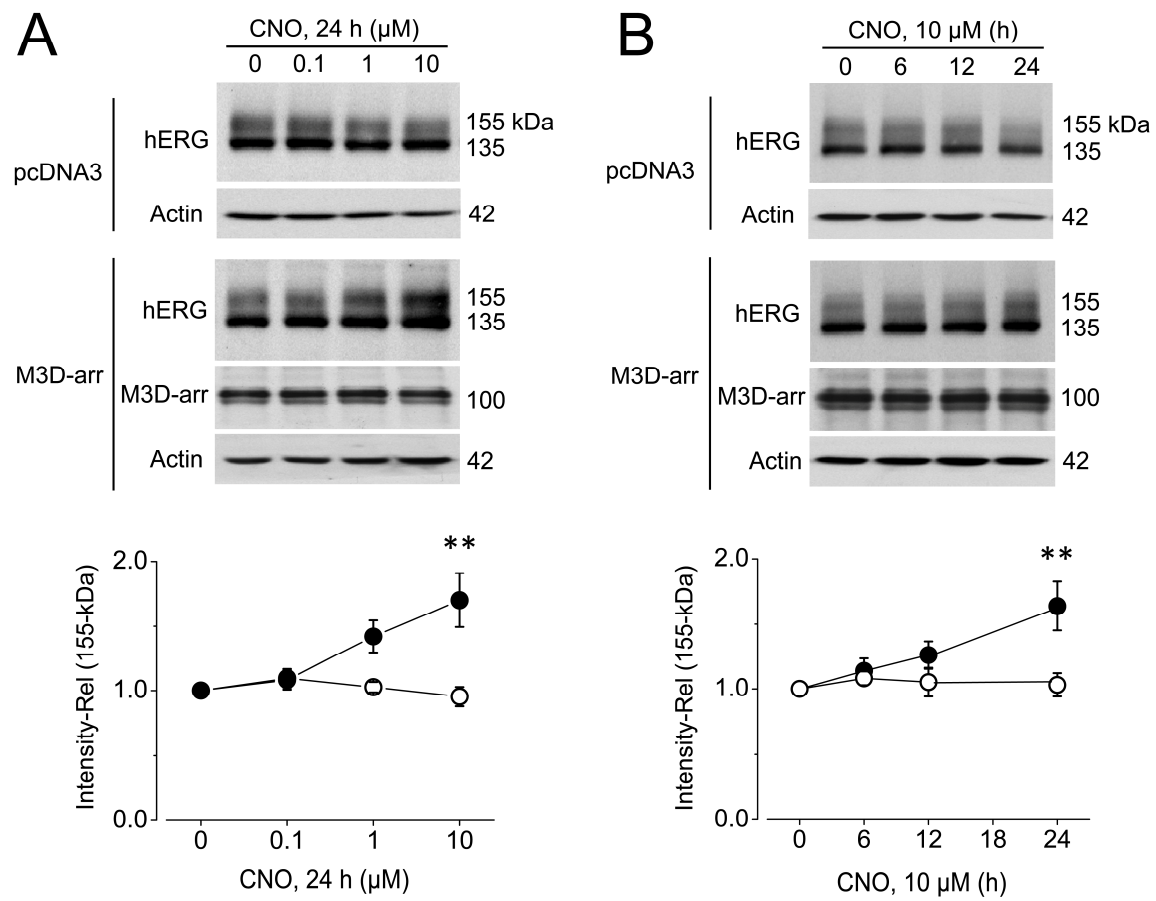


Figure 2

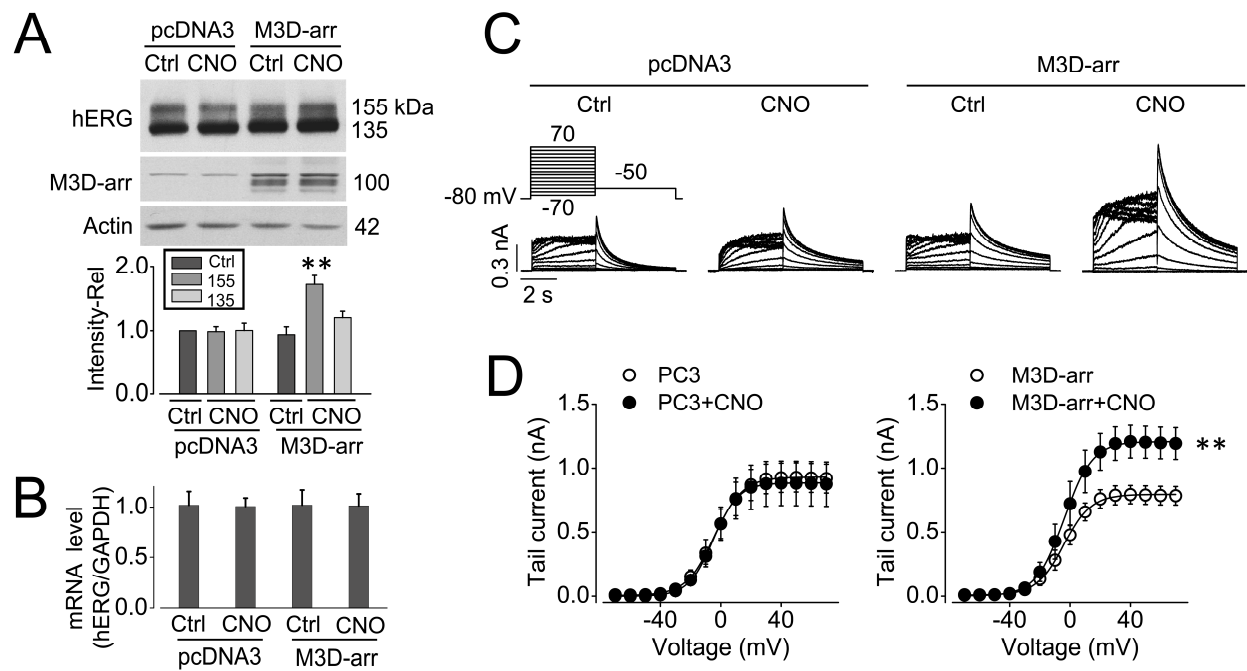
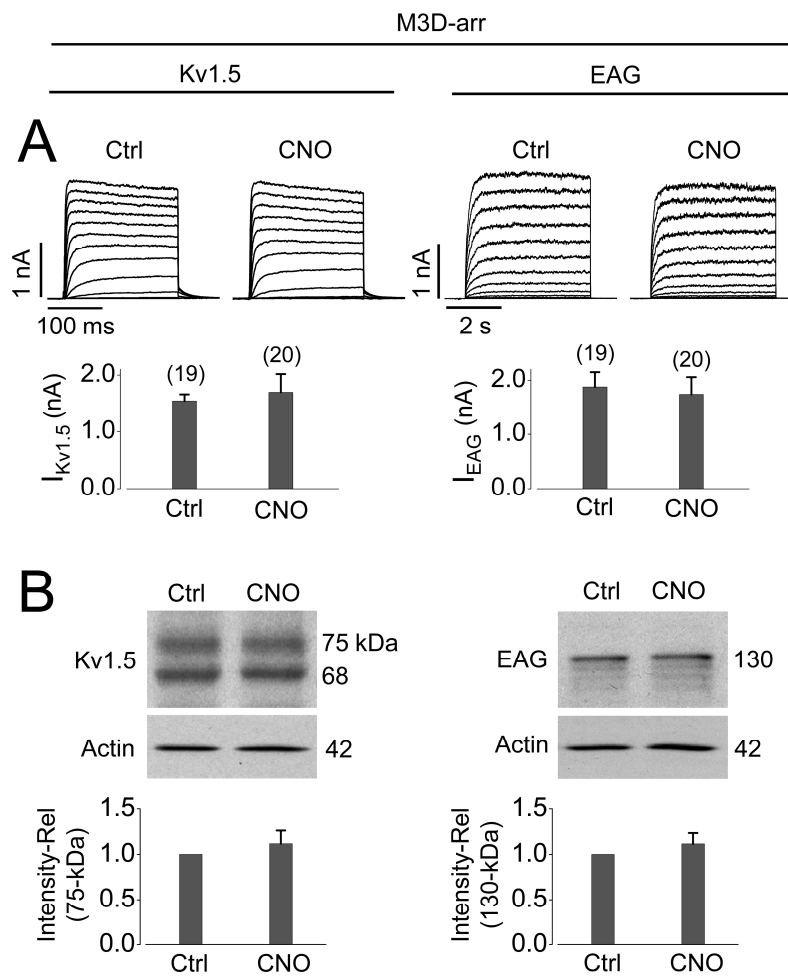


Figure 3



Western blot analysis of M3D-arr protein levels and its interaction with GAPDH and β -Arrestin-1/2. The top panel shows M3D-arr levels (100 kDa) and IgG (50 kDa) in IP: GAPDH and IP: β -Arrestin-1/2 fractions. The bottom panel shows M3D-arr levels (100 kDa) and IgG (50 kDa) in IP: GAPDH and IP: β -Arrestin-2 fractions. The CNO treatment (+) increases M3D-arr levels and its interaction with GAPDH and β -Arrestin-1/2.

Figure 5

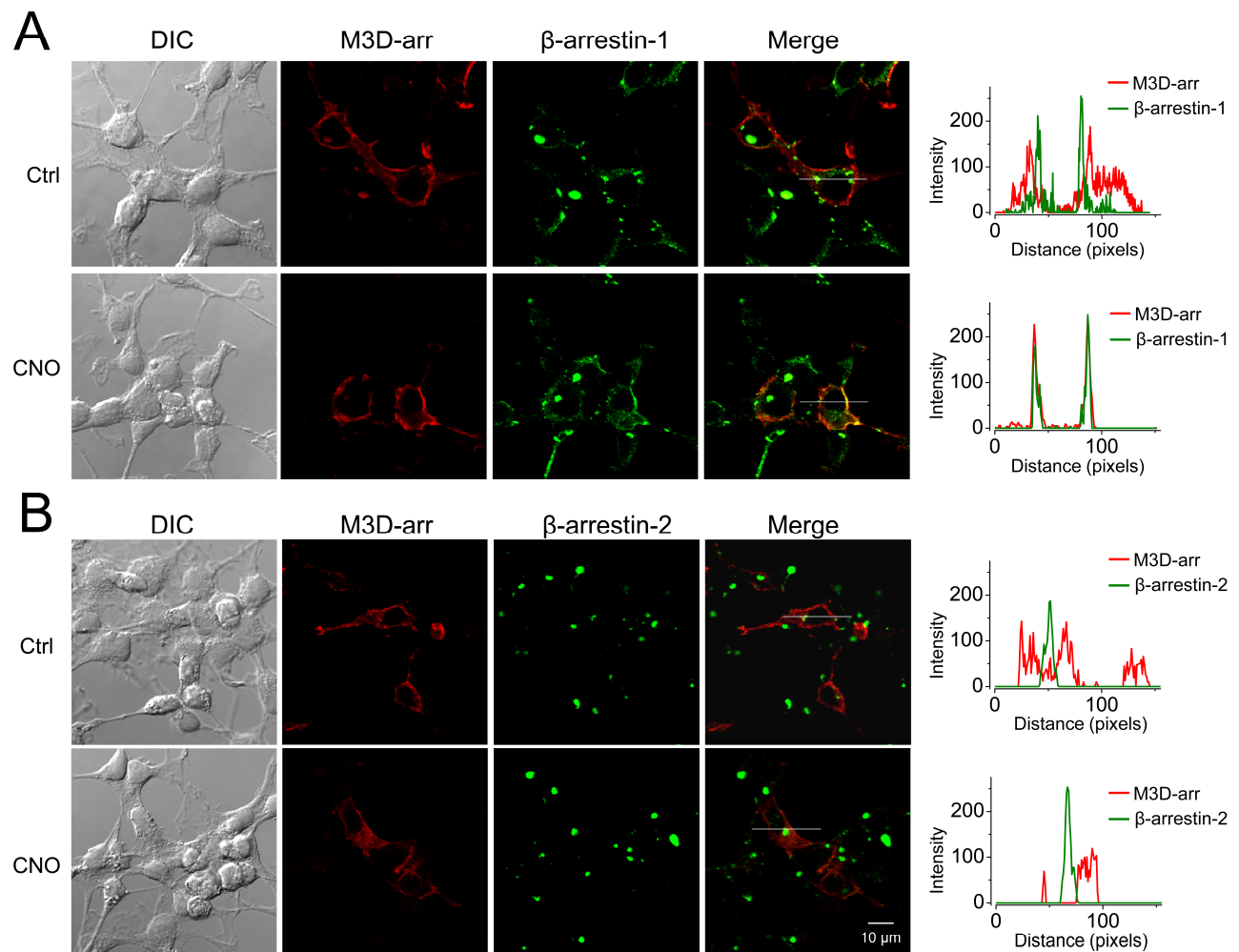


Figure 6

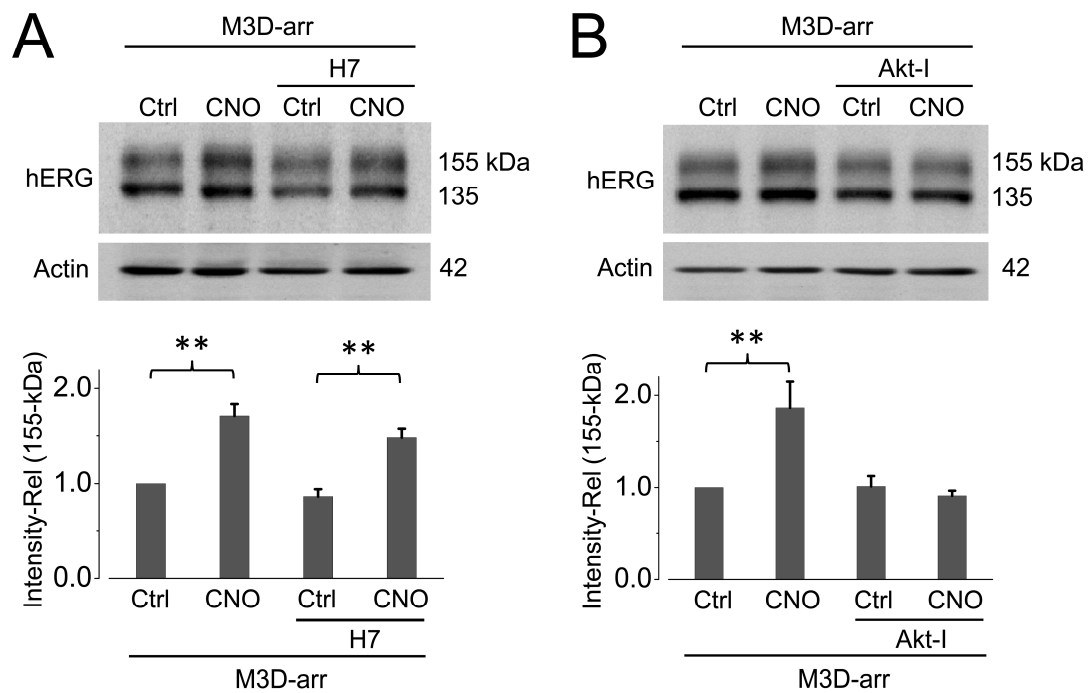


Figure 7

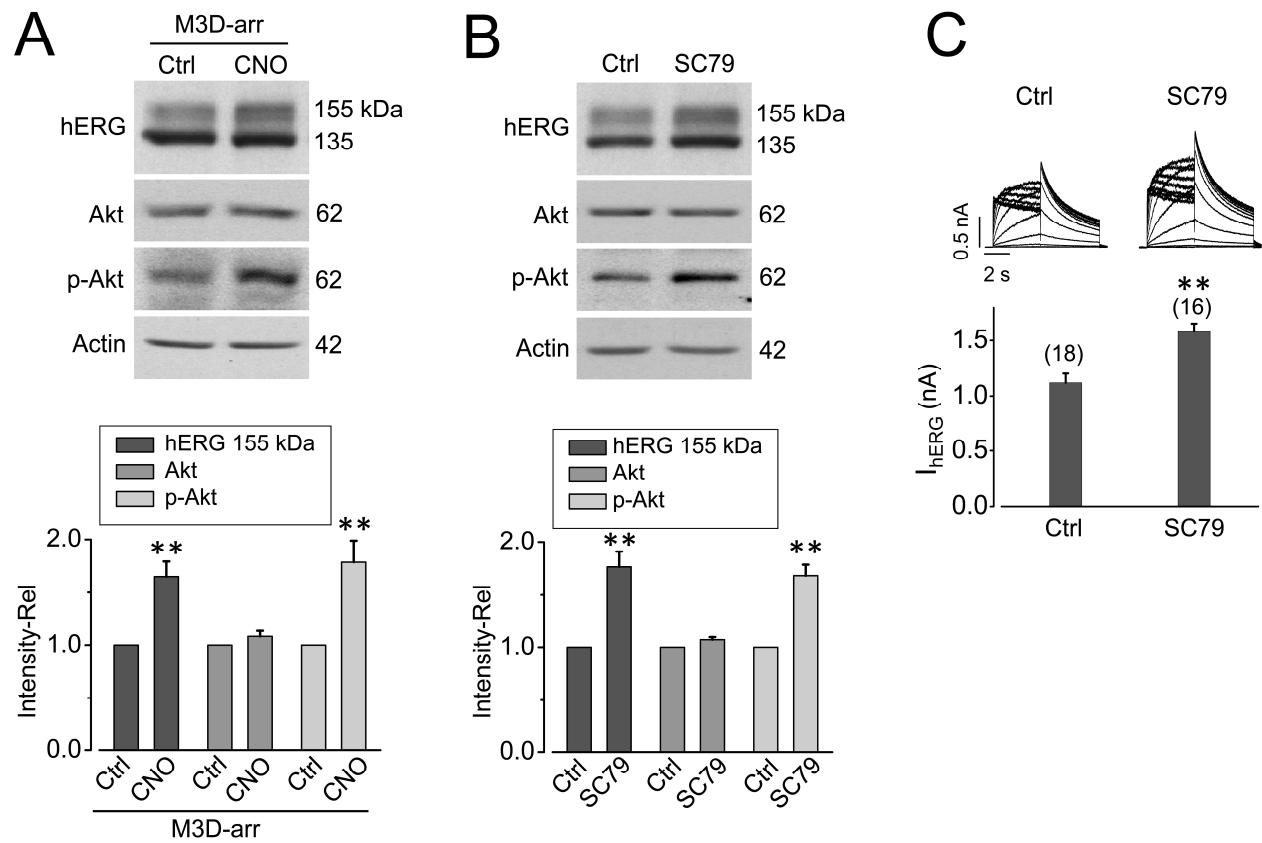


Figure 8

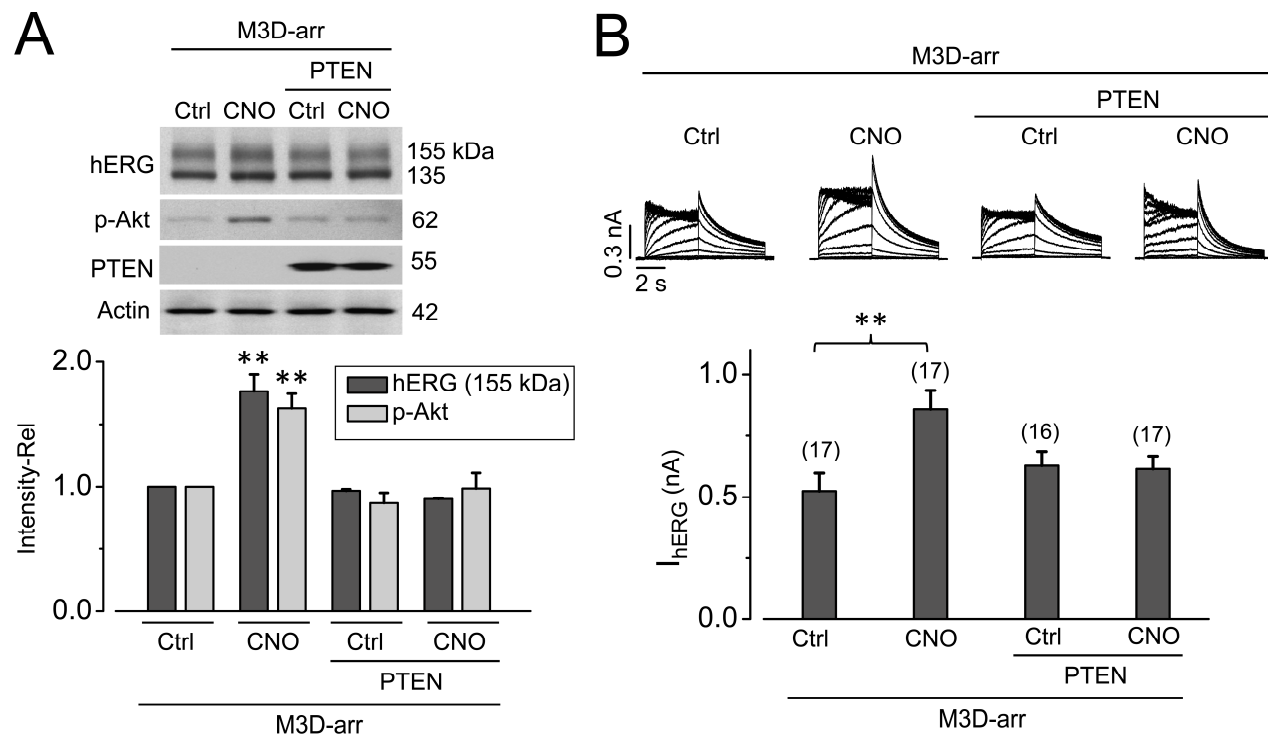


Figure 9

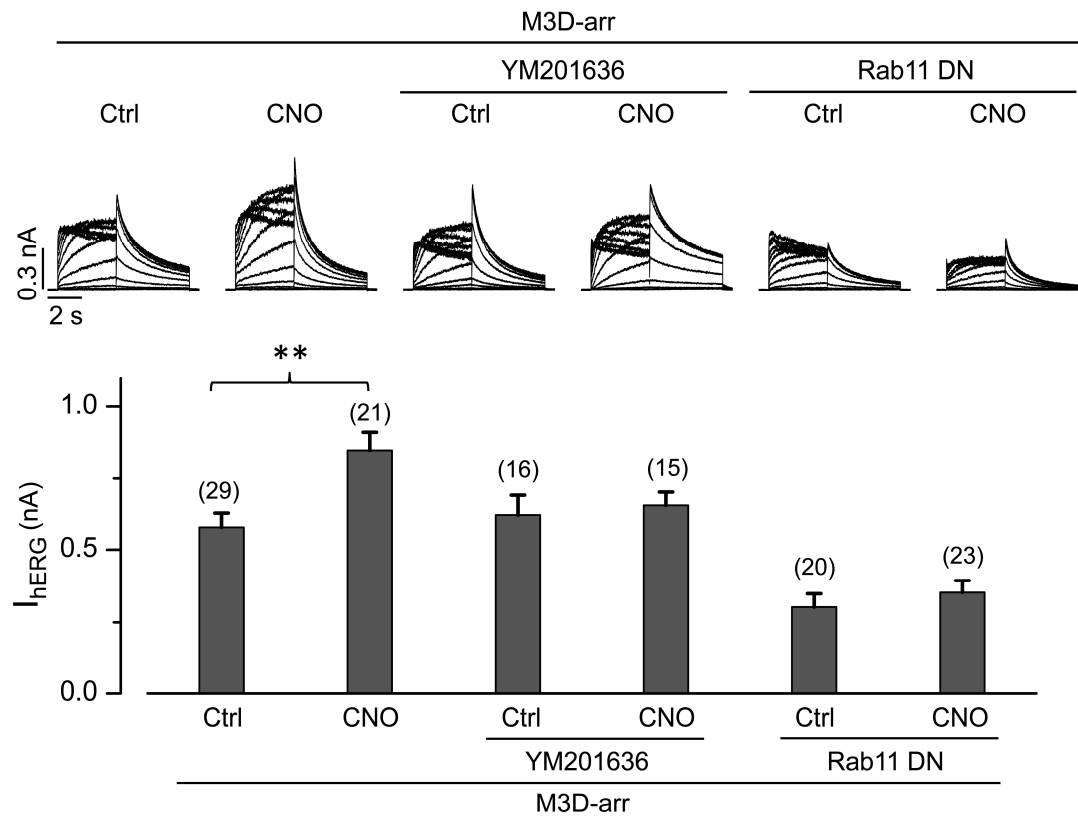


Figure 10

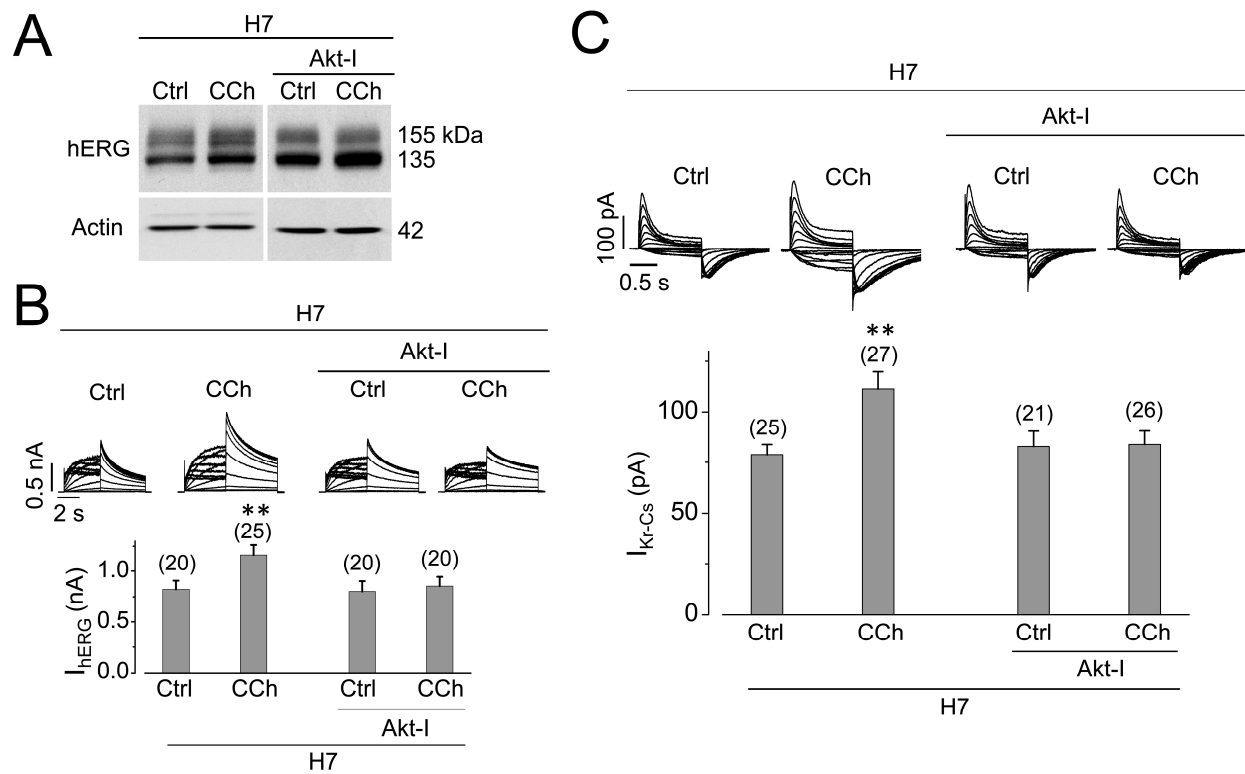


Figure 11

